The comparison of analytical methods used to measure bone mineralization across different bones, ages of pig, dietary differences, and health statuses

by

Hadley Reginald Williams

B.S., Iowa State University, Ames, IA, 2018
M.S., Kansas State University, 2020

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
College of Agriculture

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Abstract

Chapter 1 utilized a total of 2,184 pigs in a 143-d study to evaluate the effects of feeding varying analyzed calcium to phosphorus ratios (Ca:P) at two standardized total tract digestible (STTD) phosphorus to net energy ratios (STTD P:NE). The results suggested that pigs fed High STTD P:NE had improved overall ADG, G:F, and bone mineralization compared to pigs fed diets at 75% of High levels. Additionally, increasing the analyzed Ca:P ratio worsened ADG, G:F, and bone mineralization with Low STTD P:NE but had marginal impacts when adequate STTD P:NE was fed. Chapter 2 utilized a total of 360 nursery pigs in a 28-d trial to evaluate the effects of different bones and analytical methods on the assessment of bone mineralization response to dietary P and vitamin D in nursery pigs. The results suggested that bone density and bone ash responses varied depending on bone analyzed. Differences in bone density and ash in response to P and vitamin D were most apparent with fibulas and 2nd ribs. There were apparent differences in the bone ash percentage between defatted and non-defatted bone. However, differences between the treatments remain consistent regardless of the analytical procedure. For histopathology, 10th ribs were more sensitive than 2nd ribs or fibulas for detection of lesions. Chapter 3 utilized a total of 882 finishing pigs in a 112-d study to evaluate the effects of different bones and analytical methods on the assessment of bone mineralization response to changes in dietary P, phytase, and vitamin D in growing pigs. The results suggested there are differences between bone ash procedures was more apparent than the differences between diets. Differences in bone density and mineral content in response to dietary P and vitamin D were most apparent with 10^{th} ribs. Also, healthy pigs had greater serum Ca, P, and vitamin D concentrations and defatted bone ash than those exhibiting clinical signs of illness, with no difference between the two health statuses when expressed on a non-defatted bone ash basis. Chapter 4 utilized 194 pigs

from 64 commercial sites across 14 production systems in the Midwest US were used in a diagnostic survey to evaluate baseline biological measurements used to determine bone mineralization. In the diagnostic survey, there were differences in serum Ca, P, and vitamin D between healthy, lame, and unhealthy pigs. Differences in bone mineralization between pig types varied depending on the analytical procedure and bone and there was considerable range in values within pig type across the 14 production systems sampled. Chapter 5 utilized 4,887 bones from 8 different experiments to compare different bones and analytical procedures used to measure bone mineralization. The study highlights that bone density and bone ash can be used to determine bone mineralization in nursery and finishing pigs, with defatted preferred in finishing pigs due to reducing the variation. The study also suggests that the procedures for bone density are simpler and allow for a more rapid assessment of bone mineralization compared to bone ash with a reasonable degree of correlation.

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Table of Contents

List of Figures

List of Tables

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Finally, to mom and dad, you are the reason I have a passion in the swine industry. You always pushed me to set my goals high and achieve them. To Hayley, Peyton, Holden, Hudson, and Hesson I would not be who I am without your love, support, and patience. Hayden, I know it was hard for you to have your little brother in the program with you, but I cannot thank you enough for the guidance you gave me during my master's and doctoral program.

Appreciation is also expressed to DSM, PIC, Minnesota Pork Board, and Iowa Pork Board for their financial contributions to my research projects.

Dedication

This dissertation is dedicated to my parents Dr. Noel and Jacque Williams.

Chapter 1 - Impact of dietary analyzed calcium to phosphorus ratios and standardized total tract digestible phosphorus to net energy ratios on growth performance, bone, and carcass characteristics of pigs[1]

Abstract

A total of 2,184 pigs (337 × 1050, PIC; initially 12.4 ± 0.17 kg) were used in a 143-d study to evaluate the effects of feeding varying analyzed calcium to phosphorus ratios (Ca:P) at two standardized total tract digestible (STTD) phosphorus to net energy ratios (STTD P:NE). Pens of pigs (26 pigs per pen) were assigned to 1 of 6 dietary treatments in a 2 × 3 factorial with main effects of STTD P:NE and Ca:P ratio. Diets consisted of two levels of STTD P:NE; High (1.80, 1.62, 1.43, 1.25, 1.10, and 0.99 g STTD P/Mcal NE from 11 to 22, 22 to 40, 40 to 58, 58 to 81, 81 to 104, and 104 to 129 kg, respectively); or Low (75% of the High levels), and 3 analyzed Ca:P ratios (0.90:1, 1.30:1, and 1.75:1). There were 14 pens per treatment. Diets were corn-soybean meal-based and contained a constant phytase concentration within each dietary phase with levels decreasing throughout the trial (phases 1 through 3, 500 FTU/kg, assumed release of 0.13% STTD P; phase 4, 400 FTU/kg, assumed release of 0.11% STTD P; phase 5, 290 FTU/kg, assumed release of 0.09% STTD P; and phase 6, 210 FTU/kg, assumed release of 0.07% STTD P). Overall, there was a Ca:P × STTD P:NE interaction ($P < 0.05$) observed for

[1] This work has been published in *Journal of Animal Science*: H. R. Williams, M. D. Tokach, J. C. Woodworth, J. M. DeRouchey, R. D. Goodband, J. T. Gebhardt, C. M. Vier, N. Lu, R. Navales, M. Spindler, U. Orlando, L. Zaragoza, and A. M. Betlach. 2023. Impact of dietary analyzed calcium to phosphorus ratios and standardized total tract digestible phosphorus to net energy ratios on growth performance, bone, and carcass characteristics of pigs. J. Anim. Sci. skad168. doi:10.1093/jas/skad168.

average daily gain (ADG), feed efficiency (G:F), final body weight (BW), hot carcass weight (HCW), bone mineral density, bone mineral content, and bone breaking strength. When feeding Low STTD P:NE levels, increasing the analyzed Ca:P ratio decreased (linear, $P < 0.001$) ADG final BW, HCW, and tended to worsen G:F, bone mineral density, and bone mineral content (linear, $P < 0.10$). However, when feeding High STTD P:NE levels, increasing the analyzed Ca:P ratio significantly improved bone mineral content and bone mineral density (linear, $P < 0.05$), and tended to improve ADG and final BW (linear, $P < 0.10$) and G:F (quadratic $P < 0.10$). Additionally, increasing the analyzed Ca:P ratio worsened ADG, G:F, and bone mineralization with Low STTD P:NE but had marginal impacts when adequate STTD P:NE was fed.

Introduction

Calcium and phosphorus are macro minerals that are involved in various functions within the pig. Some of the main functions Ca and P have in the body are lean tissue deposition, the synthesis and maintenance of the skeletal structure, energy metabolism, muscle contraction, energy transmission, structure proteins, etc. (Crenshaw, 2001). Within the body, 96 to 99% of Ca and 60 to 80% of P are contained within skeletal tissue (Veum, 2010). Dietary P is commonly the third most expensive component of swine diets behind energy and amino acids, so feeding high levels of P leads to an increase in dietary cost. Excess P in the diet can lead to increased P excretion, which can negatively affect the environment (Maguire et al., 2005).

Dietary P requirement estimates have attained increased interest in farm animal nutrition due to environmental concerns and limited global phosphate resources (Cordell et al., 2011). In the United States, current regulations require pork producers to apply manure based on the most limiting nutrient, which is typically nitrogen (N). To meet the N requirement in corn grain, P is 3-6 times greater than crop P removal. Runoff P losses are a concern due to the negative impact

it has on surface water quality (Joern and Sutton, 2019). Vier et al. (2019b,c) observed that the STTD P requirement for nursery and finishing pigs is greater than NRC (2012) and European requirement estimates (CVB, 2020). Therefore, it appears to be a challenge to maximize growth potential yet minimize P excretion. In the current study, the High STTD P:NE levels were based on the recommendations of Vier et al. (2019b,c), and the Low STTD P:NE levels were approximately 75% of the High STTD P:NE levels. The Low STTD P:NE levels were slightly below the NRC (2012) requirement estimates for moderate lean growth genotypes, but slightly higher than the CVB (2020) requirement estimates.

Typically, when formulating swine diets, the P level is established and then the Ca level is adjusted to meet a desired Ca:P ratio. Feeding excess or insufficient Ca or P could potentially affect the utilization and metabolism of the other mineral (Veum, 2010). Therefore, swine diets are formulated to have not only adequate levels of both Ca and P, but also the ratio between the two (Ca:P ratio) minerals is routinely considered. Research has shown that a wide Ca:P ratio is detrimental to pig growth performance and bone mineralization when diets are marginal or deficient in STTD P (González-Vega et al., 2016a, 2016b; Wu et al., 2018; Vier et al., 2019a).

There is little research to compare North American recommendations for STTD P and Ca:P ratio vs European estimates from weaning to market in a commercial environment. Our hypothesis was that although European estimates might minimize P excretion, the North American values would be more representative of maximum growth performance. Therefore, the objective of this study was to evaluate the impact of varying Ca:P ratios fed at two levels of STTD P:NE on growth performance, bone mineralization, and carcass characteristics of pigs from 11 to 129 kg.

Materials and Methods

General

The Kansas State University Institutional Animal Care and Use Committee approved the protocol used in this study (IACUC no. 4525). This study was conducted at a commercial research facility in southern Minnesota (Saint Peter, MN). The barn was naturally ventilated with completely slatted concrete flooring over deep pits for manure storage. Each pen (3.0 × 5.5 m) was equipped with a cup waterer and a 3-hole stainless steel dry-self feeder (Thorp Equipment, Thorp, WI) to provide *ad libitum* access to feed and water. Feed additions to each individual feeder were made and recorded by an electronic feeding system (FeedPro; Feedlogic Corp., Wilmar, MN).

Animals, Housing, and Diets

A total of 2,184 pigs (337 × 1050, PIC, Hendersonville, TN; initially 12.4 ± 0.17 kg) were used in a 143-d growth study with 26 pigs per pen and 14 replicates per treatment. Pens of pigs were blocked by BW with an equal number of barrows and gilts in each pen, and randomly assigned to 1 of 6 treatments in a randomized complete block design. Experimental diets were corn-soybean-meal-based and fed in 6 phases with targeted STTD P:NE and Ca:P ratios (Table 1). Treatments consisted of two levels of STTD P:NE, including a relatively high set of STTD P requirement estimates (Vier et al., 2019c) of 1.80, 1.62, 1.43, 1.25, 1.10, and 0.99 g STTD P/Mcal NE from 11 to 22, 22 to 40, 40 to 58, 58 to 81, 81 to 104, and 104 to 129 kg, respectively or a relatively low set of diets formulated to be 75% of the high diets and 3 analyzed Ca:analyzed P ratios: 0.90:1, 1.30:1, and 1.75:1. The P content of the diet was modified with the inclusion of monocalcium phosphate and the Ca content of the diet was modified with the inclusion of calcium carbonate. To achieve the 1.30:1 Ca:P ratio treatment, the 0.90:1 and 1.75:1 Ca:P ratio

diets for each STTD P/Mcal NE were blended using FeedPro (Feedlogic Corp., Wilmar, MN). Choice white grease was adjusted between the experimental diets to make them isocaloric. Experimental diets were fed from d 0 to 20, 21 to 44, 45 to 62, 63 to 87, 88 to 107, and 108 to 143 for phases 1 to 6, respectively (Tables 2 to 4). Prior to diet formulation, corn, soybean meal, and monocalcium phosphate were analyzed for Ca, P, and proximate analysis (Midwest Laboratories, Omaha, NE) in duplicate, and these values were used in diet formulation. Phytase (Quantum Blue 2G AB Vista, Marlborough, Wiltshire, UK) was included in all diets with the inclusion rate decreasing with subsequent dietary phase. For diet formulation, the NRC (2012) net energy values for ingredients were used, except for SBM. The energy value for SBM was 2,352 kcal/kg (88% the net energy value of corn). Complete diet samples were collected directly from the feeder. Three samples per dietary treatment were submitted for duplicate Ca analysis at the KSU soils lab in Manhattan, KS. For P and proximate analysis, two samples per dietary treatment were submitted for duplicate analysis at Midwest Laboratories in Omaha, NE. Standard procedures from AOAC (2006) were followed for analysis of moisture (Method 934.01), CP (Method 990.03), ether extract (Method 920.39), and Ca and P were analyzed using inductively coupled plasma spectroscopy-optical emission spectroscopy after wet ash sample preparation (Method 985.01 A, B, and D; Method 942.05). All treatment diets were fed in meal form and were manufactured at a commercial feed mill (Klossner Feed Mill, New Ulm, MN).

Pens of pigs were weighed, and feed disappearance was recorded approximately every 14 d to determine ADG, ADFI, and G:F. On d 124 and 131 of the study, the 5 heaviest pigs from each pen were selected and transported to a U.S. Department of Agriculture-inspected packing facility (JBS; Worthington, MN) for carcass data collection. At the completion of the study, the final individual weights were taken, and pigs were transported to the same commercial packing

plant. At the plant, hot carcass weight (HCW), loin depth, backfat, and percentage lean were measured. Loin depth and backfat were measured using an optical probe (SFK; Herlev, Denmark) at the 10th rib. Percentage lean was calculated from a plant proprietary equation. Carcass yield was calculated by dividing the individual pig HCW by the individual pig body weight obtained at the farm. A weighted average of the 1st (d 124), 2nd (d 131), and final marketing event (d 143) was used for carcass data.

Bone collection

On d 138 of the study, two pigs per pen (1 barrow and 1 gilt) that represented the average weight of pigs in the pen were sent to a commercial abattoir. The left front metacarpus was collected from each pig at the junction of the carpal bones and radius and was further dissected to obtain the third metacarpal within 24 h after slaughter. Bones were stored in a -20°C freezer until analysis. Each bone was analyzed for mineral content and density using a DXA machine [Hologic QDR4500A Dual Fan Beam X-Ray Bone Densitometer (DXA); Hologic, INC., Waltham, MA] and bone-breaking strength via the Instron machine (Instron 5569; NV Lab, Norwood, MA). For bone-breaking strength, the bones were held by two supports spaced 30 mm apart and were broken by a wedge lowered on the center of the bone at a speed of 100 mm/min and a maximal pressure of 5,000 kg. The force was measured by a pressure-sensitive cell, and peaks of maximum force were recorded.

Statistical analysis

Data were analyzed as 2 × 3 factorial for two-way ANOVA using the lmer function from the lme4 package in R (version 3.5.1 (2018-07-02), R Foundation for Statistical Computing, Vienna, Austria), with pen considered as the experimental unit, body weight as blocking factor, and treatment as a fixed effect with 14 replicates per treatment. The main effects of Ca:P ratio

and STTD P:NE, as well as their interactions, were tested. Preplanned linear and quadratic contrasts were used to determine the effects of increasing analyzed Ca:P ratios. For carcass characteristics, HCW was used as a covariate in the statistical model for back fat, loin depth, and percent lean. Results were considered significant at $P \leq 0.05$ and marginally significant at $0.05 < P \leq 0.10$.

Results

The analyzed dietary Ca and P concentrations were slightly greater than the formulated values but followed similar patterns as the designed treatment structures (Tables 2 to 4).

For final BW (Table 5), a Ca:P × STTD P:NE interaction (linear, $P < 0.001$) was observed. Increasing Ca:P from 0.90:1 to 1.75:1 linearly decreased ($P < 0.001$) final BW for pigs fed the Low PIC STTD P:NE levels, whereas BW tended to increase (linear, $P = 0.063$) as the Ca:P increased for pigs fed the High STTD P:NE levels recommendations.

For overall performance, a Ca:P × STTD P:NE interaction (linear, $P < 0.050$) was observed for ADG, ADFI, and G:F. For ADG, increasing Ca:P from 0.90:1 to 1.75:1 linearly decreased ($P < 0.05$) ADG for pigs fed the Low STTD P:NE levels, whereas ADG tended (linear, $P < 0.10$) to increase as the Ca:P increased for pigs fed the High STTD P:NE levels. Increasing Ca:P from 0.90:1 to 1.75:1 linearly reduced ($P < 0.05$) intake of pigs fed the Low P:NE levels while intake decreased from 0.90:1 to 1.30:1 and then increased from 1.30:1 to 1.75:1 (quadratic, $P < 0.05$) for pigs fed the High STTD P:NE levels. Increasing Ca:P from 0.90:1 to 1.75:1 tended to decrease (linear, $P < 0.10$) G:F for pigs fed the Low STTD P:NE concentrations. Increasing the Ca:P from 0.90:1 to 1.30:1 improved G:F and then G:F decreased (quadratic, $P < 0.10$) as the Ca:P increased from 1.30:1 to 1.75:1 for pigs fed the High STTD P:NE levels.

For bone characteristics, there was linear Ca:P × STTD P:NE interaction ($P < 0.05$) observed for mineral content, mineral density, and bone-breaking strength. Bone mineral content and density tended to decrease (linear, $P < 0.10$) with increasing Ca:P for pigs fed the Low STTD P:NE levels, but increased (linear, $P < 0.05$) for pigs fed the High STTD P:NE levels. Increasing Ca:P ratio did not influence bone-breaking strength for pigs fed Low STTD P:NE levels; however, increasing the Ca:P for pigs fed the High STTD P:NE recommendation increased (linear, $P < 0.05$) bone-breaking strength.

For carcass characteristics, there was a Ca:P × STTD P:NE interaction ($P < 0.05$) for HCW and carcass yield. Increasing the Ca:P for pigs fed Low STTD P:NE linearly reduced HCW ($P < 0.001$), while there was no difference for HCW between Ca:P ratios for pigs fed the High STTD P:NE ($P > 0.10$). Increasing the Ca:P for pigs fed the Low STTD P:NE concentration did not influence carcass yield; however, for pigs fed High STTD P:NE , increasing the Ca:P from 0.90:1 to 1.30:1 reduced carcass yield, with no difference as Ca:P further increased from 1.30:1 to 1.75:1 (quadratic, $P < 0.05$). Pigs fed the Low STTD P:NE levels had increased ($P < 0.05$) back fat and reduced ($P < 0.05$) carcass lean and HCW compared to the pigs fed High STTD P:NE. Increasing the Ca:P from 0.90:1 to 1.75:1 tended to increase loin depth (linear, $P < 0.10$).

Discussion

Calcium and phosphorus have multiple physiological roles in the body for growth and the development and maintenance of the skeletal system. When P is fed at or above the pigs' requirement, there is an isometric relationship between bone ash and protein, with both increasing at the same rate; whereas, when P is deficient in the diet, body protein increases while bone ash is reduced. This has led to a considerable amount of research being conducted to

determine the requirements for Ca and P for different phases of production (Misiura et al., 2020). The STTD P requirements estimated by NRC (2012) for moderate lean growth genotype pigs with the same weight ranges of the current study are 0.33, 0.31, 0.29, 0.26, 0.23, and 0.21%, which is close to 75% of the High STTD P levels used in the present study. However, commercial breeding stock suppliers establish nutrient requirements to maximize performance for their genetic lines for various dietary nutrients, some based on Vier et al. (2019a,c) data for STTD P and Ca:P ratios.

Research has shown that increasing the Ca in the diet can affect growth performance when dietary P is marginal or deficient (Merriman et al., 2017; Wu et al., 2018; Lagos et al., 2019a,b). In our experiment, we observed a linear reduction in ADG when the Ca:P ratio is widened from 0.90:1 to 1.75:1 for pigs fed the Low STTD P level, or 75% of the STTD P High estimates, but these detrimental effects were not observed when the high P levels were fed. The reduction in ADG can be attributed to a reduction in feed intake. A similar result was observed by González-Vega et al. (2016b) when pigs were fed with deficient P diets and increasing levels of STTD Ca. They also observed increased BW, ADG, ADFI, and G:F when P was fed at or above suggested requirement estimates, while STTD Ca percentage was increased. Vier et al. (2019a) observed that widening the Ca:P ratio above 1.25:1 decreased pig performance. However, in a second experiment, the addition of phytase to increase P concentrations improved growth performance as Ca:P ratio increased from 0.75:1 to 2.00:1. Including phytase in the diets in experiment 2 caused the analyzed Ca levels to be approximately 30% lower than analyzed Ca concentrations in experiment 1, which explains why the pigs in experiment 2 were able to tolerate a wider analyzed Ca:P ratio when phytase was included in the diet. These results

corroborate with the tendency we found for an increase in ADG as the Ca:P ratio widened when adequate P level is fed.

The ratio of dietary Ca:P affects the absorption of each mineral and is an important ratio to consider when formulating swine diets. In a study conducted by Stein et al. (2011), the authors found that increasing the Ca level of the diet from 0.33% to 1.04% linearly reduced the apparent total tract digestible of P from 56.9% to 46.2% when P was held constant in the diet. The observed response has been reported in research with phytase-supplemented diets (Selle et al., 2009) due to the formation of calcium-phytate complexes in the small intestine. Stein et al. (2011) suggested that the cause of reduced absorbed P from increased dietary Ca could be the result of the binding of the P by Ca in the intestinal tract to form Ca-P complexes. Vier et al. (2019a) conducted research to establish the analyzed Ca:P ratio of pigs from 26-to 127 kg. The STTD P was set for all treatments at 122% of the NRC (2012) estimates for pigs with moderate lean growth genotypes. The analyzed Ca:P ratios ranged from 0.75:1 to 2.00:1. The authors observed a significant quadratic response for final ADG, with ADG increasing when the analyzed Ca:P ratio widened from 0.75:1 to 1.50:1, and then ADG was reduced when the analyzed Ca:P ratio widened from 1.50:1 to 2.00:1.

For carcass characteristics, the results for HCW followed similar trends as ADG and final BW. Vier et al. (2019a) observed an increase in HCW when the analyzed Ca:P ratio was increased from 0.75:1 to 1.25:1 in one experiment and an increase up to 1.50:1 analyzed Ca:P ratio in another experiment. Increasing the ratio above 1.50:1 became detrimental to HCW when the STTD P level was fed at 122% of the NRC (2012) requirement estimates. Similar results were observed by Hanni et al. (2005), who observed an increase in HCW up to 1.50:1 total Ca:P ratio but reduced HCW when the Ca:P ratio was increased further when the P level was fed at the

requirement estimate based on the NRC (1998). In the present study, back fat increased, and loin depth decreased as the Ca:P ratio increased from 0.90:1 to 1.75:1. Conversely, other research has found that the ratio between Ca and P has no impact on back fat, loin depth, and percent lean (Liu et al., 1998; Hanni et al., 2005; Vier et al., 2019a). Interestingly, the present study observed similar results for carcass yield as Vier et al. (2019a). For our study, widening the Ca:P ratio when the recommended STTD P levels were fed decreased carcass yield. Similarly, in the Vier et al. (2019a) and Bertram (1995) studies, increasing the Ca:P ratio decreased yield. There is no clear explanation as to why increasing the Ca:P affects carcass yield and previous research did not observe similar results (Liu et al., 1998; Hanni et al., 2005). One explanation for the reduction in carcass yield could be the increase in bone mineralization, but further research is warranted to understand this response.

The requirement for bone mineralization is greater than for growth performance (Vier et al., 2019a,c). For proper bone mineralization, adequate concentrations of both Ca and P in the diets are needed (Crenshaw, 2001). Ca and P accumulate in bone as crystals in the form of hydroxyapatite ($Ca_{10}(PO_4)_6$) consisting of Ca and P in a constant 2.2:1 ratio. When P is deficient in the diet and the Ca content is adequate, P absorption is reduced, ultimately leading to poor bone mineralization (Sorensen et al., 2018, Lautrou et al., 2020). When Ca is elevated in diets that are deficient in P by reducing the P absorption due to the formation of insoluble Ca:P complexes. This is why the ratio between Ca and P is an important factor to consider in diet formulation (Lautrou et al., 2020). In the current study, we observed a reduction in bone mineral content, bone mineral density, and bone-breaking strength when the Ca:P ratio was widened when feeding the diets containing 75% of the High STTD P levels. In diets with High levels of STTD P, widening the Ca:P ratio increased bone mineral content and bone mineral density.

When increasing the STTD P above requirement, the pig maximizes protein deposition, allowing excess P to be utilized for bone mineralization (Létourneau-Montminy et al., 2015) due to the isometric relationship between body ash and protein deposition in pigs.

In conclusion, increasing the STTD P level to those suggested by Vier et al. (2019b,c) increased growth performance and bone mineralization compared to feeding 75% of the STTD P in the high diets. Results from this study indicate that feeding P levels below recommendations can be detrimental to growth performance, carcass characteristics, and bone mineralization, especially when the analyzed Ca:P ratio is widened from 0.90:1 to 1.75:1. Therefore, it is important to adjust the Ca content in the diet, especially if the STTD P is fed at marginal levels.

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Table 1.1. STTD P:NE, g/Mcal, ratio (STTD P) at each dietary phase

Weight range, kg	Low	High[1]
11 to 22	1.35 (0.34%)	1.80 (0.45%)
22 to 40	1.22 (0.30%)	1.62 (0.40%)
40 to 58	1.07 (0.27%)	1.43 (0.36%)
58 to 81	0.94 (0.23%)	1.25 (0.31%)
81 to 104	0.83 (0.20%)	1.10 (0.27%)
104 to 129	0.74 (0.19%)	0.99 (0.25%)

[1]Vier et al. (2019a)

Table 1.2. Basal diet composition (as-fed basis)[1]

	Phase 1				Phase 2			
STTD P:NE[2]:	1.35		1.80		1.22		1.62	
Ca:P ratio:	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1
Ingredients, %[3]								
Corn	67.50	65.85	66.35	64.37	72.20	70.83	71.26	69.43
Soybean meal, 46.5% CP	29.40	29.67	29.55	29.72	25.0	25.10	25.06	25.20
Choice white grease	0.50	1.05	0.88	1.58	0.55	1.00	0.85	1.50
Calcium carbonate	0.45	1.34	0.46	1.62	0.47	1.29	0.48	1.53
Monocalcium phosphate	0.23	0.23	0.86	0.86	0.13	0.13	0.69	0.69
Salt	0.60	0.60	0.60	0.60	0.55	0.55	0.55	0.55
L-Lys-HCl	0.48	0.48	0.48	0.48	0.45	0.45	0.45	0.45
DL-Met	0.20	0.20	0.20	0.20	0.16	0.16	0.16	0.16
L-Thr	0.24	0.23	0.24	0.23	0.21	0.21	0.21	0.21
L-Trp	0.04	0.04	0.04	0.04	0.03	0.03	0.03	0.03
L-Val	0.09	0.09	0.09	0.09	0.08	0.08	0.08	0.08
VTM[4]	0.20	0.20	0.20	0.20	0.17	0.17	0.17	0.17
Phytase[5]	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Calculated analysis								
Standardized ileal digestible amino acids, %								
Lys	1.28	1.28	1.28	1.28	1.15	1.15	1.15	1.15
Ile:Lys	55	55	55	55	55	55	55	55
Leu:Lys	117	117	117	116	122	122	122	121
Met:Lys	36	36	36	36	35	35	35	35
Met and Cys:Lys	57	57	57	57	57	57	57	57
Thr:Lys	65	65	65	65	65	65	65	65
Trp:Lys	19.0	19.0	19.0	19.0	18.2	18.2	18.2	18.2
Val:Lys	68	68	68	68	68	68	68	68

CP, %	19.4	19.4	19.4	19.3	17.7	17.6	17.6	17.5
NE, kcal/kg	2,571	2,571	2,570	2,570	2,590	2,588	2,588	2,589
Ca, %	0.37	0.72	0.48	0.93	0.34	0.66	0.44	0.85
P, %	0.41	0.41	0.54	0.53	0.38	0.38	0.49	0.48
STTD P with phytase, %	0.32	0.32	0.43	0.43	0.30	0.30	0.39	0.39
Ca:P	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1
Analyzed composition, %								
DM	86.39	86.4	86.21	86.74	86.62	86.42	86.66	86.87
CP	17.2	18.6	17.8	17.1	16.2	17.0	16.4	16.8
Fat	3.14	3.67	3.63	4.03	3.09	3.24	3.32	3.92
Ca	0.41	0.78	0.55	1.05	0.41	0.69	0.52	0.82
P	0.43	0.43	0.55	0.56	0.40	0.37	0.50	0.50
Analyzed Ca:analyzed P	0.96:1	1.82:1	1.01:1	1.87:1	1.03:1	1.85:1	1.03:1	1.65:1

[1]Experimental diets were fed from 11 to 22 kg and 22 to 40 kg for phases 1 and 2, respectively.

[2]Phosphorus levels were based on PIC Nutrition and Feedig Guidelines (2021).

[3]Corn, soybean meal, choice white grease, and calcium carbonate were adjusted to keep diets isocaloric, with similar CP and SID Lys concentrations, while maintaining the intended Ca:P ratio.

[4]Premix provided per kg of premix: 2,500,000 IU vitamin A; 500,000 IU vitamin D3; 25,352.7 IU vitamin E; 1,543.2 mg vitamin K; 18.9 mg vitamin B12; 25,352.7 mg niacin; 14,109.3 mg pantothenic acid; 3,968.2 mg riboflavin; 65,000 mg of Fe from ferrous sulphate; 65,000 mg of Zn from zinc sulfate; 25,000 mg of Mn from manganous oxide; 9,000 mg of Cu from copper sulfate; 325 mg of I; and 150 mg of Se from microgran SE.

[5]Quantum Blue 2G (AB Vista, Marlborough, Wiltshire, UK) provided 500 units of phytase (FTU/kg of diet) with the assumed release of 0.13% STTD P.

Table 1.3. Basal diet composition (as-fed basis)[1]

	Phase 3				Phase 4			
STTD P:NE[2]:	1.07		1.43		0.94		1.25	
Ca:P ratio:	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1
Ingredients, %[3]								
Corn	76.74	75.52	75.85	74.24	81.30	80.13	80.54	79.00
Soybean meal, 46.5% CP	20.40	20.49	20.46	20.58	16.21	16.30	16.27	16.38
Choice white grease	0.85	1.25	1.15	1.70	0.60	1.00	0.85	1.40
Calcium carbonate	0.48	1.21	0.49	1.43	0.50	1.19	0.50	1.39
Monocalcium phosphate	---	---	0.51	0.51	---	---	0.45	0.45
Salt	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
L-Lys-HCl	0.42	0.42	0.42	0.42	0.38	0.38	0.38	0.38
DL-Met	0.13	0.13	0.13	0.13	0.09	0.09	0.09	0.09
L-Thr	0.18	0.18	0.18	0.18	0.15	0.15	0.15	0.15
L-Trp	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
L-Val	0.06	0.06	0.06	0.06	0.04	0.04	0.04	0.04
VTM[4]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Phytase[5]	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02
Calculated analysis								
Standardized ileal digestible amino acids, %								
Lys	1.02	1.02	1.02	1.02	0.88	0.88	0.88	0.88
Ile:Lys	55	55	55	55	56	56	56	56
Leu:Lys	128	128	128	127	138	137	137	136
Met:Lys	35	35	35	35	34	33	33	33
Met and Cys:Lys	57	57	57	57	58	58	58	57
Thr:Lys	65	65	65	65	65	65	65	65
Trp:Lys	18.2	18.2	18.2	18.2	18.5	18.5	18.5	18.5
Val:Lys	68	68	68	68	69	69	69	69

CP, %	15.8	15.8	15.8	15.7	14.1	14.1	14.1	14.0
NE, kcal/kg	2,621	2,619	2,620	2,619	2,622	2,621	2,620	2,621
Ca, %	0.30	0.58	0.39	0.76	0.29	0.56	0.37	0.71
P, %	0.34	0.33	0.44	0.43	0.32	0.32	0.41	0.41
STTD P with phytase, %	0.26	0.26	0.35	0.35	0.23	0.23	0.31	0.31
Ca:P	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1
Analyzed composition, %								
DM	85.46	85.91	85.83	85.89	86.24	86.18	86.23	86.50
CP	14.7	15.1	14.6	14.8	12.5	13.8	13.2	12.5
Fat	4.76	3.51	3.51	4.08	3.70	3.82	3.55	4.19
Ca	0.31	0.56	0.41	0.74	0.35	0.57	0.33	0.66
P	0.34	0.41	0.48	0.42	0.31	0.30	0.38	0.37
Analyzed Ca:analyzed P	0.92:1	1.36:1	0.85:1	1.77:1	1.05:1	1.83:1	0.73:1	1.15:1

[1]Experimental diets were fed from 40 to 58 kg and 58 to 81 kg for phases 3 and 4, respectively.

[2]Phosphorus levels were based on PIC Nutrition and Feeding Guidelines (2021).

[3]Corn, soybean meal, choice white grease, and calcium carbonate were adjusted to keep diets isocaloric, with similar CP and SID Lys concentrations, while maintaining the intended Ca:P ratio.

[4]Premix provided per kg of premix: 2,500,000 IU vitamin A; 500,000 IU vitamin D3; 25,352.7 IU vitamin E; 1,543.2 mg vitamin K; 18.9 mg vitamin B12; 25,352.7 mg niacin; 14,109.3 mg pantothenic acid; 3,968.2 mg riboflavin; 65,000 mg of Fe from ferrous sulphate; 65,000 mg of Zn from zinc sulfate; 25,000 mg of Mn from manganous oxide; 9,000 mg of Cu from copper sulfate; 325 mg of I; and 150 mg of Se from microgran SE.

[5]Quantum Blue 2G (AB Vista, Marlborough, Wiltshire, UK) provided 500 units of phytase (FTU/kg of diet) with the assumed release of 0.13% STTD P for phase 3. For phase 4, 400 units of phytase (FTU/kg diet) with the assumed release of 0.11% STTD P.

Table 1.4. Basal diet composition (as-fed basis)[1]

	Phase 5				Phase 6			
STTD P:NE[2]:	0.83		1.10		0.74		0.99	
Ca:P ratio:	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1
Ingredients, %[3]								
Corn	84.23	83.07	83.53	82.14	84.70	83.55	84.09	82.59
Soybean meal, 46.5% CP	13.39	13.47	13.44	13.54	12.82	12.91	12.87	13.05
Choice white grease	0.65	1.05	0.90	1.35	0.95	1.35	1.15	1.65
Calcium carbonate	0.50	1.17	0.51	1.35	0.50	1.17	0.51	1.33
Monocalcium phosphate	---	---	0.39	0.39	---	---	0.36	0.36
Salt	0.56	0.56	0.56	0.56	0.56	0.56	0.56	0.56
L-Lys-HCl	0.32	0.32	0.32	0.32	0.23	0.23	0.23	0.23
DL-Met	0.04	0.04	0.04	0.04	---	---	---	---
L-Thr	0.11	0.11	0.11	0.11	0.06	0.06	0.06	0.06
L-Trp	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02
L-Val	0.03	0.03	0.03	0.03	---	---	---	---
VTM[4]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Phytase[5]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Calculated analysis								
Standardized ileal digestible amino acids, %								
Lys	0.76	0.76	0.76	0.76	0.68	0.68	0.68	0.68
Ile:Lys	58	58	58	58	64	64	64	64
Leu:Lys	150	149	150	149	166	165	166	164
Met:Lys	31	31	31	31	29	29	29	28
Met and Cys:Lys	58	57	58	57	58	58	58	57
Thr:Lys	65	65	65	65	65	65	65	65
Trp:Lys	18.7	18.7	18.7	18.7	19.1	19.1	19.1	19.1
Val:Lys	72	72	72	72	76	76	76	75

CP, %	12.9	12.9	12.9	12.8	12.5	12.5	12.5	12.5
NE, kcal/kg	2,631	2,631	2,632	2,629	2,645	2,644	2,644	2,644
Ca, %	0.28	0.54	0.35	0.67	0.28	0.54	0.34	0.66
P, %	0.31	0.31	0.39	0.38	0.31	0.31	0.38	0.38
STTD P with phytase, %	0.21	0.21	0.28	0.27	0.19	0.19	0.25	0.25
Ca:P	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1	0.90:1	1.75:1
Analyzed composition, %								
DM	85.6	85.58	85.77	85.45	85.08	85.61	85.19	85.6
CP	11.4	12.2	12.9	12.4	11.3	11.8	11.4	11.6
Fat	3.54	3.98	3.74	4.03	3.44	3.92	3.18	4.33
Ca	0.28	0.57	0.41	0.61	0.26	0.67	0.43	0.64
P	0.32	0.32	0.41	0.41	0.34	0.29	0.40	0.43
Analyzed Ca:analyzed P	0.89:1	1.79:1	0.99:1	1.49:1	0.75:1	2.32:1	1.08:1	1.50:1

[1]Experimental diets were fed from 81 to 104 kg and 104 to 129 kg for phases 5 and 6, respectively.

[2]Phosphorus levels were based on PIC Nutrition and Feeding Guidelines (2021).

[3]Corn, soybean meal, choice white grease, and calcium carbonate were adjusted to keep diets isocaloric, with similar CP and SID Lys concentrations, while maintaining the intended Ca:P ratio.

[4]Premix provided per kg of premix: 2,500,000 IU vitamin A; 500,000 IU vitamin D3; 25,352.7 IU vitamin E; 1,543.2 mg vitamin K; 18.9 mg vitamin B12; 25,352.7 mg niacin; 14,109.3 mg pantothenic acid; 3,968.2 mg riboflavin; 65,000 mg of Fe from ferrous sulphate; 65,000 mg of Zn from zinc sulfate; 25,000 mg of Mn from manganous oxide; 9,000 mg of Cu from copper sulfate; 325 mg of I; and 150 mg of Se from microgran SE.

[5]Quantum Blue 2G (AB Vista, Marlborough, Wiltshire, UK) provided 290 units of phytase (FTU/kg of diet) with the assumed release of 0.09% STTD P for phase 5. For phase 6, 210 units of phytase (FTU/kg of the diet) were used with the assumed release of 0.07% STTD P.

Table 1.5. The interactive effect of analyzed Ca:P ratio and current or 75% of the PIC STTD P:NE requirement on wean to finish pig performance[1]

								P =					
STTD P:NE:	Low			High				Interaction		Within Low		Within High	
Ca:P:	0.90	1.30	1.75	0.90	1.30	1.75	SEM	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
Body weight, kg													
d 0	12.4	12.4	12.4	12.4	12.4	12.4	0.17	0.811	0.055	0.796	0.178	0.552	0.167
d 143	130.5	127.5	125.5	132.6	134.3	134.9	0.90	0.001	0.422	0.001	0.580	0.063	0.559
Overall													
ADG, kg	0.87	0.85	0.84	0.89	0.89	0.90	0.006	0.001	0.767	0.001	0.391	0.092	0.658
ADFI, kg	2.04	1.99	2.00	2.06	2.03	2.07	0.014	0.017	0.980	0.008	0.042	0.477	0.046
G:F, g/kg	428	429	423	433	439	436	3.6	0.041	0.744	0.071	0.157	0.268	0.062
P intake g/d	4.56	4.49	4.51	6.20	6.09	6.22	0.045	0.246	0.187	0.215	0.257	0.688	0.004
P intake g/ kg gain	5.45	5.46	5.52	7.12	7.09	7.09	0.071	0.168	0.897	0.139	0.615	0.636	0.750
Bone characteristics[2]													
Mineral content, g	3.49	3.49	3.20	4.07	4.27	4.53	0.140	0.003	0.494	0.094	0.391	0.013	0.915
Mineral density, g/cm	0.28	0.28	0.26	0.30	0.31	0.33	0.007	0.001	0.591	0.068	0.476	0.002	0.966
Breaking strength, kg	297	284	282	318	333	362	12.09	0.010	0.982	0.344	0.677	0.008	0.653
Carcass characteristics[3]													
Count, n	325	320	311	329	342	333	---	---	---	---	---	---	---
HCW, kg	98.4	96.0	95.0	100.2	99.9	100.4	0.58	0.001	0.609	0.001	0.179	0.726	0.530
Yield, %	74.4	74.5	74.3	74.7	74.2	74.3	0.13	0.399	0.026	0.471	0.364	0.057	0.025
Back fat, mm	18.8	18.4	19.4	18.1	17.9	17.9	0.25	0.100	0.174	0.055	0.025	0.699	0.721
Loin depth, mm	68.5	68.0	67.5	68.8	68.3	68.4	0.41	0.460	0.652	0.080	0.921	0.449	0.641
Lean, %	55.6	55.8	55.1	56.1	56.1	56.1	0.18	0.104	0.195	0.032	0.059	0.908	0.941

[1]A total of 2,184 pigs were used in a 143-d study to evaluate the interactive effects of different levels of analyzed Ca:P ratio in diets formulated to the current PIC STTD P:NE or 75% of the current PIC STTD P:NE. There were 14 pens per treatment, with 26 pigs per pen. Phase 1, 2, 3, 4, 5 and 6 were fed from d 0 to 20, d 21 to 44, d 45 to 62, d 63 to 87, d 88 to 107, and d 108 to 143, respectively.

[2]The left 3rd metacarpal was used for bone characteristics analysis. The results from bone mineral content and bone mineral density are from analyzing the bones with a DXA machine [Hologic QDR4500A Dual Fan Beam X-Ray Bone Densitometer (DXA); Hologic, INC., Waltham, Massachusetts, USA].

[3]Pigs were marketed on d 124, 131, and dumped on d 143. On d 124 and 131, the 5 heaviest pigs were removed from the pen and marketed. On d 143, the remaining pigs in the pen were marketed. The carcass data is a weighted average of the 1st cut, 2nd cut, and dump. Hot carcass weight was used as a covariate in the statistical model for back fat, loin depth, and percent lean.

Table 1.6. Main effect of Ca:P ratios and STTD P:NE ratio on wean-to finish-pig growth performance[1]

	Ca:P				*P* =		STTD P:NE			
Item	0.90	1.30	1.75	SEM	Linear	Quadratic	Low	High	SEM	*P* =
Body weight, kg										
d 0	12.4	12.4	12.4	0.01	0.546	0.982	12.4	12.4	0.01	0.322
d 143	131.5	130.9	130.2	0.84	0.137	0.982	127.8	133.9	2.06	< 0.001
Overall										
ADG, kg	0.88	0.87	0.87	0.01	0.070	0.358	0.86	0.89	0.011	< 0.001
ADFI, kg	2.05	2.01	2.03	0.01	0.157	0.005	2.01	2.05	0.027	< 0.001
G:F, g/kg	430	434	430	2.0	0.615	0.022	427	436	4.8	< 0.001
P intake g/d	5.38	5.29	5.36	0.039	0.550	0.004	4.52	6.17	0.037	< 0.001
P intake g/kg gain	6.28	6.28	6.31	0.066	0.473	0.562	5.48	7.10	0.065	< 0.001
Bone characteristics[2]										
Mineral content, g	3.78	3.88	3.87	0.088	0.503	0.593	3.39	4.29	0.073	< 0.001
Mineral density, g/cm	0.29	0.30	0.30	0.004	0.279	0.634	0.27	0.31	0.004	< 0.001
Breaking strength, kg	308	308	322	8.1	0.194	0.541	289	338	6.5	< 0.001
Carcass characteristics[3]										
HCW, kg	99.3	98.0	97.7	0.49	0.002	0.165	96.5	100.2	1.20	< 0.001
Yield, %	74.6	74.3	74.3	0.10	0.065	0.331	74.4	74.4	0.08	0.872
Back fat, mm	18.4	18.2	18.7	0.23	0.263	0.064	18.9	18.0	0.57	< 0.001
Loin depth, mm	68.7	68.2	68.0	0.50	0.075	0.555	68.0	68.5	0.96	0.104
Lean, %	55.9	56.0	55.6	0.14	0.141	0.162	55.5	56.1	0.12	< 0.001

[1]A total of 2,184 pigs were used in a 143-d study to evaluate the interactive effects of different levels of analyzed Ca:P ratio in diets formulated to the current PIC STTD P:NE or 75% of the current PIC STTD P:NE. There were 42 pens per treatment for the main effect of PIC STTD P:NE and 28 pens per treatment for the main effect of Ca:P, with 26 pigs per pen. Phase 1, 2, 3, 4, 5 and 6 were fed from d 0 to 20, d 21 to 44, d 45 to 62, d 63 to 87, d 88 to 107, and d 108 to 143, respectively.

[2]The left 3rd metacarpal was used for bone characteristic analysis.

[3]Pigs were marketed on d 124, 131, and dumped on d 143. On d 124 and 131, the 5 heaviest pigs were removed from the pen and marketed. On d 143, the remaining pigs in the pen were marketed. The carcass data is a weighted average of the 1st cut, 2nd cut, and dump. Hot carcass weight was used as a covariate in the statistical model for back fat, loin depth, and percent lean.

Chapter 2 - The effect of bone and analytical methods on the assessment of bone mineralization response to dietary phosphorus, phytase, and vitamin D in nursery pigs

Abstract

A total of 360 pigs (DNA 600 × 241, DNA; initially 11.9 ± 0.56 kg) were used in a 28-d trial to evaluate the effects of different bones and analytical methods on the assessment of bone mineralization response to dietary P, vitamin D, and phytase in nursery pigs. Pens of pigs (6 pigs per pen) were randomized to 6 dietary treatments in a randomized complete block design with 10 pens per treatment. Dietary treatments were designed to create differences in bone mineralization and included: 1) 0.19% STTD P (deficient), 2) 0.33% STTD P (NRC (2012) requirement) using monocalcium phosphate, 3) 0.33% STTD P including 0.14% release from phytase (Ronozyme HiPhos 2700, DSM Nutritional Products, Parsippany, NJ), 4) 0.44% STTD P using monocalcium phosphate, phytase, and no vitamin D, 5) diet 4 with vitamin D (1,653 IU/kg), 6) diet 5 with an additional 50 µg/kg of 25(OH)D_3 (HyD, DSM Nutritional Products, Parsippany, NJ) estimated to provide an additional 2,000 IU/kg of vitamin D3. After 28 d on feed, eight pigs per treatment were euthanized for bone (metacarpal, 2nd rib, 10th rib, and fibula), blood, and urine analysis. The response to treatment for bone density and ash was dependent upon the bone analyzed (treatment × bone interaction for bone density, $P = 0.044$; non-defatted bone ash, $P = 0.060$; defatted bone ash, $P = 0.068$). Thus, the response related to dietary treatment differed depending on which bone (metacarpal, fibula, 2nd rib, or 10th rib) was measured. 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 25(OH)D_3. Pigs fed 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 bone analyzed. Differences in bone density and ash in response to P and vitamin D were most apparent with fibulas and 2nd ribs. There were apparent differences in the bone ash percentage between defatted and non-defatted bone. However, differences between the treatments remain consistent regardless of the analytic procedure. For histopathology, 10th ribs were more sensitive than 2nd ribs or fibulas for detection of lesions.

Introduction

Lameness is defined as impaired movement or deviation from normal gait (Pairis et al., 2011). There are many factors that can contribute to lameness, including but not limited to: infectious disease, genetic and conformational anomaly, and toxicity that affects the bone, muscle, and nervous systems. Metabolic bone disease is another cause of lameness in swine production and can be caused by inappropriate levels of essential vitamins or minerals.

A deficiency in bone mineralization results in weak bones that can more easily fracture when force is applied to them. Appropriate mineral supply is essential for bone structure to become sufficiently strong and to ensure optimal bone expansion and bone mass accumulation during growth. It is important to have a continuous supply of calcium (Ca) and phosphorus (P) for the formation of hydroxyapatite (Calvo, 1993). Several studies have established the requirement estimates for Ca and P in growing pigs (NRC, 2012) including recent investigations using modern genotypes (Vier et al., 2019a,b; Wu et al., 2019). Currently, many nutritionists

feed greater Ca and P concentrations (120 to 125% of NRC, 2012) in swine diets to maximize growth and bone mineralization (Vier et al., 2019a,b).

The diagnosis of metabolic bone disease can be made by radiographic imaging and/or assessment of pathological changes on the articular surfaces, synovium, and growth plates as well as ancillary assays to evaluate endogenous molecules that regulates bone metabolism and the bone integrity. These diagnostic tests include measurements of serum concentrations of Ca, P, and vitamin D due to the potential involvement of vitamin D deficiency with metabolic bone disease. Over the years, there have been methods established to measure bone mineralization, including bone ash. However, bone ash can differ whether analyzed with the defatted vs non-defatted procedure (Wensley et al., 2020).

The effects of Ca and P deficiency are similar to those of vitamin D deficiency and include reduced growth and poor bone mineralization. The current standard for determining vitamin D status in swine has been circulating 25-hydroxycholecalciferol [25(OH)D_3], however, there has been recent interest in evaluating the activated form of 25(OH)D_3. Another ante-mortem, non-invasive diagnostic sample which may have utility in evaluating Ca and P homeostasis is urine. Urinary Ca and P can be analyzed, and when incorporated as a ratio to creatinine to standardize for potential differences in glomerular filtration rate, this has been shown to be a promising non-invasive measure for the evaluation of calcium, phosphorous, and potentially vitamin D deficiencies (Hagemoser et al., 2000).

There is a great deal of literature available regarding the concentrations of Ca and P necessary to optimize growth performance and evidence related to bone ash, but less information comparing the mineralization between multiple bones, different bone ash techniques, or relating those results to histology and serum chemistry. Therefore, our objective was to create wide

variations in dietary Ca, P, and vitamin D status in order to understand the range of biological measurements that would be expected when analyzing different bones with different analytical procedures to understand which techniques and bones are most appropriate for diagnostic analysis.

Materials and Methods

General

The Kansas State University Animal Care and Use Committee approved the protocol used in this study (IACUC no. 4485). This study was conducted at the Kansas State University Swine Teaching and Research Center in Manhattan, KS. Each pen contained a 4-hole, dry self-feeder and nipple waterer for ad libitum access to feed and water. Pens were 1.22 by 1.22 m providing approximately 0.30 m^2 per pig.

Animals, Housing, Diets

A total of 360 pigs [DNA 600 (Duroc) × DNA 241 (Landrace × Yorkshire), DNA Genetics, Columbus, Nebraska; initially 11.9 ± 0.56 kg] were used in a 28-d trial. Pigs were weaned at approximately 19 days of age and placed in pens of 6 pigs based on initial weight and sex. Pigs were fed common phase 1 then 2 diets from d 0 to 10 and d 10 to 24, respectively. Dietary Ca, P, and vitamin D, levels fed during phases 1 and 2 were at or above the NRC (2012) requirement estimates. On d 24 post weaning (considered d 0 of the feeding trial), pens of pigs were blocked by body weight (BW) and randomly allotted to 1 of 6 dietary treatments. There were 6 pigs per pen (3 barrows and 3 gilts) and 10 pens per treatment. The dietary treatments were: 1) 0.19% standardized total tract digestibility (STTD) P (deficient), 2) 0.33% STTD P (NRC, 2012 requirement estimate) using monocalcium phosphate, 3) 0.33% STTD P including 0.14% release from phytase (Ronozyme HiPhos 2700, DSM Nutritional Products, Parsippany,

NJ), 4) 0.44% STTD P using monocalcium phosphate, phytase, and no vitamin D, 5) diet 4 with 1,653 IU/kg vitamin D_3, 6) diet 5 with 50 µg/kg of 25(OH)D_3 (HyD, DSM Nutritional Products, Parsippany, NJ) estimated to provide an additional 2,000 IU/kg of vitamin D3. All diets were manufactured in meal form at Hubbard Feeds in Beloit, KS (Table 1). Treatment 1 had a STTD P level of 0.19%, which was 57% of the NRC (2012) requirement estimate for pigs in this weight range. Treatments 2 and 3 had a STTD P level of 0.33%, which was 100% of the NRC (2012) requirement estimate for this weight range. Treatments 4, 5, and 6 had a STTD P level of 0.44%, which was approximately 133% of the NRC (2012) requirement estimate for this weight range, similar to the levels found by Vier et al. (2019a) to maximize growth performance and bone mineralization. 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% from the phytase. 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 providing 1,653 IU/kg of vitamin D_3. 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 equivalency from 50 µg/kg of 25(OH)D_3. All diets were formulated to a total Ca:total P ratio of 1.20:1. Experimental diets were fed for 28 d.

Growth performance and sample collection

On d 0 of the study, 8 pigs were randomly selected for serum analysis of Ca, P, and 25(OH)D_3 to establish the baseline levels before feeding dietary treatments. Throughout the 28-d experiment, pig and feeder weights were measured every 7 d to determine average daily gain (ADG), average daily feed intake (ADFI), and gain-to-feed (G:F). On d 28, 1 pig from 8 pens per treatment was euthanized and used for the analysis of bones, blood, and urine. A blood sample

was taken from the jugular vein from each pig prior to euthanasia. Blood samples (10 mL) were centrifuged (3000 × *g*, 30 min) within 2 h after collection and serum was stored at -20˚C until analysis. Serum was split into three, 2 mL aliquots and analyzed for Ca and P via large animal serum chemistry profile (Iowa State University Veterinary Diagnostic Lab), $25(OH)D_3$, and $1,25-(OH)_2D_3$ (Heartland Assays, Ames, IA). A 10 mL urine sample was collected shortly after euthanasia via cystocentesis using a 20 gauge × 2.5 cm needle and a 12 mL syringe, then frozen at -20˚C until analyzed for Ca, P, and creatinine (Iowa State University Veterinary Diagnostic Lab).

Bone measurements

The metacarpal, 2nd, 3rd, 5th, 7th, and 10th rib, and fibula from both sides of each were collected for a total of 8 bones per pig. The left fibula, left 2nd, and left 10th rib were processed for histological examination. Briefly, the bones were fixed in 10% neutral buffered formalin for at least 24 h and decalcified using DeltaCAL (Delta Medical, Inc., Aurora, IL, USA) for at least 4-6 h. The costochondral junction and the body of the 2nd and 10th rib, and the head of the left fibula were embedded in paraffin blocks, sectioned at 4 µm thickness, and stained with hematoxylin and eosin. Microscopic examination was performed by blinded assessment from three pathologists at Iowa State University Veterinary Diagnostic Laboratory. Bones were scored for failure of endochondral ossification of the physis and microscopic fractures (infarctions). Histological grading of each bone consisted of physis grading, infarctions/fracture lines, cortical bone thickness measurements, and trabeculae bone thickness. The physis grading consisted of: 0) no histologic findings of significance, 1) multifocal small tongues or islands of viable cartilage extend into the primary spongiosa, 2) moderate-sized tongues or islands of viable cartilage extend into the primary and secondary spongiosa, and 3) extensive areas of the zone of

hypertrophy are expanded and extend down into the primary and secondary spongiosa. The infarctions/fracture scoring consisted of: 0) no evidence of infarctions, 1) small infarctions covering < 50% of the diameter of the bone, 2) large infarctions covering > 50% of the diameter of the bone, and 3) cortical fracture. Cortical bone thickness measurements were taken at 10 locations in the diaphysis. Trabeculae bone thickness was taken at 10 locations in the diaphysis, 4 cm distal to the physis, and 1 cm in from the edge of the cortical bone.

The metacarpal, 2nd rib, 10th rib, and fibulas were analyzed for dual-energy X-ray absorptiometry (DEXA) scan, bone density via Archimedes principle, Instron bone-breaking strength, bone ash (defatted and non-defatted), and bone Ca and P. The 3rd, 5th, and 7th ribs were analyzed for bone density and non-defatted bone ash. The leftover extraneous soft tissues and cartilage were removed from the bones prior to assessment. Bone mineral content and bone mineral density were determined using dual-energy X-ray absorptiometry (DXA) scans of each bone (Hologic QDR4500A Dual Fan Beam X-Ray Bone Densitometer (Hologic, INC., Waltham, MA)). The machine is routinely used to measure animal bones, with an average run time of 2 minutes per scan, with the capability of measuring 4 unique bones per scan. Archimedes principle was used for bone density. Briefly a dry bone weight was collected, then bones were submerged in ultra-purified water under a negative pressure vacuum with 1.06 kg per cubic centimeter for a minimum of 4 h. Bones were then weighed while suspended in a vessel of ultra-purified water, and the weight used to calculate bone density. Bone-breaking strength was determined with an Instron (Instron 5569, NV Lab, Norwood, MA). Briefly each bone was held by two supports spaced 30 mm apart and were broken by a wedge lowered on the center of the bone at a speed of 100 mm per min and a maximal pressure of 5,000 kg. The force was measured by a pressure-sensitive cell, and peaks of maximum force were recorded. For bone ash, both the

defatted and the non-defatted methods were used (Wensley, et al., 2020). For defatted bone ash, all bones were placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105˚C for 7 d and then ashed in a muffle furnace at 600˚C for 24 h. For non-defatted bone ash, all bones were dried at 105˚C for 7 d and then ashed in a muffle furnace at 600˚C for 24 h. These methods were used to determine total bone ash weight and percentage ash relative to dried bone weight. For bone Ca and P, a portion of the residual defatted bone ash (0.05 g) was placed in a tube with nitric acid and incubated at 60˚C for 6 h. The solution was then diluted using ultra-purified water and Ca (AOAC 985.01, 2006) and P (AOAC 985.01, 2006) quantity measured by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Concentrations of Ca and P (%) were calculated as a percentage of the total bone ash.

Diet chemical analysis

Three diet samples per treatment were submitted for duplicate Ca (AOAC 985.01, 2006) and P (AOAC 985.01, 2006) analysis at the Kansas State University Soils Lab in Manhattan, KS.

Statistical analysis

For growth performance, blood, and urine analysis, data were analyzed as a randomized complete block design using a one-way ANOVA with pen considered the experimental unit, treatment as a fixed effect, and weight block as a random effect. For the statistical analysis for bone measurements and histopathology, data were analyzed as a completely randomized design for two-way ANOVA with pen considered the experimental unit, treatment, bone, and the associated interactions included as fixed effects and pen as a random effect. A Tukey multiple comparison adjustment was used to control Type I error rate. All

growth and analytical data were analyzed using the lmer function from the lme4 package in R from the stats package in R version 3.5.1 (2018-07-02).

Results

Growth performance

On d 28, pigs fed 0.19% STTD P had decreased ADG and final body weight ($P < 0.05$) compared to pigs fed 0.33% and 0.44% STTD P, with no differences observed between the high P treatments ($P > 0.05$ Table 2). 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 G:F 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 and urine analysis

Initial mean serum concentrations on d 0 were 14.6 ng/mL for 25(OH)D_3, 10.5 mg/dL for Ca, and 9.3 mg/dL for P. For serum Ca on d 28, there was a tendency for a difference between treatments ($P = 0.096$), but no significant mean separation was observed using Tukeys adjustment. For serum P on d 28, pigs fed 0.19% STTD P had decreased concentrations compared to pigs fed 0.33% and 0.44% STTD P ($P < 0.05$). Pigs fed no dietary vitamin D had the lowest ($P < 0.05$) circulating 25(OH)D_3, and pigs fed added 25(OH)D_3 had the greatest ($P < 0.05$) with all other pigs fed 1,653 IU/kg vitamin D_3 intermediate. Pigs fed no dietary vitamin D had the lowest ($P < 0.05$) circulating level of 1,25-$(OH)_2D_3$ and pigs fed 0.19% STTD P had the highest. Pigs fed 1,653 IU/kg of vitamin D with 0.33% STTD P, 0.44% STTD P, and added 25(OH)D_3 were intermediate. There were no differences observed between dietary treatments for urine creatinine concentrations ($P = 0.670$). Pigs fed P deficient diets and 0.33% STTD P with no

phytase excreted greater Ca in urine compared to pigs fed all other treatments ($P < 0.05$). Pigs fed 0.19% STTD P had reduced P excreted in urine compared to pigs fed 0.44% STTD P and added vitamin D in the diet from 25(OH)D_3 ($P < 0.05$) with all other treatments intermediate. Pigs fed 0.19% STTD P diet had increased Ca:creatinine ratio ($P < 0.001$) in urine compared to pigs fed diets with 0.44% STTD P, with pigs fed 0.33% STTD P with no phytase intermediate. For P:creatinine ratio, pigs fed no vitamin D or high vitamin D in the diet from 25(OH)D_3 had increased P:creatinine levels in the urine ($P = 0.004$) compared to pigs fed 0.19% STTD P levels, with all other treatments intermediate.

Bone analysis

The response to treatment for bone density and ash was dependent upon the bone that was analyzed (treatment × bone interaction for bone density, $P = 0.044$; non-defatted bone ash, $P = 0.060$; defatted bone ash, $P = 0.068$; Table 3). Thus, the response related to dietary treatment differed depending on which bone (metacarpal, fibula, 2nd rib, or 10th rib) was measured. Pigs fed 0.19% STTD P had decreased ($P < 0.05$) bone density and ash (non-defatted and defatted) for all bones compared to pigs fed 0.44% STTD P, with those fed 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 25(OH)D_3. 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 P, phytase, and vitamin D concentrations in the diet ($P > 0.10$). Pigs fed 0.19% STTD P had reduced g of P and Ca in the bone ash compared to pigs fed 0.33% and 0.44% STTD P ($P < 0.05$).

For defatted percentage bone ash, the 2nd rib had the lowest, fibulas the highest, and the 10th rib and metacarpal were intermediate ($P < 0.001$; Table 4). For non-defatted percentage bone ash, bone ash changed by bone as the metacarpal was the lowest, then greatest for 10th and 2nd rib ($P < 0.001$), with fibulas, 3rd, 5th, and 7th ribs between metacarpals and 2nd and 10th ribs. When comparing rib location for bone density, there was a significant main effect of bone ($P = 0.001$), with the 10th rib having greater bone density than all other ribs and the 3rd rib having greater bone density than the 5th rib. The bone density of the 2nd and 7th ribs did not differ ($P > 0.05$) from either the 3rd or 5th ribs. For non-defatted bone ash, 10th rib had the greatest ($P < 0.05$) bone ash, followed by the 2nd rib which was greater ($P < 0.05$) than the 3rd rib, with the 5th and 7th ribs having the lowest percentage non-defatted bone ash.

Histopathologic evaluation of the physes of the fibula, 2nd, and 10th ribs showed that lesions of failure of endochondral ossification, consistent with metabolic bone disease, were most frequently observed in the 10th rib (bone × treatment, $P = 0.03$). Pigs that were fed a phosphorous deficient diet were significantly more likely to have both lesions of failure of endochondral ossification as well as infarctions (microscopic fractures) compared to most of the other dietary treatment groups with the notable exception being the vitamin D deficient group ($P < 0.05$). This group contained a single outlier animal that, compared to the other evaluated animals, had the most severe lesions of both failure of endochondral ossification as well as infarction (Figure 1 to 4). The medullary trabecular bone thickness of the tenth rib was thinner in pigs fed a P deficient diet compared to the other dietary treatment groups ($P < 0.05$). Cortical and trabeculae bone thickness measurements were not significant between treatment groups ($P > 0.10$).

Discussion

Diagnostic investigation of metabolic bone disease in pigs is challenging due to an incomplete understanding of the samples and assays used for diagnosis. Although there are diagnostic references for percentage bone ash, bone density, and vitamin D, many of the references stem from research conducted on different genotypic pigs than are raised currently (Field et al., 1974; Arnold et al., 2015). Currently, reference values used by diagnosticians for bone ash do not differ across bones or age of the animal. Lee et al. (2021) evaluated percentage bone ash across different bones in growing pigs and found differences in percentage bone ash across the various bones measured in the study. Thus, the bone that is used for diagnostic testing has an influence on results generated and there has been limited research to describe which bone should be measured to accurately assess bone mineralization in lameness cases.

Calcium and P are essential for multiple physiological roles in the body, including growth, development, and maintenance of the skeletal system. Due to the majority of P in plant-based grains being bound to phytate, making it unavailable for the pig, phytase has become widely used in swine diets (Dersjant-Li et al., 2014). If inadequate digestible P is included in nursery diets, there is a potential for restricted growth, lameness, and decreased bone P reserves for later growing and finishing phases (Buhler et al., 2010; She et al., 2017; Wensley et al., 2020). It has been reported that maximum bone mineralization levels are achieved at greater P levels than required for optimal growth performance (Vier et al., 2019a,b). Vitamin D is also an important factor in the absorption and retention of Ca and P (O'Doherty et al., 2010). When vitamin D_3 is fed, it must undergo a hydroxylation step in the liver to be converted to 25(OH)D_3. Once converted to 25(OH)D_3 it enters circulation or is stored and can be further hydroxylated in the kidney or extra-renal tissues to the active form, 1,25-$(OH)_2$-D_3, when needed. By feeding

25(OH)D_3 in the diet, it can bypass this first step in the liver and be directly available to the kidney or other tissues to be hydroxylated to the active form of vitamin D [1,25-$(OH)_2D_3$] and be utilized by the pig. Currently, there are commercially available 25(OH)D_3 products that can be fed to pigs and increase serum levels of 25(OH)D_3, which is the primary vitamin D metabolite used to assess an animal's status (Flohr et al., 2016). Vitamin D can also be available to the pig through synthesis in the skin from 7-dehydrocholesterol when exposed to sunlight. When skin is exposed to ultraviolet-B (UV-B) sunlight, 7-dehydrocholesterol is converted to vitamin D_3 (Crenshaw, 2001). The timing of the year and barn design can limit the amount of sunlight to the pigs (Arnold et al., 2015) and cause the need for dietary vitamin D supplementation.

In the current study, 3 different levels of STTD P were fed. The first was a P-deficient diet (0.19% STTD P), which was 57% of the NRC (2012) requirement estimate for pigs weighing 12 to 25 kg. The next P level was at the NRC (2012) requirement estimate of 0.33% STTD P. The last P level in the study was fed at 133% of the NRC (2012) requirement estimate based on recent findings of Vier et al. (2019a,b). Vitamin D was also omitted or fed in excess and phytase was included in some diets considering expected STTD P release values. Our objective was to ensure there would be differences in bone mineralization among the treatments. By collecting several different bones, any significant dietary treatment × bone interactions would be able to suggest which bone is more sensitive to dietary differences in bone mineralization. When comparing the different bones used for bone density, fibulas and 2nd ribs were more sensitive to differences in mineralization compared to metacarpals and 10th ribs (bone by treatment interaction $P < 0.05$). In the current study, we observed differences in percentage bone ash and bone density across the 4 bones analyzed. Lee et al. (2021) also observed differences across various bones for defatted bone ash.

Calcium and P homeostasis is a complicated interaction of multiple factors which include absorption from the gut, bone deposition and resorption, and renal excretion, which is controlled largely by circulating levels of parathyroid hormone (PTH; Rosol et al., 1995). Parathyroid hormone increases Ca resorption and decreases P resorption from the renal tubules in the kidney. Calcium and P status of pigs can be measured in serum and urine. Due to efficient homeostatic mechanisms regulating serum Ca and P, changes in serum values due to dietary mineral status generally lack sensitivity to detect differences (Koch and Mahan, 1985). In the current study, we observed no differences in serum Ca levels across dietary treatments. Although analyzed dietary Ca levels differed between treatments, we did not expect to see differences in serum Ca levels due to all dietary levels being at or above the requirement of the pigs (Votterl et al., 2021). For serum P, pigs fed 0.19% STTD P (deficient diets) had reduced serum P compared to all other treatments. Previous research has shown that serum P can be reduced when severely deficient levels of P are fed, but changes of Ca and P level is more sensitive to detect in the urine compared to the serum (Hagemoser et al., 2000). Recent research has shown that urine Ca and P can be used to monitor dietary excesses or deficiencies of these minerals (Grez-Capdeville and Crenshaw 2021; Grez-Capdeville and Crenshaw 2022). The authors' recent work in sows shows an inverse relationship between urinary Ca and P excretions in response to dietary levels of P. Phosphorus excretion in urine is increased as sows consumed more dietary P. In contrast, urinary Ca levels decreased with increasing P consumption and remained low when P was fed at or above the requirements of dietary P. Similar results have been shown in growing and finishing pigs (Hagemoser et al., 2000; Gutierrez et al., 2015). Urinary Ca and P is typically reported as a ratio to creatine to determine if dietary Ca and P are in excess or deficient (Hagemoser et al., 2000). A urinary Ca:creatinine ratio < 0.25 can mean a calcium deficiency with or without a

phosphorus deficiency and a urinary Ca:creatine ratio > 0.25 can mean a phosphorus deficiency is present. In the current study, we did not have any dietary treatments that had a Ca:creatinine ratio < 0.025, meaning none of the diets were deficient in dietary calcium. The pigs fed P-deficient diets and NRC (2012) levels of STTD P with no phytase in the diet had a urinary Ca:creatinine ratio > 0.25, meaning these pigs were deficient in dietary P causing excess Ca to be excreted, thus excreting a greater amount of Ca. A urinary P:creatinine ratio > 1.0 can mean a calcium deficiency is occurring and a ratio < 1.0 is normal in growing pigs. All dietary treatments had a P:creatinine ratio < 1.0, meaning pigs were all in the normal range for urinary Ca and P ranges.

Serum or plasma 25(OH)D_3 is considered the best biomarker of vitamin D status in mammals (Jones, 2012). In the current study, there were 3 levels of vitamin D fed in the diets, the first was no added vitamin D, the second was 1,653 IU/kg of vitamin D3, and the third was 1,653 IU/kg of vitamin D3 with 50 µg of 25(OH)D_3. As expected, pigs fed no additional vitamin D in the diet had the lowest levels of 25(OH)D_3 in serum, with pigs fed the highest levels of vitamin D having the highest levels. These findings are similar to previous results when 50 µg of 25(OH)D_3 was included in the diet (Burild et al., 2016; Flohr et al., 2016; Thayer et al., 2019). Pigs fed no added vitamin D in the diet also had the lowest concentrations of 1,25-$(OH)_2D_3$, which is the active form of vitamin D in the body. Pigs fed P-deficient diets had the highest levels of 1,25-$(OH)_2D_3$ in the serum. When an animal is deficient in one nutrient, they tend to increase the levels of other nutrients in the body (Portale et al., 1986). When diets have reduced levels of P, it also affects related endocrine levels such as vitamin D (calcitriol) and PTH (Oster et al., 2016). Parathyroid hormone regulates the activation of calcitriol via 1α-hydroxylase (Freidman et al. 1996). Thus, feeding a P-deficient diet increases activation of 1α-hydroxylase

causing 25(OH)D_3 to be hydroxylated to 1,25-$(OH)_2D_3$. Similar results to our findings were observed by (Oster et al., 2018), who observed increased levels of 1,25-$(OH)_2D_3$ when low levels of P were fed.

Knowing that most Ca and P is deposited in bone tissue (Crenshaw, 2001), bone growth and synthesis in pigs can be affected by the dietary Ca and P (Lagos et al., 2019; Vier et al., 2019a,b). In the diagnosis of metabolic bone disease, bone mineralization is measured using various analytical methods and bones, with bone ash being the primary measurement. Crenshaw et al. (2009) determined that femur bone ash was a better indicator of body mineral content in 25 kg pigs compared with fibula bone ash. More recently, Lee et al. (2021) observed that metacarpals, metatarsals, and tibias were more representative of total body bone ash in growing pigs compared to femur, fibula, and ribs. Our objective in this study was to determine which bone is most sensitive to observe differences in dietary P and vitamin D. We chose to not measure the femur bone, due to the ease of collection of the fibulas, ribs, and metacarplas compared to femurs. We found that fibulas and 2nd ribs were more sensitive to observe a difference in dietary P compared to the metacarpals and 10th ribs. In the present study, we fed varying levels of STTD P, which made it possible to detect interactions between the dietary treatments and the bone being analyzed. In the Lee et al. (2021) study, the authors fed two dietary P levels, the requirement and 60% of the requirement. By only feeding two dietary P levels, the authors were not able to detect an interaction between dietary P and bone analyzed as the present study was able to do.

When analyzing bone ash, there are two procedures that can be used, non-defatted and defatted (Wensley et al., 2020). For analyzing defatted bone, bones must first be soaked in ether to extract fat and water from the bones. By doing this, variation across the bones being measured

is reduced, regardless of the dietary treatments fed or bone measured (Wensley et al., 2020). In the current study, bones measured for percentage bone ash using the defatted method had a lower standard error of mean (SEM) than bones measured using the non-defatted bone ash method. These results are consistent with Wensley et al. (2020). Interestingly, both methods can detect dietary treatment differences in bones, but differed in terms of which bone was more sensitive to detect the differences. We observed that for non-defatted bone ash, fibulas were the best bone to detect differences in dietary treatments, whereas for defatted bone ash 2nd ribs were the most sensitive bone to detect differences. Although there were significant differences between bones and dietary treatments in the current study, the greater magnitude of differences was between the different procedures used for determining the bone ash percentage. In the current study, the average defatted bone ash percent was 12% higher than the non-defatted bone ash percent across all bones and dietary treatments. These findings are consistent with Wensley et al. (2019) who observed a 10% increase in percentage bone ash for defatted bones compared to non-defatted bones. These results are important for producers and diagnosticians to understand when considering the procedures used to establish reference ranges used for lameness cases. Due to the magnitude in difference between the two procedures, misinterpretations of diagnostic results are possible when there are differences in the procedures used in the analysis compared to those used to establish the reference range.

Results from the current study demonstrated that growth performance and bone ash weight of pigs fed P-deficient diets were reduced. Pigs fed P-deficient diets (0.19% STTD P) had a 17% reduction in weight gain compared to the pigs fed adequate or increased levels of P. Also, pigs fed P-deficient diets had 35% and 40% reduced bone ash weight compared to pigs fed adequate dietary P for non-defatted and defatted bone ash weight, respectively. These results are

similar to data reported by Lee et al. (2021), which implies that a greater proportion of dietary Ca and P is used for growth and soft tissues and a lower proportion for bone tissue synthesis when P is deficient. Collectively, these data support the hypothesis that Ca and P requirements to maximize growth performance are less than the requirements to maximize bone ash. In the current study, we observed an increase in ADG as the STTD P level increased from 0.19% to 0.33%, but no further improvement in gain occurred as the STTD P level increased to 0.44%. Conversely, in a similar study Vier et al. (2019a) observed an increase in gain up to 0.43% STTD P.

Due to a majority of the Ca and P being stored in the bones (Crenshaw, 2001), analyzing the amount of Ca and P in total bone ash can explain the relationship of bone Ca and P and their impact on bone mineralization. In the current study, we observed an increase in the grams of Ca and P in bone ash for pigs fed adequate or increased levels of P compared to the pigs fed deficient levels of P. Conversely, when analyzing the percentage Ca and P in bone ash, we observed no difference across dietary treatments. Increasing bone mineralization increases the size of the entire bone, which in turn increases the weight of Ca and P in the bone without altering the percentage of Ca and P in the bone ash. This data is consistent with other published data evaluating the impact of different levels of dietary Ca and P on bone mineralization (Gonzalez-Vega et al., 2016; Lee et al., 2021).

Although percentage bone ash is typically used to measure bone mineralization (Wu et al., 2018; Vier et al., 2019a,b; Lee et al., 2020), there are other analysis that can be used to characterize the bone composition. One measurement that is less time consuming and intensive than bone ash is bone density. This can be done using the Archimedes principle or dual-energy X-ray absorptiometry (DEXA) scan. Recent research has used DEXA to observe differences in

bone mineralization across treatments (Becker et al., 2020). In the current study, by using DEXA scans to analyze bones, we observed an increase in bone mineral density as the P in the diet increased, but no interaction between treatments and bone was observed. We observed an interaction between treatment and bone using the Archimedes principle. Fibulas and 2nd rib were more sensitive to pick up differences in dietary P compared to metacarpals and 10th ribs. Our data suggests that bone density can be used as a measurement to analyze bone mineralization and detect differences in dietary P and vitamin D by using the Archimedes principle or DEXA, but the Archimedes principle is able to detect differences across different bones.

Histopathology can be a valuable tool in the evaluation of metabolic bone disease in pigs; however, it is necessary to know which bone is most sensitive when collecting samples. Here, we showed that lesions of failure of endochondral ossification and infarction were more likely to be observed in the tenth rib compared to the second rib or fibula in pigs fed a P deficient or vitamin D deficient diet. Importantly, the noted differences in lesions between the second and tenth ribs is likely representative of short ribs vs. long ribs, where the longer ribs are faster growing and thus more susceptible to manifest pathology. Future evaluation of which long rib is most likely to have lesions should be performed so that diagnosticians collecting ribs for evaluation can be confident that if lesions of metabolic bone disease are present they should be able to detect them.

Cortical and medullary trabecular bone thickness were measured as we hypothesized that dietary deficiencies of P and/or vitamin D would lead to resorption and thinning of bones. While trabecular bone thickness was significantly reduced in pigs fed a P deficient diet, the time, effort, and requirement for age-matched negative controls makes this method of evaluation impractical in everyday diagnostic medicine.

In summary, the present data suggests there are different procedures that can be used to detect dietary P and vitamin D differences in pigs. The differences in bone density and bone ash in response to P and vitamin D were most apparent with fibulas and 2nd ribs. It is important to note the differences in results depending on the procedures used to analyze bones. The difference between bone ash procedures was more apparent than the differences between treatments, with defatted bones having 10 to 12% higher bone ash compared to non-defatted bones. For histopathology, 10th ribs were more sensitive than 2nd ribs or fibulas for detection of lesions.

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Table 2.1. 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 + 25(OH)D_3
Phytase, FYT/kg:	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
L-Lys-HCl	0.50	0.50	0.50	0.50	0.50	0.50
DL-Met	0.18	0.18	0.18	0.18	0.18	0.18
L-Thr	0.20	0.20	0.20	0.20	0.20	0.20
L-Trp	0.05	0.05	0.05	0.05	0.05	0.05
L-Val	0.13	0.13	0.13	0.13	0.13	0.13
Trace mineral premix[2]	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin premix[3]	0.25	0.25	0.25	---	0.25	0.25
Vitamin premix - no vitamin D[4]	---	---	---	0.25	---	---
Phytase[5]	---	---	0.08	0.08	0.08	0.08
25(OH)D_3[6]	---	---	---	---	---	0.04
Calculated analysis, %						
SID Lys	1.30	1.30	1.30	1.30	1.30	1.30
NE, kcal/kg	2,458	2,433	2,455	2,436	2,436	2,435
Ca	0.50	0.69	0.50	0.65	0.65	0.65
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
Ca:P	1.20	1.20	1.20	1.20	1.20	1.20
Mineral analysis[7]						
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 12 to 30 kg BW.

[2]Provided per kg of diet: 110 mg Zn from zinc sulfate; 110 mg Fe from iron sulfate; 33 mg Mn from manganese oxide; 17 mg Cu from copper sulfate; 0.30 mg I from calcium iodate; 0.30 mg Se from sodium selenite.

[3]Provided per kg of diet: 4,134 IU vitamin A; 1,653 IU vitamin D; 44 IU vitamin E; 3 mg vitamin K; 0.03 mg vitamin B12; 50 mg niacin; 28 mg pantothenic acid; 8 mg riboflavin.

[4]Provided per kg of diet: 4,134 IU vitamin A; 44 IU vitamin E; 3 mg Vitamin K; 0.03 mg vitamin B12; 50 mg niacin; 28 mg pantothenic acid; 8 mg riboflavin.

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

[6]HyD provided 2,000 IU/kg of vitamin D equivalency from 50 µg/ton of 25(OH)D_3 to the diet (DSM Nutritional Products, Parsippany, NJ).

[7]A representative sample of each diet was collected from each feeder at 3 different time points throughout the study. Samples were stored at -20°C 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 2.2. 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 + 25(OH)D_3[3]		
Phytase, FYT/kg:	No	No	2,000	2,000	2,000	2,000	SEM	*P* =
Body weight, kg								
d 0	11.9	11.9	11.9	12.0	11.9	12.0	0.56	0.925
d 28	27.0^{a}	29.3^{b}	30.1^{b}	30.4^{b}	29.8^{b}	30.3^{b}	0.94	< 0.001
Overall								
ADG, g[4]	541^{a}	623^{b}	651^{b}	657^{b}	639^{b}	655^{b}	15.6	< 0.001
ADFI, g[5]	919^{a}	980^{ab}	988^{ab}	1,028^{b}	1,017^{b}	1,052^{b}	30.9	< 0.001
G:F, g/kg[4]	589^{a}	636^{bc}	660^{c}	641^{bc}	629^{bc}	627^{b}	8.2	< 0.001
Serum[6]								
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
Creatinine, mg/dL	1.31	1.17	1.15	1.14	1.20	1.01	0.084	0.208
25(OH)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-$(OH)_2D_3$, pg/mL	588^{c}	418^{b}	387^{ab}	254^{a}	320^{ab}	318^{ab}	37.4	< 0.001
Urine								
Ca, mg/dL	72.2^{b}	76.7^{b}	9.9^{a}	4.0^{a}	13.6^{a}	3.0^{a}	12.42	< 0.001
P, mg/dL	5.5^{a}	17.0^{ab}	30.2^{ab}	52.2^{ab}	30.7^{ab}	57.8^{b}	12.35	0.026
Creatinine, mg/dL	100.4	104.4	101.7	88.7	127.9	85.1	19.16	0.670
Ca:creatinine	0.80^{c}	0.68^{bc}	0.10^{a}	0.06^{a}	0.11^{ab}	0.04^{a}	0.138	< 0.001
P:creatinine	0.06^{a}	0.21^{ab}	0.33^{ab}	0.78^{b}	0.29^{ab}	0.90^{b}	0.170	0.004
Defatted Bone ash[7]								
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[8]								
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[3,2]	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
Bone ash content[9]								

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[10]								
Trabeculae bone thickness, µm	28.6	29.0	30.3	31.4	31.7	31.0	1.851	0.232
Cortical bone thickness, µm	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 360 pigs were used in a 28-d nursery trial with 6 pigs per pen and 10 pens per treatment. Pigs were placed on experimental diets 24 days post-weaning (~19 d of age). The mean values for the bone measurements are all bones (metacarpals, fibulas, 2nd and 10th ribs) within each dietary treatment.

[2]BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; G:F = gain to feed ratio

[3]HyD provided 2,000 IU/kg of vitamin D equivalency from 50 µg/ton of 25(OH)D_3 to the diet (DSM Nutritional Products, Parsippany, NJ).

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

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

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

[7]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 105°C for 7 days and then ashed in a muffle furnace at 600°C for 24 h.

[8]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 105°C for 7 days and then ashed in a muffle furnace at 600°C for 24 h.

[9] After bone ash was completed, the ash was digested and analyzed for Ca and P using ICP by the KSU Soils lab (Manhattan, KS).

[10] Histopathological evaluation of the physis of the left fibula, 2nd rib, and 10th rib was performed by three trained anatomic pathologists.

Table 2.3. Interactive effects of STTD P, phytase, and vitamin D and bone 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 + 25(OH)D_3[1]	
Phytase, FYT/kg[2]:	No	No	2,000	2,000	2,000	2,000	SEM
Bone density, g/mL[3]							
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-defatted bone ash, %[4]							
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-defatted bone ash, g[5]							
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, %[6]							
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[7]							
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}	
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[3,9]							
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	
Bone ash content							
Phosphorus, g[9]							
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[9]							
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, %[9]							
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, %[9]							
Metacarpal	20.4	20.5	19.5	19.1	19.8	20.6	1.045
Fibula	18.7	20.7	17.8	19.9	20.4	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]HyD provided 2,000 IU/kg of vitamin D equivalency from 50 µg/ton of 25(OH)D_3 to the diet (DSM Nutritional Products, Parsippany, NJ).

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

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

[4]One pig per pen (8 pens per treatment) was 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 105°C for 7 days and then ashed in a muffle furnace at 600°C for 24 h. Bone × treatment, $P = 0.060$.

[5]Bone × treatment, $P = 0.131$

[6]One pig per pen (8 pigs per treatment) was 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 105°C for 7 days, and then ashed in a muffle furnace at 600°C for 24 h. Bone × treatment, $P = 0.068$.

[7]Bone × treatment, $P = 0.063$

[8]Bone × treatment, $P = 0.045$

[9]Bone × treatment, $P > 0.10$.

Table 2.4. Effect of different bones on bone analysis of nursery pigs

			Rib number						
	Metacarpal	Fibula	2nd	3rd	5th	7th	10th	SEM	*P* =
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	1.88[d]	1.06[b]	0.70[a]	1.37[c]	2.00[d]	2.16[e]	1.34[c]	0.046	< 0.001
Bone ash, %	37.8[a]	48.0[bc]	50.0[d]	49.0[cd]	46.9[b]	47.5[b]	51.5[e]	0.416	< 0.001
Bone density, g/mL	1.18[a]	1.27[e]	1.24[d]	1.22[cd]	1.19[ab]	1.21[bc]	1.27[e]	0.006	0.001
Dual-energy X-ray absorptiometry									
Bone mineral density, g/cm[3,4]	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[3]									
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]									
Trabeculae bone thickness, µm	---	38.7[c]	28.8[b]	---	---	---	23.5[a]	1.09	< 0.001
Cortical bone thickness, µm	---	97.1	94.8	---	---	---	89.4	2.22	0.898

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

[1]The mean values for the bone measurements of each bone are the average of all dietary treatments within each bone measured.

[2]One pig per pen (8 pigs per treatment) were euthanized and the left and right metacarpal, fibula, 2nd, 3rd, 5th, 7th, and 10th ribs were collected to determine bone ash weight and percentage bone ash. The 3rd, 5th, and 7th ribs were only measured for bone density and non-defatted bone ash to compare bone mineralization across rib location. 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 105°C for 7 days and then ashed in a muffle furnace at 600°C 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 105°C for 7 days and then ashed in a muffle furnace at 600°C for 24 h.

[4]After bone ash was completed, the ash was digested and analyzed for Ca and P using ICP by the KSU Soils lab (Manhattan, KS).

[5]Histopathological evaluation of the physis of the left fibula, 2nd rib, and 10th rib was performed by three trained anatomic pathologists.

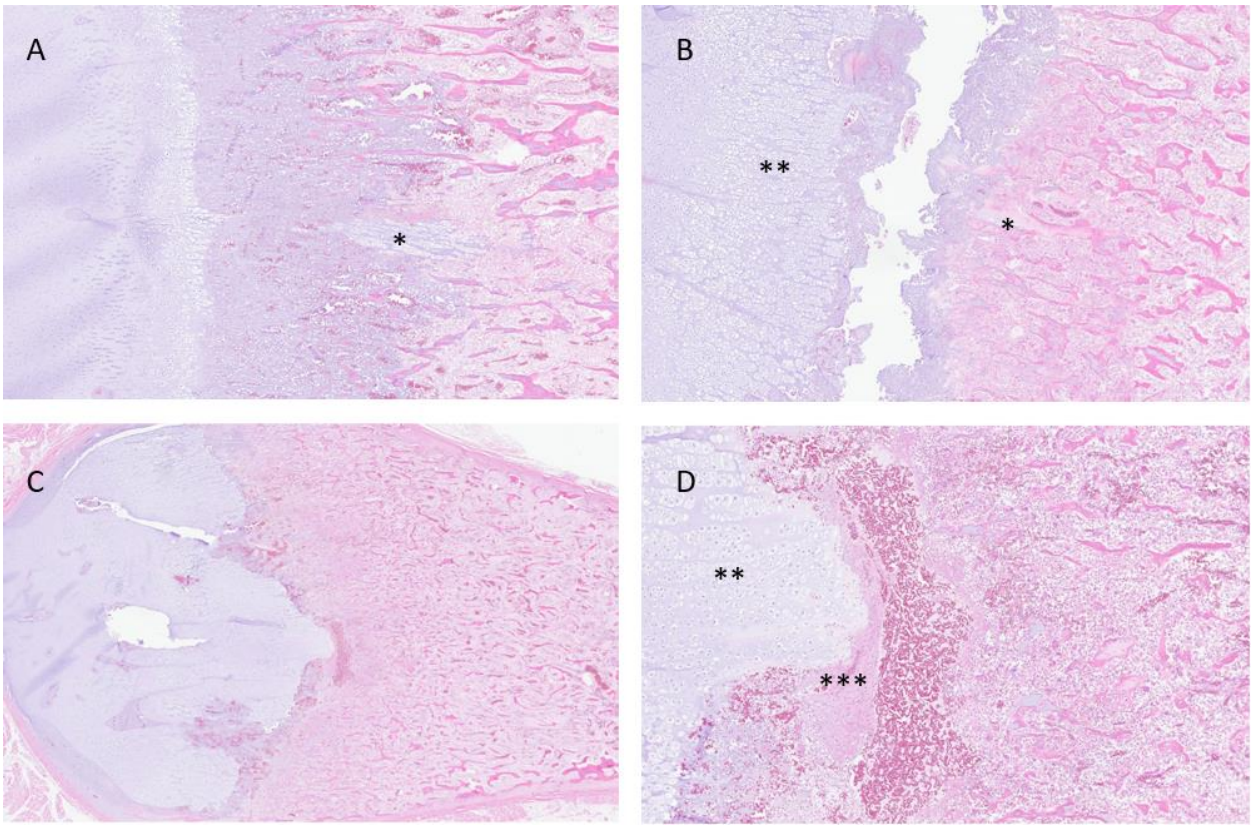

Figure 2.1. Histopathology lesions in the tenth rib.

(A) Physeal score of 1 with a single island of viable cartilage extending into the primary spongiosa (*). (100X H&E.) (B) Physeal score of 2 with an expanded zone of hypertrophy (**) and an island of cartilage extending into the secondary spongiosa (*). There is separation artifact between the zone of hypertrophy and the primary spongiosa. (100X H&E). (C) Physeal score of 3 with a markedly enlarged and jagged zone of hypertrophy (40X H&E). (D) Infarction of the primary spongiosa with an expanded and jagged zone of hypertrophy (**) and replacement of primary spongiosa by fibrin and hemorrhage (***). (200X H&E).

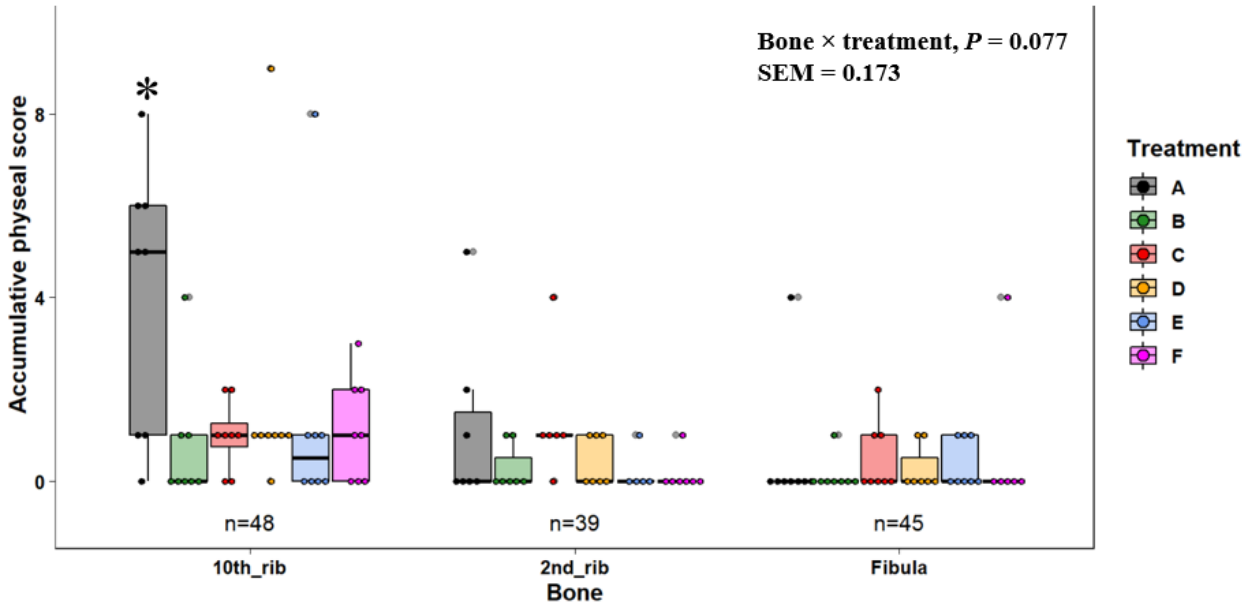

Figure 2.2. Accumulative physeal score is the score of 3 pathologists scoring the bones. The bones were scored for failure of endochondral ossification of the physis. Treatments are A) 0.19% standardized total tract digestibility (STTD) P (deficient), B) 0.33% STTD P (NRC, 2012 requirement estimate) using monocalcium phosphate, C) 0.33% STTD P including 0.14% release from phytase (Ronozyme HiPhos 2700, DSM Nutritional Products, Parsippany, NJ), D) 0.44% STTD P using monocalcium phosphate, phytase, and no vitamin D, E) diet 4 with 1,653 IU/kg vitamin D_3, F) diet 5 with 50 µg/kg of 25(OH)D_3 (HyD, DSM Nutritional Products, Parsippany, NJ) estimated to provide an additional 2,000 IU/kg of vitamin D3.

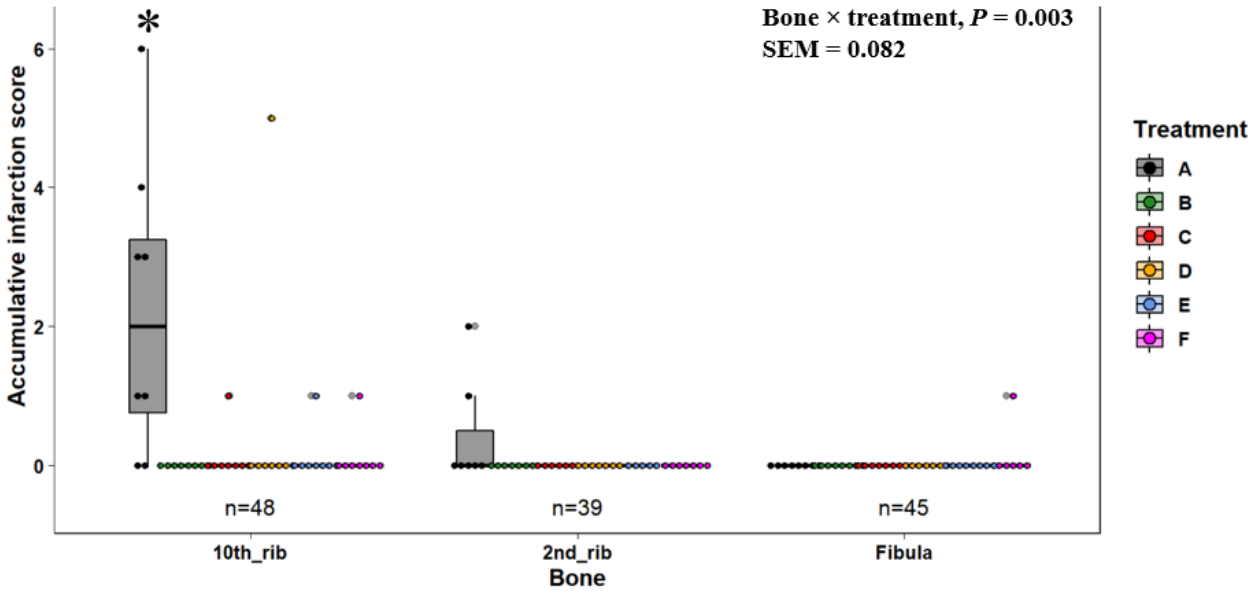

Figure 2.3. Accumulative infarction score is the score of 3 pathologists scoring the bones.

The bones infarctions/fracture scoring consisted of: 0) no evidence of infarctions, 1) small infarctions covering < 50% of the diameter of the bone, 2) large infarctions covering > 50% of the diameter of the bone, and 3) cortical fracture. Treatments are A) 0.19% standardized total tract digestibility (STTD) P (deficient), B) 0.33% STTD P (NRC, 2012 requirement estimate) using monocalcium phosphate, C) 0.33% STTD P including 0.14% release from phytase (Ronozyme HiPhos 2700, DSM Nutritional Products, Parsippany, NJ), D) 0.44% STTD P using monocalcium phosphate, phytase, and no vitamin D, E) diet 4 with 1,653 IU/kg vitamin D_3, F) diet 5 with 50 µg/kg of 25(OH)D_3 (HyD, DSM Nutritional Products, Parsippany, NJ) estimated to provide an additional 2,000 IU/kg of vitamin D3.

Chapter 3 - The effect of bone and analytical method on the assessment of bone mineralization in response to dietary phosphorus, phytase, and vitamin D in finishing pigs

Abstract

A total of 882 pigs [PIC TR4 × (Fast LW × PIC L02); initially 33.2 ± 0.31 kg] were used in a 112-d study to evaluate the effects of different bones and analytical methods on the assessment of bone mineralization response to changes in dietary P, phytase, and vitamin D in growing pigs. Pens of pigs (20 pigs per pen) were completely randomized to 1 of 5 dietary treatments with 9 pens per treatment. Dietary treatments were designed to create differences in bone mineralization and included: 1) P at 80% of NRC (2012) STTD P requirement, 2) NRC STTD P with no phytase, 3) NRC STTD P with phytase (Ronozyme HiPhos 2700, DSM Nutritional Products, Parsippany, NJ) providing an assumed release of 0.14 % STTD P from 2,000 FYT/kg, 4) high STTD P (128% of the NRC P) using monocalcium phosphate and phytase, 5) diet 4 with additional vitamin D3 from 25(OH)D_3 (HyD, DSM Nutritional Products, Parsippanny, NJ). On d 112, one pig per pen was euthanized for bone, blood, and urine analysis. Additionally, 11 pigs identified as having poor body condition which indicated a history of low feed intake (unhealthy) were sampled. There were no differences between treatments for final body weight, average daily gain, average daily feed intake, gain to feed ($P > 0.10$), or bone ash measurements (treatment × bone interaction, $P > 0.10$) regardless of bone ash method. The response to treatment for bone density and bone mineral content was dependent upon bone

sampled (density interaction, $P = 0.053$; mineral interaction, $P = 0.078$). For 10^{th} rib bone density, pigs fed high levels of P had increased ($P < 0.05$) bone density compared to pigs fed NRC levels with phytase, with pigs fed deficient P, NRC levels of P with no phytase, and high STTD P with extra $25(OH)D_3$ intermediate, with no differences for metacarpals, fibulas, or 2^{nd} ribs ($P > 0.10$). Pigs fed extra vitamin D from $25(OH)D_3$ had increased ($P < 0.05$) 10^{th} rib bone mineral content compared to pigs fed deficient P and NRC levels of P with phytase, with pigs fed industry P and vitamin D, and NRC P with monocalcium intermediate. Healthy pigs had greater ($P < 0.05$) serum Ca, P, and vitamin D concentrations and defatted bone ash than those unhealthy, with no difference between the two health statuses when expressed on a non-defatted bone ash basis ($P > 0.05$). In summary, differences between bone ash procedures was more apparent than differences between diets. Differences in bone density and mineral content in response to dietary P and vitamin D were most apparent with 10^{th} ribs.

Introduction

Metabolic bone disease is a cause of lameness in swine production, which leads to increased morbidity and mortality in growing pigs. There are many factors that can contribute to lameness, including but not limited to: infectious disease, genetic and conformational abnormality, and toxicity that affects the bone, muscle, and nervous systems. There are different diagnostic assessments that are currently used to assess bone mineralization, including bone ash, bone density, and histopathological examination of tissues.

Currently, there are reference values used by diagnosticians for bone ash, bone density, and serum vitamin D (Field et al., 1974; Arnold et al., 2015). Comparing the field observations to the reference values is difficult though because the reference values for bone ash do not describe the method used and the same bone is not always used for analysis. Wensley et al.

(2020) observed differences in percentage bone ash when comparing bones that had the lipid extracted (defatted) vs. those without lipid extraction (non-defatted) before ashing. The authors observed that not removing the lipid prior to ashing is less accurate than defatting due to an increase in standard deviation across the bones sampled. Recently, Williams et al. (2023a) evaluated different bones and various analytical measurements. The authors observed differences in percentage bone ash among fibulas, metacarpals, 2nd rib, and 10th ribs in nursery pigs and differences between analytical methods were present.

Also, there is little information regarding reference values comparing healthy and unhealthy pigs. Typically, when tissues are submitted for diagnostic assessment, they are not from a healthy pig but rather a pig that has died or was euthanized for welfare concerns. When these samples received by diagnostic labs are analyzed, the results are compared with reference values generally determined on healthy pigs. Therefore, our objective was to create ranges in bone characteristics by altering dietary Ca, P, and vitamin D in order to understand values that would be observed when analyzing bone and blood with different analytical procedures. Furthermore, this study compared different analytical procedures and compared results between healthy pigs and those diagnosed as unhealthy.

Materials and Methods

General

The Kansas State University Animal Care and Use Committee approved the protocol used in this study (4413). This study was conducted at a commercial research facility in southern Minnesota (Jackson, MN). The barn was tunnel ventilated with completely slatted concrete flooring over deep pits for manure storage. Each pen was equipped with a cup waterer and a 3-hole stainless steel dry-self feeder (Thorp Equipment, Thorp, WI) to provide *ad libitum* access to

feed and water. Pens were 2.65 × 6.125 m providing approximately 0.81 m^2 per pig. Feed additions to each individual feeder were made and recorded by an electronic feeding system (FeedPro; Feedlogic Corp., Wilmar, MN).

Animals, Housing, and Diets

A total of 882 pigs [PIC TR4 × (Fast LW × PIC L02); initially 33.2 ± 0.31 kg] were used in a 112-d trial with 20 pigs per pen and 9 pens per treatment. Pens of pigs were fed the farm's standard diets from weaning until the start of the study with dietary Ca, P, and vitamin D above NRC (2012) requirement estimates. On d 0 of the study, 8 pigs were randomly selected for blood collection and serum analysis of Ca, P, $25(OH)D_3$, and $1,25(OH)_2D_3$ to establish baseline levels before initiating dietary treatments. Pigs were randomly allotted to 1 of 5 dietary treatments in a completely randomized design. The dietary treatments were: 1) STTD P fed at 80% of the NRC (2012) requirement estimate (deficient); 2) STTD P fed at 100% of the requirement estimate (NRC requirement) using monocalcium phosphate; 3) STTD P fed at 100% of the NRC (2012) requirement estimate with phytase (Ronozyme HiPhos 2700, DSM Nutritional Products, Parsippany, NJ) providing an assumed release of 0.14 % STTD P from 2,000 FYT/kg; 4) STTD P fed at 128, 127, 121, and 114% of the NRC requirement (high) for phases 1 through 4, respectively, including monocalcium phosphate and 0.14% STTD P release from phytase; 5) diet 4 with additional vitamin D from $25(OH)D_3$ (HyD, DSM Nutritional Products, Parsippany, NJ). HyD provided 2,000 IU/kg vitamin D equivalency from 50 µg/kg in phase 1, 1,671 IU/kg vitamin D equivalency from 42 µg/kg in phase 2, 1,342 IU/kg vitamin D equivalency from 34 µg/kg in phase 2, and 1,014 IU/kg vitamin D equivalency from 25 µg/kg in phase 4, respectively. A vitamin premix was added to all diets with the inclusion rate decreasing with subsequent dietary phases. All diets were manufactured at the New Fashion Pork Feed mill in Estherville, IA

and fed in meal form (Table 1 and 2). All diets were formulated to an total Ca:total P ratio of 1.20:1. Experimental diets were fed in 4 phases from 36 to 54, 54 to 72, 72 to 99, and 99 to 127 kg, respectively.

Throughout the 112-d experiment, pens of pigs and feeders were weighed every 14 d to determine ADG, ADFI, and G:F. On d 112, 1 pig per pen (9 pigs per treatment) was euthanized and bones, blood, and urine were collected for analysis. Additionally, on d 112, bones, blood, and urine were collected from 11 unhealthy pigs, which were identified as pigs with poor body condition, indicating a history of low feed intake. Of the 11 unhealthy pigs samples, 4 were from treatment 1, 2 from treatment 2 and 3, and 3 from treatment 5. A blood sample was taken from the jugular vein of each pig prior to euthanasia. Blood samples (10 mL) were centrifuged (3000 × *g*, 30 min) within 2 h after collection and serum was stored at -20°C until analysis. Serum was split into three, 2 mL aliquots and analyzed for Ca and P via large animal serum chemistry (Iowa State University Veterinary Diagnostic Lab, Ames IA), 25(OH)D_3, and 1,25-$(OH)_2D_3$ (Heartland Assays, Ames, IA). A 10 mL urine sample was collected shortly after euthanasia via cystocentesis using a 20 gauge × 2.5 cm needle and a 12 mL syringe, then frozen at -20°C until analyzed for Ca, P, and creatinine (Iowa State University Veterinary Diagnostic Lab).

Bone measurements

The right and left metacarpal, 2nd rib, 10th rib, and fibula were collected from each pig for a total of 8 bones per pig. All bones were analyzed for dual-energy X-ray absorptiometry (DXA), bone density (Archimedes principle), Instron bone-breaking strength, bone ash (defatted and non-defatted), and bone Ca and P.

The left fibula, left 2nd, and left 10th rib were processed for histological examination. Briefly, bones were fixed in 10% neutral buffered formalin for at least 24 h and decalcified using

DeltaCAL (Delta Medical, Inc., Aurora, IL, USA) for at least 4 h. The costochondral junction and the body of the 2nd and 10th rib, and the head of the left fibula were embedded in paraffin blocks, sectioned at 4 µm thickness, and stained with hematoxylin and eosin. Microscopic examination was performed by blinded assessment from three pathologists at Iowa State University Veterinary Diagnostic Laboratory. Bones were scored for failure of endochondral ossification of the physis and microscopic fractures (infractions). Histological grading of each bone consisted of physis grading, infractions/fracture lines, cortical bone thickness measurements, and trabeculae bone thickness. The physis grading consisted of: 0) no histologic findings of significance, 1) multifocal small tongues or islands of viable cartilage extend into the primary spongiosa, 2) moderate-sized tongues or islands of viable cartilage extend into the primary and secondary spongiosa, and 3) extensive areas of the zone of hypertrophy are expanded and extend down into the primary and secondary spongiosa. The infractions/fracture scoring consisted of: 0) no evidence of infractions, 1) small infractions covering < 50% of the diameter of the bone, 2) large infractions covering > 50% of the diameter of the bone, and 3) cortical fracture. Cortical bone thickness measurements were taken at 10 locations in the diaphysis. Trabeculae bone thickness was taken at 10 locations in the diaphysis, 4 cm distal to the physis, and 1 cm in from the edge of the cortical bone.

The metacarpal, 2nd rib, 10th rib, and fibulas were analyzed for dual-energy X-ray absorptiometry (DEXA) scan, bone density via Archimedes principle, Instron bone-breaking strength, bone ash (de-fatted and non-defatted), and bone Ca and P. The leftover extraneous soft tissues and cartilage were removed from the bones prior to assessment. Bone mineral content and bone mineral density were determined using dual-energy X-ray absorptiometry (DXA) scans of each bone (Hologic QDR4500A Dual Fan Beam X-Ray Bone Densitometer (Hologic, INC.,

Waltham, MA)). The machine is routinely used to measure animal bones, with an average run time of 2 min per scan, with the capability of measuring 4 unique bones per scan. Archimedes principle was used for bone density. Briefly a dry bone weight was collected, then bones were submerged in ultra-purified water under a vacuum with 10.55 ton per square meter for a minimum of 4 h. Bones were then weighed while suspended in a vessel of ultra-purified water, and the weight used to calculate bone density. Bone-breaking strength was determined with an Instron (Instron 5569, NV Lab, Norwood, MA). Briefly each bone was held by two supports spaced 30 mm apart and were broken by a wedge lowered on the center of the bone at a speed of 100 mm per min and a maximal pressure of 5,000 kg. The force was measured by a pressure-sensitive cell, and peaks of maximum force were recorded. For bone ash, both the defatted and the non-defatted methods were used (Wensley, et al., 2020). For defatted bone ash, all bones were placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and lipid. Bones were then dried at 105˚C for 7 d and then ashed in a muffle furnace at 600˚C for 24 h. For non-defatted bone ash, all bones were dried at 105˚C for 7 d and then ashed in a muffle furnace at 600˚C for 24 h. These methods were used to determine total bone ash weight and percentage ash relative to dried bone weight. For bone Ca and P, a portion of the residual defatted bone ash (0.05 g) was placed in a tube with nitric acid and incubated at 60˚C for 6 h. The solution was then diluted using ultra-purified water and Ca (AOAC 985.01, 2006) and P (AOAC 985.01, 2006) quantity measured by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Concentrations of Ca and P (%) were calculated as a percentage of the total bone ash.

Statistical analysis

For growth performance, blood, and urine analysis, data were analyzed as a completely randomized design using a one-way ANOVA with pen considered the experimental unit and treatment as a fixed effect. For the statistical analysis of bone measurements where multiple bones were collected and analyzed from one pig per pen, data were analyzed as a split-plot design with dietary treatment, bone, and the treatment × bone interaction included as fixed effects. Pen was considered the experimental unit for dietary treatment, and bone nested within pig served as the experimental unit for bone and the bone × treatment interaction. Given the split-plot layout, pig was included in the model as a random intercept to account for multiple bones collected from each pig. A Tukey multiple comparison adjustment was used to control Type I error rate. All growth and analytical data were analyzed using the lmer function from the lme4 package in R from the stats package in R version 3.5.1 (2018-07-02). Data were summarized and reported as percentage of observations within each score category by treatment. All results were considered significant at $P \leq 0.05$ and marginally significant at $0.05 < P \leq 0.10$.

Results

Growth performance

Although dietary treatments provided a range of P, phytase, and vitamin D, there were no difference observed ($P > 0.10$ or 0.05) in growth performance between treatments (Table 3).

Serum and urine analysis

Initial mean serum concentrations for pigs sampled on d 0 of the study were 10.24 mg/dL for Ca, 9.8 mg/dL for P, 32.0 ng/mL for $25(OH)D_3$, and 193 pg/mL for $1,25(OH)_2D_3$. For serum Ca on d 112, pigs fed 80% of the NRC (2012) requirement estimate for STTD P had increased circulating Ca compared to pigs fed NRC (2012) STTD P levels with phytase ($P < 0.05$), with pigs fed high levels of STTD P, and NRC (2012) levels of STTD P with no phytase intermediate.

Pigs fed NRC (2012) levels of STTD P with no phytase had increased serum P levels compared to pigs fed high levels of STTD P with added 25(OH)D_3 in the diet ($P < 0.05$), with pigs fed the other 3 treatments intermediate. Pigs fed the diets containing added 25(OH)D_3 had increased ($P < 0.05$) circulating 25(OH)D_3 compared to all other dietary treatments. There were no differences between treatments for circulating 1,25-$(OH)_2D_3$ ($P > 0.05$). For urinary Ca and P, there were no differences between dietary treatments ($P > 0.05$). For urinary creatinine, pigs fed NRC (2012) levels of STTD P with phytase had increased ($P < 0.05$) levels compared to pigs fed high levels of STTD P, with all other treatments intermediate. For Ca:creatinine, there was a tendency for a difference between treatments ($P < 0.10$), but no significant mean separation using the Tukeys comparison was observed. For urine P:creatinine levels, there was no difference between treatments ($P > 0.05$).

Bone measurements

There were no treatment differences for defatted percentage bone ash, non-defatted percentage bone ash, non-defatted bone ash (g), or percentage and grams of bone ash Ca and P. For defatted bone ash (g), bone mineral density, and bone mineral content, pigs fed high levels of STTD P had numerically increased bone ash (g), bone mineral density, and bone mineral content compared to pigs fed the other diets, but no significant mean separation was observed ($P > 0.05$). For histopathology, there were no differences observed for cortical and trabeculae bone thickness (Table 3).

The response to treatment for bone density and bone mineral content was dependent upon the bone analyzed (density interaction, $P = 0.053$; mineral interaction, $P = 0.078$; Table 4). There were no treatment differences for bone density and bone mineral content for metacarpals, fibulas, and 2nd rib ($P > 0.05$). However, for 10th rib bone density, pigs fed high STTD P without added

vitamin D had increased ($P < 0.05$) bone density compared to pigs fed NRC (2012) levels with phytase, with pigs fed deficient STTD P, NRC (2012) STTD P with no phytase, or high STTD P with extra vitamin D from $25(OH)D_3$ intermediate. Pigs fed extra vitamin D from $25(OH)D_3$ had increased bone mineral content compared to pigs fed deficient STTD P or NRC (2012) STTD P with phytase, with pigs fed high STTD P without added vitamin D or NRC (2012) STTD P with monocalcium intermediate. There was no interaction observed for percentage bone ash, bone ash weight (g), grams of Ca and P in bone ash, and percentage of Ca and P in the bone ash ($P > 0.10$).

The defatted fibula had greater percentage bone ash compared to the 2^{nd} and 10^{th} ribs ($P < 0.05$), while the metacarpal had the lowest ($P < 0.05$) defatted percentage bone ash (Table 5). In non-defatted bones, percentage bone ash of the fibulas was greater than the 2^{nd} rib and metacarpal, and the 2^{nd} rib and 10^{th} rib both had greater ($P < 0.05$) percentage bone ash than the metacarpal. Metacarpals and 10^{th} ribs had increased ($P < 0.05$) Ca and P in the bone ash compared to the fibulas and 2^{nd} ribs.

For histopathology of the fibula, 2^{nd}, and 10^{th} ribs, there was no treatment differences observed ($P > 0.05$) for cortical bone thickness and trabeculae bone thickness; however, trabeculae and cortical bone thickness were greater for the fibula when compared to the 2^{nd} and 10^{th} ribs.

Health status

For serum analysis, pigs categorized as healthy had increased ($P < 0.05$; Table 6 and Figure 1) Ca, P, and $25(OH)D_3$ compared to pigs categorized as unhealthy, with no difference ($P > 0.05$) between the two categories for $1,25\text{-}(OH)_2D_3$. For urine Ca, there was no difference between health status ($P > 0.05$). Pigs identified as unhealthy, excreted more P and

creatinine in urine compared to healthy pigs ($P < 0.05$), which led to greater P:creatinine ratio than the healthy pigs ($P < 0.05$). Healthy pigs had increased bone density compared to unhealthy pigs ($P < 0.05$). There was no difference between healthy and unhealthy pigs for non-defatted bone ash ($P > 0.05$; Figure 2); conversely, healthy pigs had increased defatted bone ash ($P < 0.05$) compared to pigs classified as unhealthy. Healthy pigs had increased ($P < 0.05$) grams of Ca and P in bone ash compared to unhealthy pigs. There was no difference ($P > 0.05$) between healthy and unhealthy pigs for percentage Ca and P in bone ash.

Discussion

Because bone mineralization plays an important role in the development of lameness, (Crenshaw, 2001), it is important to understand differences between and variation within analytical procedures used to measure bone mineralization. Research in the mid-2000s demonstrated that femur bones were the most accurate bone used to predict whole body bone mineral content in growing pigs (Crenshaw et al., 2009). Due to the difficulty for diagnosticians to collect the femurs, we chose to collect fibulas, metacarpals, and ribs from all of the pigs sued in the analysis. More recently, Lee et al. (2021) observed that metacarpals, metatarsals, and tibias can be used to predict total body mineral content in 65 kg pigs. It is important to note, these authors only used 2 dietary levels of P to compare differences in bone mineralization. To more fully understand dietary variables that can affect bone mineralization, it is important to have a variety of dietary levels of P, phytase, and vitamin D. Previous research by Williams et al. (2023a) evaluated varying dietary levels of STTD P, phytase, and vitamin D for their impact on bone mineralization across different bones in nursery pigs. The authors observed that fibulas and 2nd ribs are more sensitive to differences in bone density and bone ash across dietary treatments compared to metacarpals and 10th ribs. Interestingly, they observed that differences in

histopathological lesions were more apparent in 10th ribs compared to the 2nd ribs and fibulas. Instead of nursery pigs, the current study was designed to understand the impact that various nutrients have on mineralization of different bones in growing and finishing pigs.

One common method to evaluate bone mineralization is bone density. The standard procedure for determining density has been the application of Archimedes' principle. This procedure uses the bone weight and bone weight fully submerged in water to calculate density of the bone. Dual-energy X-ray absorptiometry (DXA) can also determine bone mineral content (g) and bone mineral density (g/cm^2). This technique can differentiate lean muscle tissue from bone, so it does not require removing all of the extraneous soft tissues like the Archimedes principle does. Research in rats has shown a linear relationship when comparing bone density in femurs using DXA and Archimedes' principle (Keenan et al., 1997). A study in nursery pigs (Williams et al., 2023a) observed similar results across dietary treatments between Archimedes principle and DXA scans. For bone density using Archimedes principle, the authors observed a bone × treatment interaction, with fibulas and 2nd ribs being more sensitive to detect dietary P differences compared to metacarpals and 10th ribs. For DXA scans, there was no bone × treatment interaction observed, but DXA still detected significant dietary treatment differences. In the current study, utilizing similar dietary treatments as those used in the previous nursery pig study, we observed a tendency for bone × treatment interaction for bone density using either method. For bone density utilizing Archimedes principle or bone density via DXA scans, there was no difference between dietary treatments when analyzing metacarpals, fibulas, or 2nd rib. For 10th ribs, pigs fed high levels of STTD P and vitamin D had the greatest bone density, while pigs fed NRC (2012) levels with phytase had the lowest bone density. Based on the results from the current study, both methods can be utilized to measure bone density in finishing pigs to detect

dietary differences across different bones, with 10th ribs being the preferred bone to measure. Conversely, in nursery pigs, bone density using the Archimedes principle is the preferred method to detect dietary differences between bones, with fibulas and 2nd ribs being more sensitive to detect these differences compared to metacarpals and 10th ribs. The reason for the Archimedes principle being more sensitive in the 2nd ribs and fibulas to detect P differences could be due to the size and fat accumulation in the bone being lower than the metacarpals and 10th ribs.

Historically, bone ash has been the preferred measurement used to determine bone mineralization (Wu et al., 2018; Vier et al., 2019a,b; Lee et al., 2020). There are two ways to conduct bone ash analysis, one extracts all of the lipid and water from the bone using ether in a Soxhlet extractor (defatted), while the non-defatted method does not and the lipid remains inside the bone for analysis. Wensley et al. (2020) compared the two procedures in nursery and finishing pigs and observed that defatting reduces variation across the bones compared to non-defatting and that this is more apparent in finishing pigs than in nursery pigs. By reducing variation, this allows for smaller differences to be detected when conducting experiments. In the study by Williams et al. (2023a), when analyzing bones using the non-defatted method, there was no difference between percentage bone ash between pigs fed NRC (2012) STTD P levels and pigs fed 133% of the NRC requirement estimates. Conversely, when the same bones were analyzed using the defatted method, pigs fed 133% of the NRC (2012) requirement estimates had greater percentage bone ash compared to pigs fed the NRC (2012) requirement estimates. This demonstrates that by reducing the amount of variation across bones, a smaller magnitude of change can result in statistical differences. It is also important to understand which procedure is used when interpreting bone ash results from diagnostic labs. The difference between the two procedures is greater than the difference between dietary treatments. In the current study, the

defatted percentage bone ash was 14 to 15 percentage units greater than the same bone measured using the non-defatted method. Similar results were observed by Williams et al. (2023a) in nursery pigs. The authors observed that defatted bones had 11 to 12 percentage units greater ash than the same bones measured using the non-defatted method, which is similar to results observed by Wensley et al. (2020).

Knowing there are differences among bones that can be used to measure bone mineralization, previous research along with the current observations demonstrate the need to specify which bone is used for diagnostic references. Currently, there is limited data on reference values for different analyses and different bones for bone mineralization. In the current study, fibulas had the greatest defatted and non-defatted percentage bone ash, with metacarpals lowest, and 2nd and 10th ribs intermediate. These results were similar to the results observed by Williams et al. (2023a) in nursery pigs. Currently, reference values used by diagnosticians do not specify the procedures used and do not have different reference values for different bones measured. Depending on the bone measured, there can be a wide range of mineralization values across bones. When using the defatted bone ash method, the range between the metacarpal and the fibula was approximately 6 percentage units. Due to the range for defatted bone ash between bones, this may cause misinterpretation between diagnosticians, nutritionists, and producers depending on the bone analyzed. The majority of diagnosticians have adapted their reference values used for bone density and bone ash from 50 years ago with different genotype pigs than currently being produced (Field et al., 1974).

Currently, most nutritionists feed higher STTD P than NRC (2012) requirement estimates (Vier et al., 2019b). Research has shown that increasing dietary STTD P to approximately 125% of the NRC (2012) estimates increased growth performance and bone mineralization of growing

pigs raised in a commercial environment (Vier et al., 2019b). In the current study, the high STTD P levels were fed at approximately 130% the NRC (2012) estimate in each weight range. To observe bone mineralization differences across dietary treatments, it is routine to feed a P deficient diet. In the current study, the deficient diets were 80% of the NRC (2012) STTD P estimate, with their grams of P intake similar to NRC (2012) estimate requirements on a g/d basis. Interestingly, for growth performance and bone mineralization, there were limited differences between the pigs fed the deficient diets, recommended levels, or high levels. For bone density, there were no dietary differences observed for metacarpals, fibulas, or 2nd ribs, but there were differences observed within 10th ribs. Based on these results, we speculate that 80% of NRC (2012) estimates was not low enough for all response criteria. Other researchers had fed diets with P levels at 74% (Pokharel et al., 2017), 60% (Lee et al., 2021), or 57% (Williams et al., 2023a) of the recommended P levels for a shorter period of time. Each of these studies were able to detect significant reductions in growth performance and bone mineralization. In our study, there were concerns that providing low STTD P for an extended time in a commercial research facility where feed intake may be lower than in university settings had the potential to compromise animal welfare. Thus, 80% of STTD P requirement was used.

Measuring Ca, P, and vitamin D in serum and urine are potential non-invasive diagnostic techniques which can be used to assess mineral status of pigs. Calcium and P in urine can be used to monitor dietary excesses or deficiencies of these minerals (Grez-Capdeville and Crenshaw 2021; 2022). Phosphorus in urine is increased as dietary P is increased, whereas urinary Ca level decreases with increasing P consumption and remains low when P is fed at or above the requirement. In the current study, we observed no difference between dietary treatments for urinary Ca and P meaning there was no excess or deficient Ca and P being fed in

the diet. For vitamin D analysis, the standard measurement in serum is $25(OH)D_3$; however, there is recent interest in evaluating $1,25(OH)_2D_3$, the active form of $25(OH)D_3$, (Hurst et al., 2020). After absorption in the small intestine, vitamin D_3 undergoes an activation step in the liver by 25-hydroxylase. A second bioconversion process then takes place in the kidney by 1-alpha hydroxylase to convert to the active form, $1,25(OH)D_2D_3$. Research has shown that feeding $25(OH)D_3$ can increase $25(OH)D_3$ serum levels faster compared to feeding vitamin D_3 (Flohr et al., 2016; Thayer et al., 2018; Williams et al., 2023a). In the current study, pigs fed added $25(OH)D_3$ in the diet had the highest serum vitamin D compared to all other pigs, which were fed 1,654 IU/Kg of vitamin D_3. Currently, there are reference ranges used by diagnosticians for $25(OH)D_3$ but not the active form, $1,25(OH)D_3$. The primary role of $1,25(OH)D_3$ is the maintenance of Ca and P homeostasis. Due to this, $1,25(OH)D_3$ is tightly controlled via parathyroid hormone and fibroblast growth factor 23 (Hurst et al., 2020), which makes it difficult to establish a reference range. The reference range used for growing pigs for $25(OH)D_3$ is between 18 and 30 ng/mL (Arnold et al., 2015). Based on these reference values, all dietary treatments were within this range for serum 25(OH) except for pigs fed NRC STTD P with phytase.

Currently, there is limited research that compares expected diagnostic results for healthy vs unhealthy pigs. In the current study, 11 pigs diagnosed as unhealthy were sampled and results compared with healthy pigs. Unhealthy pigs were identified as those that were ineligible for marketing based on poor body condition, indicating a history of low feed intake. Gross necropsy was performed on all of these pigs, with varying gross lesions observed. The most common gross pathology was evidence of respiratory disease including cranioventral bronchopneumonia and/or interstitial pneumonia. No further histopathological or ancillary diagnostic testing was performed

on these pigs. By collecting all of the same samples from healthy and unhealthy pigs, we were able to compare diagnostic results. In serum, healthy pigs had greater Ca, P, and $25(OH)D_3$ compared to the unhealthy pigs. Based on reference values used by diagnosticians adapted from Arnold et al. (2015), the unhealthy pigs were below the reference value for $25(OH)D_3$. There was no difference between clinically healthy and unhealthy pigs for serum $1,25(OH)_2D_3$ or urine Ca. The unhealthy pigs were excreting excess P and creatinine in the urine compared to the healthy pigs. Creatinine is a waste product created from muscle tissue breakdown. Creatinine is produced from creatine and creatine is a protein needed to generate the energy for muscle contractions. When pigs have reduced energy intake, they will begin to use muscle tissue as a source of energy. Due to the unhealthy pigs having reduced feed intake, muscles were likely catabolized to use as an energy source. Due to the unhealthy pigs likely being in a catabolic state, the P is not being used for lean tissue deposition, which results in the P being excreted in the urine.

The second and tenth rib and fibula were collected for histopathologic evaluation; however, a large proportion of these bones, particularly in the ribs, lacked cartilage due to an error in sampling. In many cases, this precluded interpretation of the bone for lesions of metabolic bone disease. This may have prevented meaningful analysis of the usefulness of histopathology for the evaluation of metabolic bone disease in finishing pigs. More importantly, it highlights the necessity of proper sample collection in diagnostic investigations, as without appropriate animal and tissue selection and sampling a diagnosis may not be possible.

For bone mineralization measurements, unhealthy pigs had lower bone density and defatted bone ash compared to healthy pigs. Interestingly, a recent diagnostic survey comparing healthy, lame, and unhealthy pigs observed different results for bone ash comparing the two methods for percentage bone ash (Williams et al., 2023b). The authors observed a similar result

for bone density, with healthy pigs having increased bone density compared to unhealthy pigs. Interestingly, the authors found no statistical difference between the healthy and unhealthy pigs using the defatted bone ash method, but they did observe a statistical difference for the non-defatted bone ash, with the unhealthy pigs having increased percentage bone ash compared to the healthy pigs. The authors hypothesized this result is due to the unhealthy pigs mobilizing the lipid from their bones, due to a reduction in feed intake, and utilizing the lipid as an energy source leading to greater ash percentage compared to healthy pigs. This further shows the need to utilize the defatting method for percentage bone ash to standardize results across pigs with different health status.

Similar to the bone ash results, we observed that unhealthy pigs had lower grams of Ca and P in the bone ash compared to the healthy pigs. Conversely, when analyzing the percentage Ca and P in bone ash, there was no difference between the healthy and unhealthy pigs. Due to increased overall growth of healthy pigs compared to the unhealthy pigs, healthy pigs have increased bone mineralization. Increasing bone mineralization increases the size of the bone, which in turn increases the grams of Ca and P in the bone ash content but does not alter the percentage Ca and P in the bone. Due to bone hydroxyapatite, the bone maintains a constant Ca and P ratio of 2.01:1 (Crenshaw, 2001), which is consistent with recent data evaluating bone ash Ca and P mineral content (Blavi et al., 2019; Lee et al., 2020; Williams et al., 2023a).

In summary, the present data shows results from different analytical procedures and bones used to detect dietary P and vitamin D differences in finishing pigs. Bone density and bone mineral content responses varied depending on the bone. Similar results were observed for bone density regardless of whether Archimedes principle or DXA scans were used. Differences in bone density and mineral content in response to P and vitamin D were most apparent with the

10^{th} ribs. The difference between bone ash procedures was more apparent than the differences between diets. Bones that are analyzed using the defatted methods have 12 to 13 percentage units greater ash compared to analyzing with the non-defatted procedure. Defatting the bones reduces the amount of variation compared to non-defatted and in our opinion is the preferred method to be used when measuring percentage bone ash. Also, there are significant differences in diagnostic measurements between healthy and unhealthy pigs. This data demonstrates there are clear differences between analytical methods and bone measured. These results between healthy and unhealthy pigs warrant further diagnostic research to update reference values used by diagnosticians, nutritionists, and producers.

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Table 3.1. Diet composition, phases 1 and 2 (as-fed basis)[1]

	Phase 1					Phase 2				
STTD P:	80% NRC	NRC	NRC	High	High + 25(OH)D_3	80% NRC	NRC	NRC	High	High + 25(OH)D_3
Phytase:	No	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes
Ingredients, %										
Corn	70.15	69.75	70.60	70.05	70.05	74.85	74.50	75.25	74.85	74.85
Soybean meal, 46.5% CP	27.45	27.45	27.45	27.45	27.45	22.95	22.95	22.95	22.95	22.95
Calcium carbonate	0.65	0.71	0.56	0.65	0.65	0.66	0.72	0.59	0.65	0.65
Monocalcium phosphate	0.45	0.75	---	0.45	0.45	0.35	0.65	---	0.28	0.28
Salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
L-Lys-HCl	0.30	0.30	0.30	0.30	0.30	0.28	0.28	0.28	0.28	0.28
DL-Met	0.09	0.09	0.09	0.09	0.09	0.05	0.05	0.05	0.05	0.05
L-Thr	0.12	0.12	0.12	0.12	0.12	0.10	0.10	0.10	0.10	0.10
L-Trp	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Trace mineral premix[2]	0.15	0.15	0.15	0.15	0.15	0.13	0.13	0.13	0.13	0.13
Vitamin premix[3]	0.15	0.15	0.15	0.15	0.15	0.13	0.13	0.13	0.13	0.13
Phytase[4]	---	---	0.08	0.08	0.08	---	---	0.03	0.08	0.08
25(OH)D_3[5]	---	---	---	---	0.04	---	---	---	---	0.03
Calculated analysis										
SID Lys, %	1.03	1.03	1.03	1.03	1.03	0.91	0.91	0.91	0.91	0.91
NE, kcal/kg	2,465	2,465	2,478	2,463	2,462	2,494	2,485	2,505	2,495	2,494
Ca, %	0.54	0.62	0.43	0.54	0.54	0.50	0.57	0.41	0.48	0.48
P, %	0.45	0.52	0.36	0.45	0.45	0.41	0.48	0.34	0.40	0.40
Ca:P	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20
STTD P, %[6]	0.23	0.29	0.29	0.37	0.37	0.20	0.26	0.26	0.33	0.33

[1]Phase 1 was fed from 36 to 54 kg. Phase 2 was fed from 54 to 72 kg.

[2] Provided per kg of premix: 73.41 g of Zn from zinc sulfate; 73.41 g Fe from iron sulfate; 22.05 g Mn from manganese oxide; 11.02 mg Cu from copper sulfate; 1.98 g I from calcium iodate; 1.98 mg Se from sodium selenite.

[3]Vitamin premix contributed 992 IU/kg of vitamin D_3 in the diet when the premix was included at 0.15% and 827 IU/kg of vitamin D_3 in the diet when the premix was included at 0.13%. Provided per kg of premix: 340,203 IU vitamin A; 136,081 IU vitamin D; 3,628 IU vitamin E; 272 mg vitamin K; 2.7 mg vitamin B12; 4082 mg niacin; 2,268 mg pantothenic acid; 680 mg riboflavin.

[4]Ronozyme HiPhos 2700 (DSM Nutritional Products, Parsippany, NJ) was included at 2,000 FYT/kg with an assumed release of 0.14% STTD P when included in the diet at 0.08%. When phytase was provided in the diet at 0.03% it contributed 770 FYT/kg with an assumed release of 0.12% STTD P.

[5]HyD provided 2,000 IU/kg of vitamin D equivalency from 50 µg/kg of 25(OH)D_3 to the diet (DSM Nutritional Products, Parsippany, NJ) when included in the diet at 0.04%. When HyD was included in the diet at 0.03%, it provided 1,680 IU/kg of vitamin D equivalency from 42 µg/kg of 25(OH)D_3.

[6]Standardized total tract digestible phosphorus.

Table 3.2. Diet composition, phases 3 and 4 (as-fed basis)[1]

	Phase 3					Phase 4				
STTD P:	80% NRC	NRC	NRC	High	High + 25(OH)D_3	80% NRC	NRC	NRC	High	High + 25(OH)D_3
Phytase[3]:	No	No	Yes	Yes	Yes	No	No	Yes	Yes	Yes
Ingredients, %										
Corn	82.65	82.40	83.15	82.90	82.85	85.30	85.00	85.70	85.60	85.60
Soybean meal, 46.5% CP	14.95	14.95	14.95	14.95	14.95	12.50	12.50	12.50	12.50	12.50
Calcium carbonate	0.71	0.75	0.63	0.65	0.65	0.73	0.76	0.65	0.65	0.65
Monocalcium phosphate	0.42	0.63	---	0.15	0.15	0.35	0.57	---	---	---
Salt	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
L-Lys-HCl	0.35	0.35	0.35	0.35	0.35	0.33	0.33	0.33	0.33	0.33
DL-Met	0.05	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.03	0.03
L-Thr	0.12	0.12	0.12	0.12	0.12	0.10	0.10	0.10	0.10	0.10
L-Trp	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
L-Val	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.02
Trace mineral premix[2]	0.10	0.10	0.10	0.10	0.10	0.08	0.08	0.08	0.08	0.08
Vitamin premix[3]	0.10	0.10	0.10	0.10	0.10	0.08	0.08	0.08	0.08	0.08
Phytase[4]	---	---	0.03	0.08	0.08	---	---	0.02	0.08	0.08
25(OH)D_3[5]	---	---	---	---	0.02	---	---	---	---	0.02
Calculated analysis										
SID Lys, %	0.79	0.79	0.79	0.79	0.79	0.72	0.72	0.72	0.72	0.72
NE, kcal/kg	2,541	2,534	2,554	2,548	2,547	2,557	2,550	2,568	2,567	2,566
Ca, %	0.48	0.53	0.37	0.41	0.41	0.45	0.51	0.36	0.36	0.36
P, %	0.40	0.44	0.31	0.34	0.34	0.38	0.42	0.30	0.30	0.30
Ca:P	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20
STTD P, %[6]	0.20	0.24	0.24	0.29	0.29	0.18	0.22	0.22	0.25	0.25

[1]Phase 3 was fed from 72 to 99 kg. Phase 4 was fed from 99 to 127 kg.

[2]Provided per kg of premix: 73.41 g of Zn from zinc sulfate; 73.41 g Fe from iron sulfate; 22.05 g Mn from manganese oxide; 11.02 mg Cu from copper sulfate; 1.98 g I from calcium iodate; 1.98 mg Se from sodium selenite.

[3]Vitamin premix contributed 661 IU/kg of vitamin D_3 in the diet when the premix was included at 0.10% and 496 IU/kg of vitamin D_3 in the diet when the premix was included at 0.08%. Provided per kg of premix: 340,203 IU vitamin A; 136,081 IU vitamin D; 3,628 IU vitamin E; 272 mg vitamin K; 2.7 mg vitamin B12; 4082 mg niacin; 2,268 mg pantothenic acid; 680 mg riboflavin.

[4]Ronozyme HiPhos 2700 (DSM Nutritional Products, Parsippany, NJ) was included at 2,000 FYT/kg with an assumed release of 0.14% STTD P when included in the diet at 0.08%. When phytase was included in the diet at 0.03% it provided 770 FYT/kg with an assumed release of 0.12% STTD P. When phytase was included in the diet at 0.02% it provided 490 FYT/kg with an assumed release of 0.10 STTD P.

[5] HyD provided 1,340 IU/kg of vitamin D equivalency from 34 µg/kg of 25(OH)D_3 to the diet (DSM Nutritional Products, Parsippany, NJ) when included in the diet at 0.02%. When HyD was included in the diet at 0.0185%, it provided 1,013 IU/kg of vitamin D equivalency from 25 µg/kg of 25(OH)D_3.

[6]Standardized total tract digestible phosphorus.

Table 3.3. Effect of STTD P, phytase, and vitamin D on growth performance, serum, and bone analysis of finishing pigs[1,2]

STTD P:	80% NRC	NRC	NRC	High	High + 25(OH)D_3[3]		
Phytase:	No	No	Yes	Yes	Yes	SEM	*P* =
Body weight, kg							
d 0	33.2	33.2	33.2	33.1	33.1	0.31	0.994
d 112	130.3	130.4	130.1	130.4	132.5	1.87	0.891
Overall							
ADG, kg	0.90	0.90	0.90	0.90	0.92	0.014	0.810
ADFI, kg	2.35	2.35	2.38	2.35	2.39	0.046	0.945
G:F	0.38	0.38	0.38	0.39	0.39	0.004	0.782
Serum[4]							
Ca, mg/dL	11.6^{a}	11.0^{ab}	10.9^{b}	11.4^{ab}	11.0^{ab}	0.17	0.030
P, mg/dL	8.4^{ab}	9.4^{a}	8.9^{ab}	9.0^{ab}	8.0^{b}	0.33	0.036
25(OH)D_3, ng/mL	19.3^{b}	18.1^{b}	16.6^{b}	20.7^{b}	37.8^{a}	1.80	< 0.001
1,25-$(OH)_2D_3$, pg/mL	213	151	160	161	133	23.6	0.147
Urine							
Ca, mg/dL	63.6	44.2	37.7	11.8	47.9	17.73	0.294
P, mg/dL	4.0	23.5	19.4	7.3	7.5	8.08	0.311
Creatinine, mg/dL	250^{ab}	292^{ab}	365^{a}	174^{b}	272^{ab}	46.8	0.074
Ca:creatinine	0.38	0.16	0.13	0.08	0.18	0.078	0.081
P:creatinine	0.02	0.09	0.04	0.06	0.03	0.026	0.378
Defatted bone ash[5]							
Bone ash, g[9]	6.26	6.61	6.33	6.98	6.90	0.260	0.017
Bone ash, %	62.8	63.9	63.1	63.3	63.4	0.26	0.219
Non-defatted bone ash[6]							
Bone ash, g	6.88	7.39	6.79	7.46	7.53	0.328	0.168
Bone ash, %	48.0	49.8	47.1	49.9	49.7	0.94	0.327
Bone density, g/mL	1.35	1.37	1.34	1.39	1.37	0.013	0.059
Dual-energy X-ray absorptiometry							
Bone mineral density, g/cm^{2}[2,9]	0.18	0.19	0.19	0.20	0.21	0.007	0.028
Bone mineral content, g[9]	2.07	2.15	2.17	2.31	2.39	0.111	0.002
Bone ash content[7]							
Ca, g	2.60	2.54	2.39	2.51	2.71	0.131	0.119

P, g	1.25	1.23	1.16	1.21	1.31	0.064	0.115
Ca, %	39.5	39.1	38.4	37.2	39.4	1.51	0.758
P, %	19.1	18.9	18.7	18.1	19.1	0.75	0.803
Histopathology[8]							
Trabeculae bone thickness, µm	46.8	48.6	48.3	55.5	48.4	2.16	0.244
Cortical bone thickness, µm	139.1	141.2	141.8	147.8	145.6	5.21	0.729

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

[1]A total of 882 pigs were used in a 112-d finishing trial with 20 pigs per pen and 9 pens per treatment. Pigs were placed on experimental diets at approximately 33 kg. One pig per pen (9 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.

[2]BW = body weight. ADG = average daily gain. ADFI = average daily feed intake; G:F = gain to feed ratio.

[3]High STTD P levels were fed at 128, 127, 121, and 114% of the NRC (2012) recommendation based on data from Vier et al. (2019b) for diets 1 through 4, respectively. HyD provided 2,000 IU/kg of vitamin D equivalency from 50 µg/kg of 25(OH)D_3 to the diet (DSM Nutritional Products, Parsippany, NJ) when included in the diet at 0.04%. When HyD was included in the diet at 0.03%, it provided 1,680 IU/kg of vitamin D equivalency from 42 µg/kg of 25(OH)D_3. HyD provided 1,340 IU/kg of vitamin D equivalency from 34 µg/kg of 25(OH)D_3 when included in the diet at 0.02%. When HyD was included in the diet at 0.0185%, it provided 1,013 IU/kg of vitamin D equivalency from 25 µg/kg of 25(OH)D_3.

[4]Serum Ca and P were measured at the Iowa State University Veterinary Diagnostic Lab (Ames, IA). The vitamin D serum analysis was conducted at Heartland Assays (Ames, IA).

[5]All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105°C for 7 d and then ashed in a muffle furnace at 600°C for 24 h.

[6]All bones were cleaned of tissue and then dried at 105°C for 7 d and then ashed in a muffle furnace at for 600°C 24 h.

[7]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.

[8]Histopathological evaluation of the physis of the left fibula, 2nd rib, and 10th rib was performed by three trained anatomic pathologists.

[9]No mean separation was detected due to Tukey adjustment.

Table 3.4. Interactive effects of STTD P, phytase, and vitamin D on bone mineral assessment

STTD P:	80% NRC[1]	NRC	NRC	High	High + 25(OH)D_3[2]	
Phytase:	No	No	Yes	Yes	Yes	SEM
Non-defatted bone ash, %[3]						1.28
Metacarpal	39.8	42.9	41.1	42.6	42.8	
Fibula	51.2	53.0	52.6	53.4	54.3	
2nd rib	49.2	51.0	50.0	51.9	49.6	
10th rib	51.8	52.3	48.9	51.5	52.1	
Non-defatted bone ash, g[4]						0.421
Metacarpal	8.50	9.01	8.50	9.04	9.08	
Fibula	4.86	5.37	4.78	5.32	5.42	
2nd rib	4.91	5.36	5.16	5.77	5.50	
10th rib	9.24	9.81	8.74	9.71	10.12	
Defatted bone ash, %[5]						0.488
Metacarpal	60.8	61.0	60.6	60.5	61.6	
Fibula	66.3	67.1	66.5	66.9	66.8	
2nd rib	62.1	63.7	62.5	62.9	62.1	
10th rib	62.2	63.7	62.8	63.1	63.2	
Defatted bone ash, g[4]						0.337
Metacarpal	8.54	8.62	8.82	9.08	9.07	
Fibula	4.26	4.56	4.18	4.76	4.49	
2nd rib	4.01	4.27	4.11	4.84	4.49	
10th rib	8.22	8.98	8.23	9.25	9.55	
Bone density, g/mL[6]						0.016
Metacarpal	1.28	1.31	1.30	1.32	1.31	
Fibula	1.43	1.46	1.45	1.48	1.49	
2nd rib	1.30	1.33	1.30	1.34	1.30	
10th rib	1.37^{ab}	1.37^{ab}	1.33^{b}	1.40^{a}	1.38^{ab}	
Dual-energy X-ray absorptiometry						
Bone mineral content, g[7]						0.200
Metacarpal	5.09	5.10	5.69	5.33	5.26	
Fibula	0.69	0.82	0.77	0.77	0.93	
2nd rib	0.12	0.16	0.12	0.23	0.21	
10th rib	2.39^{bc}	2.53^{abc}	2.09^{c}	2.92^{ab}	3.18^{a}	
Bone mineral density, g/cm[4]						0.012
Metacarpal	0.33	0.34	0.37	0.36	0.34	
Fibula	0.12	0.13	0.13	0.12	0.15	
2nd rib	0.11	0.13	0.14	0.14	0.14	
10th rib	0.15^{ab}	0.16^{ab}	0.13^{b}	0.17^{ab}	0.19^{a}	
Bone ash content						
P, g[4]						0.118

Metacarpal	1.71	1.57	1.50	1.37	1.66	
Fibula	0.90	0.86	0.67	0.95	0.81	
2nd rib	0.83	0.87	0.72	0.86	0.82	
10th rib	1.56	1.61	1.57	1.67	1.95	
Ca, g[4]						0.254
Metacarpal	3.61	3.29	3.14	2.89	3.50	
Fibula	1.84	1.79	1.76	1.97	1.66	
2nd rib	1.69	1.79	1.46	1.76	1.66	
10th rib	3.25	3.31	3.20	3.40	4.00	
P, %[4]						1.57
Metacarpal	18.4	18.6	17.1	15.3	18.6	
Fibula	21.1	19.0	20.4	20.0	17.8	
2nd rib	18.1	20.1	18.2	18.7	19.5	
10th rib	18.7	18.0	19.1	18.2	20.4	
Ca, %[4]						3.15
Metacarpal	38.9	39.0	35.9	32.4	39.3	
Fibula	43.3	39.5	41.3	41.3	36.8	
2nd rib	36.9	41.1	37.1	38.3	39.6	
10th rib	39.0	36.9	39.2	36.9	41.8	

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

[1] National Research Council. 2012.

[2] High STTD P levels were fed at 128, 127, 121, and 114% of the NRC (2012) recommendation based on data from Vier et al. (2019b) for diets 1 through 4, respectively. HyD provided 2,000 IU/kg of vitamin D equivalency from 50 µg/kg of 25(OH)D_3 to the diet (DSM Nutritional Products, Parsippany, NJ) when included in the diet at 0.04%. When HyD was included in the diet at 0.03%, it provided 1,680 IU/kg of vitamin D equivalency from 42 µg/kg of 25(OH)D_3. HyD provided 1,340 IU/kg of vitamin D equivalency from 34 µg/kg of 25(OH)D_3 when included in the diet at 0.02%. When HyD was included in the diet at 0.0185%, it provided 1,013 IU/kg of vitamin D equivalency from 25 µg/kg of 25(OH)D_3.

[3]All bones were cleaned of tissue and then dried at 105°C for 7 d and then ashed in a muffle furnace at 600°C for 24 h. Bone × treatment, $P = 0.542$.

[4]Bone × treatment, $P > 0.10$.

[5]All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105°C for 7 d, and then ashed in a muffle furnace at 600°C for 24 h. Bone × treatment, $P = 0.702$.

[6]Bone density was measured on each bone based on Archimedes' principle. Bone × treatment, $P = 0.053$.

[7]Bone × treatment, $P = 0.078$.

Table 3.5. Effect of different bones on analysis of finishing pigs[1]

	Metacarpal	Fibula	2nd rib	10th rib	SEM	*P* =
Defatted bone ash[2]						
Bone ash, g	8.82[a]	4.45[b]	4.34[b]	8.85[a]	0.152	< 0.001
Bone ash, %	60.9[c]	66.7[a]	62.6[b]	63.0[b]	0.208	< 0.001
Non-defatted bone ash[3]						
Bone ash, g	8.83[b]	5.15[c]	5.34[c]	9.52[a]	0.190	< 0.001
Bone ash, %	41.8[c]	52.9[a]	50.4[b]	51.3[ab]	0.57	< 0.001
Dual-energy X-ray absorptiometry						
Bone mineral density, g/cm^2	0.35[a]	0.13[c]	0.13[c]	0.16[b]	0.005	< 0.001
Bone mineral content, g	5.29[a]	0.80[c]	0.17[d]	2.62[b]	0.090	< 0.001
Bone ash content[4]						
Ca, g	3.29[a]	1.80[b]	1.67[b]	3.43[a]	0.108	< 0.001
P, g	1.56[a]	0.88[b]	0.82[b]	1.67[a]	0.053	< 0.001
Ca, %	37.1	40.4	38.6	37.8	1.34	0.482
P, %	17.6	19.7	18.9	18.9	0.67	0.472
Histopathology[5]						
Trabeculae bone thickness, mm	---	65.2[a]	41.4[b]	42.0[b]	1.34	< 0.001
Cortical bone thickness, mm	---	167.1[a]	130.7[b]	131.4[b]	3.27	0.001

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

[1]A total of 882 pigs were used in a 112-d finishing trial with 20 pigs per pen and 9 pens per treatment. Pigs were placed on experimental diets at approximately 33 kg. One pig per pen (9 pigs per treatment) was euthanized and the left and right metacarpal, fibula, 2nd rib, and 10th rib were collected to determine bone ash weight and percentage bone ash.

[2]All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105°C for 7 d and then ashed in a muffle furnace at 600°C for 24 h.

[3]All bones were cleaned of tissue and then dried at 105°C for 7 d and then ashed in a muffle furnace at 600°C for 24 h.

[4]After bone ash was completed, the ash was digested and analyzed for Ca and P using ICP by the KSU Soils lab (Manhattan, KS).

[5]Histopathological evaluation of the physis of the left fibula, 2nd rib, and 10th rib was performed by three trained anatomic pathologists.

Table 3.6. Comparison of healthy and unhealthy pigs on bone mineralization assesment[1]

	Healthy	Unhealthy	SEM	*P* =
Serum[2]				
Ca, mg/dL	11.2	10.2	0.20	< 0.001
P, mg/dL	8.7	7.2	0.34	< 0.001
25(OH)D_3, ng/mL	22.5	11.0	2.71	< 0.001
1,25-$(OH)_2D_3$, pg/mL	162.5	151.8	23.02	0.674
Urine				
Ca, mg/dL	41.1	36.4	17.42	0.804
P, mg/dL	12.2	96.1	14.86	< 0.001
Creatinine, mg/dL	268	520	99.7	0.025
Ca:creatinine	0.19	0.11	0.079	0.396
P:creatinine	0.05	0.31	0.061	< 0.001
Bone density, g/cm^3	1.36	1.31	0.014	0.001
Non-defatted bone ash, %[4]	49.1	47.5	0.971	0.144
Defatted bone ash, %[4]	63.3	60.1	0.327	< 0.001
Bone ash content[5]				
Ca, g	2.55	2.07	0.134	0.002
P, g	1.23	0.99	0.065	0.001
Ca, %	38.7	38.9	1.32	0.892
P, %	18.8	18.7	0.659	0.975

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

[1]A total of 882 pigs were used in a 112-d finishing trial with 20 pigs per pen and 9 pens per treatment. Pigs were placed on experimental diets at approximately 33 kg. On d 112, 45 clinically healthy and 11 clinically unhealthy pigs were euthanized, and their blood, urine, and bones were analyzed. Of the 11 unhealthy pigs, 4 pigs came from the 80% NRC STTD P treatment, 2 from both the NRC P treatments, and 3 from the High STTD P and high 25(OH)D_3 treatment.

[2]Serum Ca and P were measured at Iowa State Veterinary Diagnostic Lab (Ames, IA). The vitamin D serum analysis was conducted at Heartland Assays (Ames, IA).

[3]Bone Density was measured on each bone based on Archimedes principle.

[4]All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105℃ for 7 d and then ashed in a muffle furnace at 600℃ for 24 h.

[5]After bone ash was completed, the ash was digested and analyzed for Ca and P using ICP by the KSU Soils lab (Manhattan, KS).

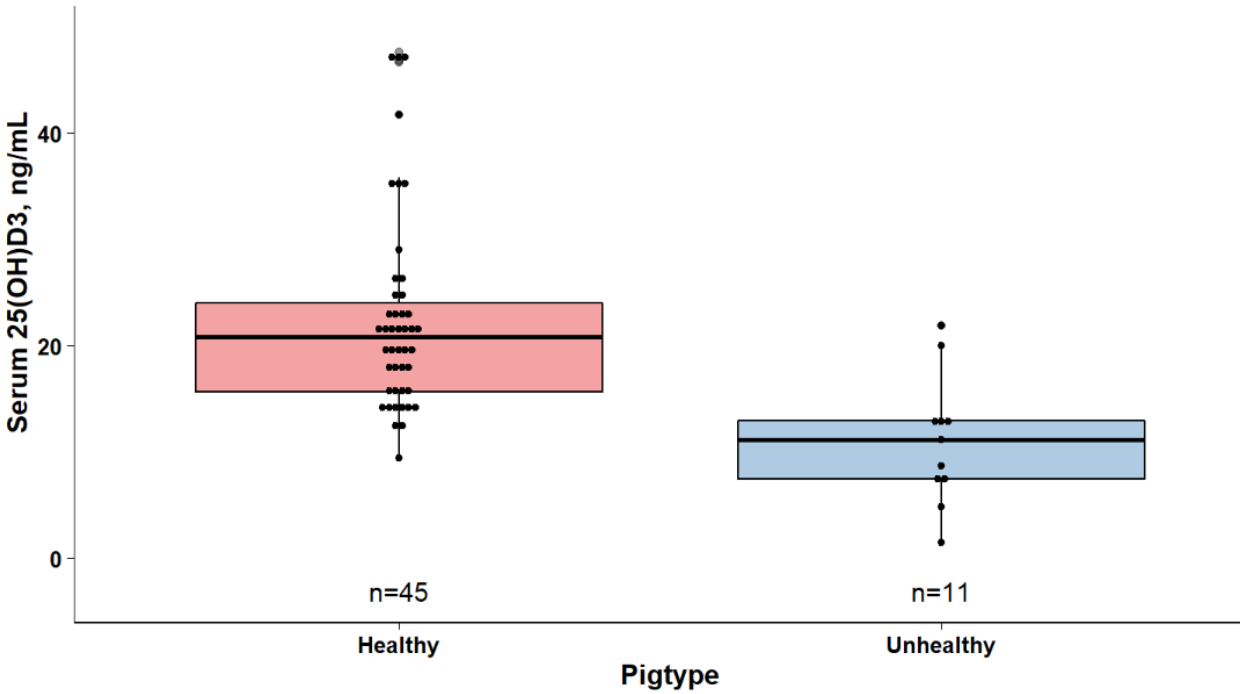

Figure 3.1. Serum 25(OH)D_3 of healthy and unhealthy pigs. A total of 45 and 11 pigs were used for the healthy and unhealthy pigs, respectively.

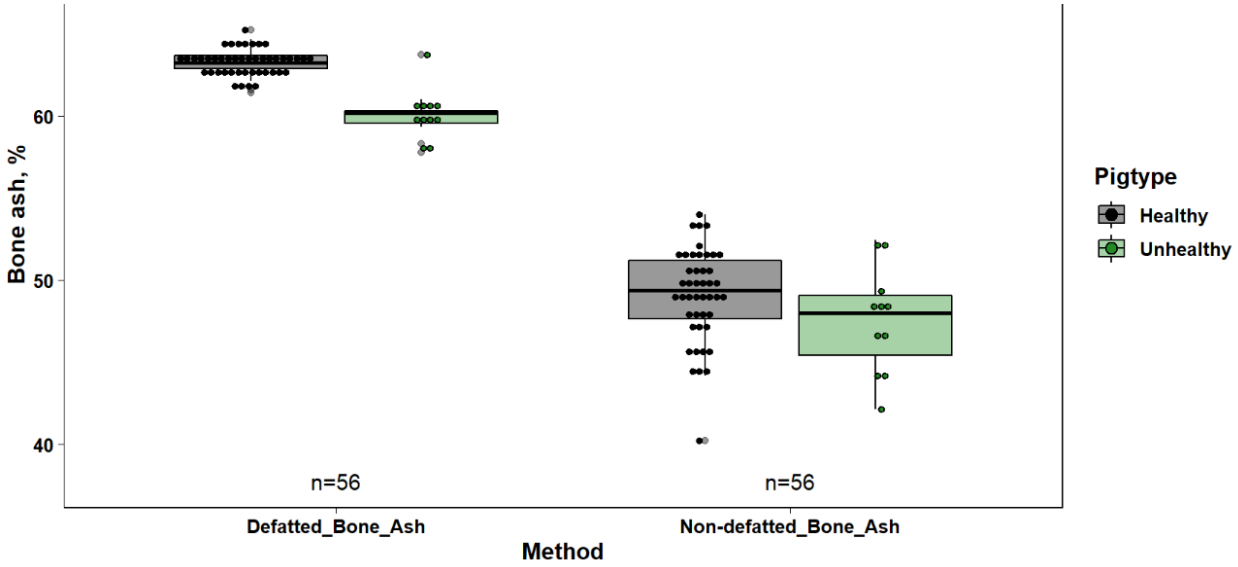

Figure 3.2. Percentage bone ash of healthy and unhealthy pigs within non-defatted and defatted bone ash procedures. A total of 45 and 11 pigs were used for the healthy and unhealthy pigs, respectively.

Chapter 4 - Diagnostic survey of biological measurements used to determine bone mineralization in pigs across the US swine industry

Abstract

Pigs from 64 commercial sites across 14 production systems in the Midwest US were used to evaluate baseline biological measurements used to determine bone mineralization. There were 3 pigs selected from each commercial site representing: 1) a clinically normal pig (healthy), 2) a pig with evidence of clinical lameness (lame), and 3) a pig from a hospital pen that is assumed to have recent low feed intake (unhealthy). Pigs ranged in age from nursery to market weight, with the three pigs sampled from each site representing the same age or phase of production. Blood, urine, metacarpal, fibula, 2nd rib, and 10th rib were collected and analyzed. Serum was analyzed for Ca, P, and 25(OH)D_3, and urine was collected and analyzed for Ca, P, and creatinine. Each bone was measured for density, ash (defatted and non-defatted technique), and breaking strength. A bone × pig type interaction ($P < 0.001$) was observed for defatted and non-defatted bone ash, density, and breaking strength. For defatted bone ash, there were no differences between pig types for the fibulas, 2nd rib, and 10th rib ($P > 0.10$), but metacarpals from healthy pigs had greater ($P < 0.05$) percentage bone ash compared to unhealthy pigs, with the lame pigs intermediate. For non-defatted bone ash, there were no differences between pig types for metacarpals and fibulas ($P > 0.10$), but unhealthy pigs had greater ($P < 0.05$) non-defatted percentage bone ash for 2nd and 10th ribs compared to healthy pigs, with lame pigs intermediate. Healthy and lame pigs had greater ($P < 0.05$) bone density than unhealthy pigs for metacarpals and fibulas, with no difference observed for ribs ($P > 0.10$). Healthy pigs had increased breaking strength compared to lame and unhealthy pigs for metacarpals and 10th ribs

($P < 0.05$) with no differences between pig types for fibula and 2nd rib ($P > 0.05$). Healthy pigs had greater ($P < 0.05$) serum Ca and 25(OH)D_3 compared to unhealthy pigs, with lame pigs intermediate. Healthy pigs had greater ($P > 0.05$) serum P compared to unhealthy and lame pigs, with no differences between the unhealthy and lame pigs. Unhealthy pigs excreted more ($P < 0.05$) P and creatinine in the urine compared to healthy pigs with lame pigs intermediate. In summary, there are differences in serum Ca, P, and vitamin D between healthy, lame, and unhealthy pigs. Differences in bone mineralization between pig types varied depending on the analytical procedure and bone. There was considerable range in values within pig type across the 14 production systems sampled.

Introduction

Due to improper bone mineralization in pigs, producers in the US swine industry experience economic losses and animal welfare concerns. Diagnosticians rely on bone mineralization as a diagnostic tool for identifying cases of lameness in pigs. However, because numerous factors can contribute to lameness, identifying the underlying cause can be difficult. There are different diagnostic assessments to measure bone mineralization, including bone ash, density, and histopathological examination; however, there is limited data comparing these different measurements across pigs with different health status. Recent work evaluating bone mineralization has compared the difference between bone ash procedures available for use. Wensley et al. (2020) observed differences in percentage bone ash when comparing bones that had the lipid extracted (defatted) vs. those without the lipid extraction (non-defatted) before ashing of the bone. The authors observed a reduction in standard deviation when the bone is defatted before ashing, and this response was more apparent in finishing pigs compared to nursery pigs due to the increased fat accumulation in bones as pigs grow. Also, Williams et al.

(2023a) observed differences in percentage bone ash and bone density among fibulas, metacarpals, 2nd ribs, and 10th ribs in nursery pigs and differences between analytical methods. Similar to Wensley et al. (2020), defatting bones prior to ashing reduced the amount of variation between the bones compared to not extracting the lipid.

Currently, reference values used by diagnosticians for bone mineralization and serum vitamin D have been adapted from Field et al. (1974) and Arnold et al. (2015). Typically, the pigs submitted to diagnostic laboratories are unhealthy or lame and their recent dietary intakes are unknown but likely reduced and highly variable, possibly causing their nutritional status or bone mineralization to be lower than that of healthier pigs in the general population. Thus, interpretation of these results may potentially lead to misdiagnosis relative to the entire population. Recently, Williams et al. (2023b) evaluated different bones and various analytical measurements between healthy and unhealthy pigs. The authors observed that healthy pigs have increased serum Ca, P, and vitamin D compared to unhealthy pigs. They also observed no statistical difference between the health statuses for non-defatted bone ash; however, healthy pigs had increased defatted bone ash compared to the unhealthy pigs. Therefore, our objective in the current study was to evaluate the effect of different pig types (healthy, lame, or unhealthy) from pigs in commercial production sites across the Midwestern US on the assessment of bone mineralization. Furthermore, this study evaluated the effect of different bones and analytical methods on the assessment of bone mineralization in growing pigs.

Materials and Methods

General

The Kansas State University Animal Care and Use Committee approved the protocol used in this study (IACUC no. 4595). The diagnostic survey utilized pigs from commercial production facilities in the Midwest USA.

A total of 192 pigs from 64 commercial production sites across 14 different production systems in Minnesota and Iowa were used in the diagnostic survey. All samples were collected from December 2021 to February 2022. At each site, the staff veterinarian selected 3 pigs to be euthanized and used in the diagnostic survey: 1) a clinically normal pig, 2) a pig with locomotive issues and evidence of clinical lameness, and 3) a pig from within a hospital pen with an assumed recent low feed intake (unhealthy pig). Pigs ranged in age from nursery to market weight, with the three pigs sampled from each site representing the same age and phase of production.

Sample collection

A blood sample was taken from the jugular vein of each pig prior to euthanasia. Blood samples (10 mL) were centrifuged (3000 × *g*, 15 min) within 2 h after collection and serum was stored at -20°C until analysis. Serum samples were split into three, 2 mL aliquots and analyzed for Ca and P (Iowa State University Veterinary Diagnostic Lab) and 25(OH)D_3 (Heartland Assays, Ames, IA). A 10 mL urine sample was collected shortly after euthanasia via cystocentesis using a 20 gauge × 2.5 cm needle and a 12 mL syringe, then frozen at -20°C until analyzed for Ca, P, and creatinine (Iowa State University Veterinary Diagnostic Lab, Ames IA).

The metacarpal, 2nd rib, 10th rib, and fibulas were analyzed for bone density, bone-breaking strength, bone ash (de-fatted and non-defatted), and bone Ca and P. The leftover extraneous soft tissues and cartilage were removed from the bones prior to assessment. Archimedes principle was used for bone density. Briefly a dry bone weight was collected, then

bones were submerged in ultra-purified water under a vacuum with 1.06 kg per cubic centimeter for a minimum of 4 h. Bones were then weighed while suspended in a vessel of ultra-purified water, and the weight used to calculate bone density. Bone breaking strength was determined with an Instron (Instron 5569, NV Lab, Norwood, MA). Briefly each bone was held by two supports spaced 30 mm apart and were broken by a wedge lowered on the center of the bone at a speed of 100 mm per min and a maximum pressure of 5,000 kg. The force was measured by a pressure-sensitive cell, and peak of maximum force was recorded. For bone ash, both the defatted and the non-defatted methods were used (Williams et al., 2023c). For defatted bone ash, all bones were placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105˚C for 7 d and then ashed in a muffle furnace at 600˚C for 24 h. For non-defatted bone ash, all bones were dried at 105˚C for 7 d and then ashed in a muffle furnace at 600˚C for 24 h. These methods were used to determine total bone ash weight and percentage ash relative to dried bone weight. For bone Ca and P, a portion of the residual defatted bone ash (0.05 g) from the metacarpal and 10^{th} ribs were placed in a tube with nitric acid and incubated at 60˚C for 6 h. The solution was then diluted using a 20x dilution of ultra-purified water and the Ca (AOAC 985.01, 2006) and P (AOAC 985.01, 2006) quantity were measured by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Concentrations of Ca and P were calculated as a percentage of the total bone ash.

Survey information

Information regarding the health status of each pig and building design from which it was obtained was recorded. The building design was used to determine if the pigs had access to UV light or not. The two main forms of building types were curtain sided or tunnel ventilated. Pigs in

curtain barns had limited access to sunlight due to the time of the year of sample collections, while those in tunnel ventilated barns had minimal access to sunlight.

Information was gathered to characterize the formulated dietary levels of total Ca, standardized total tract digestible (STTD) P, vitamin D_3, and total vitamin D in the diets for all phases of production of the pigs sampled. The total vitamin D in the diet included vitamin D provided in the form of D_3 and any vitamin D equivalency provided by supplementing $25(OH)D_3$ (HyD, DSM Nutritional Products, Parsippany, NJ) in the diet. When added, 50 µg/kg of $25(OH)D_3$ was estimated to provide an additional 2,000 IU/kg of vitamin D activity (NRC, 2012). The weight range for each phase was provided by the production system, and the same average daily gain (ADG) curve was used to calculate the number of days on feed for each phase for each pig all the systems. With the numbers of days on feed in each phase, a weighted average intake of dietary Ca, P, and vitamin D was calculated until the time of sample collection. Each participant also included the phytase source, inclusion, and release values given for each dietary phase when applicable.

Total calcium, STTD P, and vitamin D within each phase of production were compared to NRC (2012) total dietary requirement estimates to quantify supplementation rates compared to published requirement estimates. A weighted average of each dietary nutrient as a percentage of the NRC (2012) requirement estimate was calculated, in which the dietary nutrient percentage compared to NRC requirement estimate was multiplied by the days on feed in each phase and divided by the total number of days on feed until the time of collection occurred. If any pig was collected during the middle of the phase, partial phase consumption was calculated. The weighted average of each dietary nutrient for each pig was used to correlate the relationship between dietary nutrients and bone analysis measured. The interaction between multiple

nutrients and bone analysis was also tested to understand the relationship these nutrients have on different bones and different measurements.

Statistical analysis

For the statistical analysis, bone data were analyzed by fitting a linear mixed model using the lmer function from the lme4 package in R (version 3.5.1 (2018-07-02), R Foundation for Statistical Computing, Vienna, Austria), pig type, bone, and the associated interaction included as fixed effects. System and pig type within system were included as random effects to account for systems having multiple sites sampled and multiple bones being collecred from each pig type with each site. Correlation coefficients (r) between dietary levels of Ca and P and individual bone analysis were determined using the lm function in R with dietary levels being the predictor variables and individual bone analysis being the response. Results were considered significant at $P \leq 0.05$ and marginally significant at $0.05 < P \leq 0.10$.

Results

Diagnostic survey

For the bone analysis, there was a significant bone × pig type interaction ($P < 0.001$; Table 1) observed for percentage bone ash (defatted and non-defatted), defatted bone ash weight, bone density, non-defatted bone ash weight, and bone-breaking strength. For each analysis, the response to pig type varied depending on the bone analyzed. For defatted percentage bone ash, there was no difference between the pig types for the fibulas, 2nd rib, or 10th ribs ($P > 0.10$). For the metacarpals, the bones from healthy pigs had greater ($P < 0.05$) defatted percentage bone ash than unhealthy pigs, with the lame pigs intermediate. For the 10th rib and metacarpal defatted bone ash weight, healthy and lame pigs had greater bone ash weight compared to unhealthy pigs ($P < 0.05$) with no difference between healthy and lame pigs ($P > 0.05$). For the fibulas and 2nd

ribs, healthy pigs had greater bone ash weight compared to unhealthy pigs ($P < 0.05$) with lame pigs being intermediate. For non-defatted percentage bone ash, there was no difference between pig types for metacarpals and fibulas ($P > 0.05$). For 2nd and 10th ribs, unhealthy pigs had greater non-defatted percentage bone ash compared to healthy pigs, with lame pigs intermediate ($P < 0.05$). For non-defatted bone ash weight, metacarpals and 10th ribs from healthy and lame pigs were greater than unhealthy pigs ($P > 0.05$) but no difference between healthy and lame pigs ($P > 0.10$). For fibulas and 2nd ribs, healthy pigs had greater non-defatted bone ash weight compared to unhealthy pigs, with lame pigs being intermediate ($P < 0.05$). Metacarpals and 10th ribs from healthy and lame pigs had greater non-defatted bone ash weight compared to unhealthy pigs ($P < 0.05$) with no difference between healthy and lame pigs. Healthy pigs had greater fibula non-defatted bone ash weight compared to unhealthy pigs ($P < 0.05$) with lame pigs intermediate. For bone density, there was no difference between pig types when 2nd and 10th ribs were analyzed ($P > 0.10$). Healthy pigs had greater bone density for metacarpals and fibulas compared to unhealthy, with no difference between healthy and lame pigs ($P < 0.05$). There was no difference between pig types when fibulas and 2nd ribs were analyzed for bone breaking strength ($P > 0.10$). For the 10th ribs, healthy and lame pigs had increased bone-breaking strength compared to unhealthy pigs ($P < 0.05$) with no difference between healthy and lame pigs ($P > 0.10$). For metacarpals, healthy pigs had greater breaking strength compared to unhealthy pigs ($P < 0.05$) with lame pigs being intermediate, but statistically different than healthy and unhealthy pigs ($P < 0.05$).

Healthy pigs had greater serum 25(OH)D_3 and serum Ca levels compared to unhealthy pigs, with lame pigs intermediate, but significantly different than the healthy and unhealthy pigs ($P < 0.05$; Table 2). Healthy pigs had greater serum P compared to unhealthy and lame pigs ($P <$

0.05) with no difference between unhealthy and lame pigs ($P > 0.10$). There was no significant difference for urine Ca between the different pig types ($P > 0.10$). Unhealthy pigs were excreting greater concentrations ($P < 0.05$) of P and creatinine in urine compared to healthy pigs, with lame pigs intermediate. There was no difference observed between the pig types for Ca:creatinine and P:creatinine ratios ($P > 0.10$). There was no difference in serum $25(OH)D_3$, between pigs sampled from curtain barns (21.7 ng/mL) compared to pigs sampled from tunnel barns (19.8 ng/mL; $P > 0.10$).

For the main effect of pig type, there was no difference between pig types for defatted bone ash percentage ($P = 0.122$; Table 3). Healthy and lame pigs had increased defatted bone ash weight compared to unhealthy pigs ($P < 0.05$). There was a significant difference between pig types for percentage non-defatted bone ash, non-defatted bone ash weight, bone density, and breaking strength ($P < 0.05$). For non-defatted bone ash, unhealthy pigs had greater percentage bone ash compared to healthy pigs, with lame pigs intermediate; however, healthy and lame pigs had greater bone ash weight compared to unhealthy pigs. Healthy and lame pigs had greater bone density compared to unhealthy pigs. Healthy and lame pigs required greater force to break bones compared to unhealthy pigs ($P < 0.05$). Healthy and lame pigs had increased grams of Ca and P in defatted bone ash compared to unhealthy pigs ($P < 0.05$).

For the main effect of bone, there were significant differences for defatted and non-defatted bone ash weight, defatted and non-defatted percentage bone ash, bone density, and bone breaking strength between bones ($P < 0.05$; Table 4). For defatted bone ash weight, metacarpals and 10^{th} ribs had greater bone ash weight compared to the fibula and 2^{nd} rib ($P < 0.05$). Fibulas had the greater defatted percentage bone ash compared to the 2^{nd} and 10^{th} ribs, with the metacarpals having the lowest defatted percentage bone ash ($P < 0.05$). Metacarpals and 10^{th} ribs had greater non-defatted

bone ash weight compared to fibulas and 2nd ribs ($P < 0.05$), with the 2nd ribs being the lowest. For non-defatted percentage bone ash, 10th ribs had the greatest percentage bone ash, metacarpals the lowest, with 2nd ribs and fibulas intermediate ($P < 0.05$). Fibulas had the greatest bone density, with metacarpals having the lowest bone density, with 2nd and 10th ribs intermediate, with 10th ribs having greater bone density than 2nd ribs ($P < 0.05$). Metacarpals had the greatest bone breaking strength ($P < 0.05$), with the 2nd ribs being the lowest, and fibulas and 10th ribs intermediate, with 10th ribs having greater bone breaking strength than fibulas. There were no differences between defatted bone ash of metacarpals and 10th ribs for grams and percent Ca and P ($P > 0.10$).

Presented in Tables 5 to 10 are descriptive statistics comparing serum 25(OH)D_3, Ca and P, bone density, defatted bone ash, and non-defatted bone ash across healthy, unhealthy, and lame pigs for each bone and across all systems. For serum 25(OH)D_3, there is variation across the different pig types across all systems (Figure 3). Average serum 25(OH)D_3 was 25.2 ng/mL for healthy pigs, 20.9 ng/mL for lame pigs, and 15.3 ng/mL for unhealthy pigs (Figure 1). The lowest value for 25(OH)D_3 for all samples was 2.8 ng/mL with the maximum serum 25(OH)D_3 at 78.9 ng/mL. For serum Ca and P, there was less variation across the systems compared to serum vitamin D. The average serum Ca was 10.4 mg/dL for healthy pigs, 9.7 mg/dL for lame pigs, and 9.2 mg/dL for unhealthy pigs. The average serum P was 10.2 mg/dL for healthy pigs, 9.4 mg/dL for lame pigs, and 8.8 mg/dL for unhealthy pigs. For bone density, there was little variation across the different pig types for the different systems, with healthy pigs having greater bone density compared to unhealthy pigs. Considering the non-defatted and defatted bone ashing methods, some systems observed no difference between the pig types of healthy, lame, and unhealthy pigs, compared to other systems observing differences between the pig types. The average non-defatted bone ash was

55.4% for healthy pigs, 56.6% for lame pigs, and 57.2% for unhealthy pigs. The average defatted bone ash was 62.3% for healthy pigs, 62.5% for lame pigs, and 62.7% for unhealthy pigs (Figure 2).

Dietary correlations

For non-defatted bone ash, increasing the STTD P of the diet was correlated with a linear increase ($P < 0.05$) in percentage bone ash for all bones from healthy pigs, whereas there was no significant correlation for all bones from lame or unhealthy pigs ($P > 0.10$; Table 11). For the non-defatted bone ash in fibulas of lame pigs, increasing the STTD P of the diets tended to be correlated with an increase in non-defatted percentage bone ash ($P < 0.10$). Increasing STTD P was correlated with a linear increase ($P < 0.05$) in defatted percentage bone ash in 2nd and 10th ribs for healthy pigs, but no significant correlation was observed between STTD P and defatted percentage bone ash for metacarpals and fibulas. For lame pigs, increasing the STTD P of the diets tended to be correlated with a linear increase in defatted percentage bone ash ($P < 0.10$) for metacarpals, with no significant correlation for any bones of unhealthy pigs ($P > 0.10$). Dietary STTD P was not correlated ($P > 0.10$) with density of any bones for lame and unhealthy pigs; but for healthy pigs, increasing the STTD P of the diets tended to be correlated with a linear increase in bone density ($P < 0.10$) in 10th ribs, with no correlation for metacarpals, fibulas, or 2nd ribs. For all pig types, there was no significant correlation ($P > 0.10$) between dietary STTD P and bone ash (g), percentage bone ash, Ca and P, and bone ash Ca and P (g) in all bones.

For non-defatted bone ash, total dietary Ca was not correlated ($P > 0.10$) for all bones from healthy and lame pigs but tended to be correlated with a linear decrease for unhealthy pig 10th ribs ($P <0.10$). For defatted bone ash, total dietary Ca was correlated with a linear increase ($P < 0.05$) in fibulas, 2nd ribs, and 10th ribs of healthy pigs, with no impact on unhealthy pigs for all bones ($P > 0.10$). For lame pigs, increasing total dietary Ca was correlated with a

linear increase ($P < 0.05$) in defatted bone ash for metacarpals, with no impact on fibulas, 2nd ribs, and 10th ribs ($P > 0.10$). For healthy, lame, and unhealthy pigs, increasing the total Ca of the diet was correlated with a linear increase ($P < 0.10$) in bone breaking strength, bone ash weight, and bone ash Ca and P (g). Increasing total dietary Ca was correlated with a linear increase ($P < 0.05$) in bone density for metacarpals and fibulas in healthy, lame, and unhealthy pigs. For healthy pigs, increasing the total Ca of the diet was correlated with a linear increase ($P < 0.05$) in bone density for 2nd and 10th ribs, with no impact on lame and unhealthy pig 2nd and 10th ribs ($P > 0.10$). For lame pigs, increasing the total dietary Ca tended to be correlated with a linear decrease in percentage bone ash Ca and P ($P < 0.10$) for 10th ribs, with no difference in bones of healthy and unhealthy pigs ($P > 0.10$).

For serum analysis and bone measurements, increasing dietary vitamin D3 ($P > 0.10$; Table 12) was not correlated for all bones from healthy, lame, and unhealthy pigs, but bone Ca and P percentage tended to be correlated with a linear increase for lame pig 10th ribs and unhealthy pig metacarpals ($P < 0.10$). For unhealthy pigs, increasing the vitamin D3 of the diet was correlated with a linear decrease ($P < 0.10$) in defatted bone ash for metacarpals and 2nd ribs, with no impact on unhealthy fibulas and 10th ribs. For lame and unhealthy pigs, increasing serum $25OHD_3$ was correlated with a linear decrease in non-defatted bone ash of metacarpals, fibulas, 2nd ribs, and 10th ribs of unhealthy pigs ($P < 0.10$), with no impact on lame pig 10th ribs ($P > 0.10$). For lame pig metacarpals, 2nd ribs, and 10th ribs, increasing serum $25OHD_3$ was correlated with a linear increase in bone density ($P < 0.10$), but increasing serum $25OHD_3$ of unhealthy pigs was correlated with a linear decrease in 10th rib bone density ($P < 0.10$). For lame pigs, increasing serum $25OHD_3$ levels was correlated with a linear increase ($P < 0.05$) in serum Ca and P, with no impact on healthy and unhealthy serum Ca and P ($P > 0.10$).

Discussion

The measurement of bone mineralization is routinely conducted alongside histopathologic evaluation in diagnostic cases to assess metabolic bone disease in pigs. The most common measurement to assess bone mineralization is bone density and percentage bone ash. Reference values adapted by diagnosticians have been adopted from research generated in the last 50 years (Field et al., 1974; Hagemoser et al., 2000; Arnold et al., 2015). Recent work has evaluated different analytical methods to assess bone mineralization in different ages of pigs (Williams et al., 2023a,b). Currently, there is limited research comparing the differences between bone mineralization and serum analysis across pigs of different health statuses. This diagnostic survey is one of the first large-scale studies to compare different bones and analytical measurements between healthy, lame, and unhealthy pigs. The goal of the diagnostic survey is to compare the results from different analytical measurement across systems, health statuses, and diet diet formulation strategies in pigs raises in the Midwestern US.

Previous research has demonstrated which bone may be most appropriate to assess total bone mineralization. Crenshaw et al. (2009) demonstrated the femur was a better indicator to predict whole body mineral content in young pigs (25 to 30 kg) compared to fibulas. The authors observed that fibulas tended to overestimate whole body bone mineral content at low P intakes, but underestimated bone mineral content at high P intake. In older pigs, there was not as drastic a difference in whole body bone mineral content between the different bones. Femurs, metacarpals, and metatarsals all showed similar results as predictors for whole body bone mineral content; but, similar to younger pigs, fibulas tended to over-estimate whole-body mineral content. A more recent study observed that metacarpals, metatarsals, and tibias can be used to predict total body mineral content in pigs weighing 65 kg (Lee et al., 2021). Fibulas have

routinely been used to measure bone mineralization due to the ease of collection compared to the femurs. To fully understand which bone should be used to assess bone mineralization, it is important to feed a range of dietary P levels to determine which bones are more sensitive to detect these dietary differences. In nursery pigs, the fibulas and 2nd ribs are more sensitive to detect differences, using bone density and bone ash as the measurements, across dietary treatments when compared to metacarpals and 10th ribs (Williams et al., 2023a). In finishing pigs, it is more difficult to detect these differences, but the differences in dietary P levels were able to be detected in 10th ribs when analyzing bone density (Williams et al., 2023b).

Percentage bone ash has been the preferred measurement to determine bone mineralization in growing pigs (Vier et al., 2019a,b; Lee et al., 2021). The two main procedures used to measure percentage bone ash are the defatted and non-defatted techniques (Wensley et al., 2020). The major difference between the two methods is with defatting the bones, the lipid and water is extracted from the bone using ether in a Soxhlet device which circulates petroleum ether around the submerged bones for 7 d, while the non-defatted technique bypasses this step and leaves the lipid inside the bone during the ashing process. Previous research has shown the bone of a mature animal is comprised of 10% fat (Lian et al., 1999). By extracting the lipid from the bone, the variation across all bones measured is reduced, and this variation is larger in finishing pigs compared to nursery pigs (Wensley, et al., 2020; Williams et al., 2023b). In finishing pigs, the defatted percentage bone ash is 14 to 15 percentage units greater than the same bone measured using the non-defatted bone ash method (Williams et al., 2023b); whereas in nursery pigs, the defatted percentage bone ash is 11 to 12 percentage units greater than non-defatted bones (Williams et al., 2023a). Similar results were observed by Wensley et al. (2020) who analyzed nursery and finishing bones using both methods for bone ash.

Limited data has been generated comparing the different methods to analyze bone ash between pigs of different health statuses. Williams et al. (2023b) compared the percentage bone ash between healthy and unhealthy finishing pigs and observed a significant difference for defatted bone ash between healthy and unhealthy pigs, with unhealthy pigs having lower defatted bone ash compared to healthy pigs. Due to the number of unhealthy pigs measured in that study (n = 11), the authors were not able to detect differences for non-defatted bone ash between the two different pig types. By defatting bones, variation across bones is reduced, allowing for a smaller magnitude of change in percentage bone ash to be detected. In the current study, we had approximately 64 pigs within each of the 3 pig types. We observed no difference between healthy, lame, and unhealthy pigs when defatting the bones. For non-defatted bone ash, there was a significant difference between the pig types. Unhealthy pigs had greater non-defatted bone ash than healthy pigs, with lame pigs intermediate. Unhealthy pigs have reduced feed intake relative to their healthy cohorts, resulting in unhealthy pigs mobilizing fat from the skeletal system as a source of energy. By mobilizing fat from bones, unhealthy pigs have less fat in the bone and thus greater percentage bone ash than healthy pigs when measured using the non-defatted bone ash technique.

The magnitude of change in percentage bone ash between defatted and non-defatted bone ash method varies across different bones. For fibulas, 2nd rib, and 10th ribs, the absolute change between the defatted and non-defatted percentage bone ash was 6 to 8%, while for metacarpals, the absolute change was 16%, with the defatted method yielding higher percentage bone ash. In nursery pigs, the magnitude of change was similar for 2nd and 10th ribs but was greater for metacarpals and fibulas, with approximately 20% and 13% absolute difference between defatted and non-defatted methods, respectively (Williams et al., 2023a). Similar results for metacarpals

are observed in finishing pigs (Williams et al., 2023b), whereas the magnitude of difference between the two procedures was greater in finishing pigs than in nursery pigs for the 2nd and 10th ribs. These findings suggest that fat accumulation in metacarpals and fibulas is consistent between nursery and finishing pigs, while ribs show increased accumulation of fat as pigs grow. While the accumulation of fat in metacarpals and fibulas of nursery and finishing pigs is consistent, it is advisable to remove fat when analyzing for percentage bone ash, as this leads to a reduction in variation. Therefore, defatting bones prior to ashing is recommended for more accurate results.

Although bone ash is the most common method to determine bone mineralization, other methods, such as bone density, can be used. The two main procedures for measuring bone density are the Archimedes principle and Dual-energy X-ray absorptiometry (DXA). The equation used to calculate density using the Archimedes principle is density = (A/A-B) × P, where A is bone weight, B is bone weight submerged in water, P is density of distilled water, and A-B is the difference in weight, which is equivalent to the volume. With DXA, bone mineral density and bone mineral content can be determined by scanning the bone. Keenan et al. (1997) compared the two methods and observed that the densities from these two techniques are highly correlated (r = 0.82). Previous research in nursery and finishing pigs (Williams et al., 2023a,b) compared both methods. The authors found similar results in finishing pigs between the two methods but found using the Archimedes principle in nursery pigs was more sensitive to find differences in the dietary STTD P response in fibulas and 2nd ribs compared to metacarpals and 10th ribs. In the current study, Archimedes principle was used to calculate bone density. There were differences in bone density across different bones measured and health status of the pigs. Unlike defatted percentage bone ash, bone density was capable of detecting differences between

healthy and unhealthy pigs with healthy pigs having greater bone density than unhealthy pigs. These results are similar to Williams et al. (2023b) who also observed greater bone density for healthy pigs compared to unhealthy pigs.

Some analytical procedures used to measure bone mineralization are purely dependent on bone size. For grams of bone ash and bone breaking strength, the larger the bone, the greater the value. These measurements make it difficult to provide reference ranges for diagnosticians because the analytical measurement is heavily influenced by animal size. This can also be observed when comparing grams of Ca and P in the ash content of bones. As a pig increases in size, bones increase in size, which results in an increase in grams of bone ash as well as an increase in grams of Ca and P in the bone (Gonzalez-Vega et al., 2016a; Lee et al., 2021; Williams et al., 2023a). Thus, interpreting the results of bone ash (g) may not be a useful approach for diagnosticians, as the outcome is known to be correlated with animal size. Instead, diagnosticians are recommended to utilize bone density, percentage bone ash, and percentage Ca and P in the bone ash, as these measurements can be applied to pigs of all sizes.

When investigating bone metabolic disease cases in pigs, it is common practice to measure serum vitamin D due to its critical role in the absorption and retention of Ca and P (O'Doherty et al., 2010). Unlike measuring bone mineralization, this procedure is non-invasive and can provide important insights into the pig's metabolic status. Vitamin D can be obtained by pigs through dietary supplementation or synthesized in the skin from 7-dehydrocholesterol upon exposure to sunlight. Upon exposure to ultraviolet-B (UV-B), 7-dehydrocholesterol is converted to vitamin D_3 (Crenshaw, 2001). Vitamin D_3 must then undergo a hydroxylation step in the liver to be converted to the primarily inactive form, $25(OH)D_3$. The $25(OH)D_3$ enters circulation and can be stored and later hydroxylated in the kidney to the active metabolite, $1,25\text{-}(OH)_2D_3$, as

needed. To assess vitamin D status in pigs, diagnosticians primarily measure serum levels of $25(OH)D_3$, which can be increased through greater dietary supplementation with vitamin D_3 or $25(OH)D_3$ (Arnold et al., 2015; Burild et al., 2016; Flohr et al., 2016). Several factors can affect serum vitamin D levels in pigs, including the dietary form of vitamin D and supplementation levels, growth stage, and sunlight exposure.

Previously, a survey was conducted to determine vitamin D status across stages of swine production (Arnold et al., 2014). The authors analyzed 1,200 serum samples over a 9 - month period for $25(OH)D_3$ concentrations. The authors observed numerous samples across all ages of pigs that had serum $25(OH)D_3$ levels below the reference range (18 to 30 ng/mL; Arnold et al., 2012). In the current study, we observed similar results with values below the reference range provided by diagnosticians. Across the different pig types, there were several samples below the current reference range, with some samples being well below the reference range. Although the mean concentration of serum $25(OH)D_3$ for healthy pigs in the current study was above the reference range, there were also healthy pigs below the reference range. Conversely, mean serum $25(OH)D_3$ levels for unhealthy pigs were below the reference range, but there were also unhealthy pigs with values above the reference range. In the Arnold et al. (2014) study, authors also compared serum levels of pigs raised indoors vs outdoors. The authors observed that pigs raised outdoors had 2 to 3 times greater serum $25(OH)D_3$ levels compared to pigs raised indoors. In modern commercial swine production, pigs are typically raised indoors with limited access to sunlight. In the current study, we compared pigs from curtain sided barns (that had limited exposure to sunlight during the winter months when the study was conducted) and pigs raised in mechanically ventilated “tunnel” barns with limited exposure to sunlight and found no difference between the different types of barns.

Along with serum vitamin D levels, Ca and P can also be analyzed in the serum to determine circulating levels. Due to efficient homeostatic mechanisms of serum Ca and P, it is difficult to detect differences due to dietary changes (Koch and Mahan, 1985). Feeding deficient levels of P to pigs for an extended period can lead to low serum P. Williams et al. (2023a) observed reduced serum P in nursery pigs fed 57% of their P requirement estimate for 28-d. Similar results were observed by Hagemoser et al. (2000), who observed reduced serum P when dietary P levels were deficient. In the current study, lame and unhealthy pigs had lower serum P than healthy pigs, with the healthy pigs having 16% greater levels of P in serum compared to unhealthy pigs. The reduction in serum P for unhealthy pigs is speculated to be due to a feed intake reduction compared to the healthy pigs. Serum P ranged from 4.5 to 18.0 mg/dL across the different pig types.

Hagemoser et al. (2000) suggested that changes in urine Ca and P levels is a more sensitive method than serum Ca and P, due to serum levels being highly regulated in the body, unless there is a severe deficiency. Typically, urine Ca and P values are reported as a ratio to creatinine, due to creatinine levels being a waste product produced in muscle cells. Creatinine concentrations in the urine can be altered based on water intake of the pig (Luginbuhl and Weinmann, 2017). It was speculated that the unhealthy pigs used in the analyses had reduced feed and water intake compared to healthy pigs, which could lead to differences in urinary creatinine concentrations. In the current study, we observed increased creatinine and P levels in urine from unhealthy pigs compared to healthy pigs. This shows that unhealthy pigs are in a catabolic state, causing increased muscle tissue breakdown, which leads to increased creatinine and P in the urine. When comparing urine Ca:creatinine and P:creatinine ratios, we did not observe any differences between different types of pigs. Using the results and suggested

references from Hagemoser et al. (2000), there appears to be no signs of Ca and P deficiencies, but unhealthy pigs were close to the ratio used to determine Ca deficiency. A P:creatinine ratio greater than 1.0 demonstrates a Ca deficiency with normal P status or moderate P deficiency. A P:creatinine ratio below 1.0 is typically normal but can be compatible with severe P deficiency or vitamin D deficiency. Calcium and P concentrations within urine are typically not directly interpreted due to excretion levels varying based on glomerular filtration rate from the kidneys, which is why they are most often compared as a ratio to creatinine. However, given the differences in metabolic state data are reported both as ratios and concentrations. The data herein presents evidence that there are differences in P and creatinine excretion in urine between different pig types, which is a result of muscle catabolism and P not being utilized for lean tissue deposition.

In the current study, dietary information for Ca, P, and vitamin D fortification offered to each pig was provided by each system, which allowed correlations between the formulated dietary levels and bone responses to be analyzed. In the study, the dietary levels were compared to the NRC (2012) requirement estimates for each weight range. Across all systems, the average STTD P level fed to pigs when compared to the NRC (2012) requirement estimates was 136%, whereas the average total Ca level fed to pigs when compared to the NRC (2012) requirement was 96%. Recently, Vier et al. (2019b) observed that a P requirement of 24 to 130 kg pigs ranged from 116 to 131% of the NRC (2012) requirement estimates. For percentage bone ash, Vier et al. (2019b) observed the maximum defatted bone ash for metacarpals at 131% of the NRC (2012) estimates. In the current study, we observed few correlations of higher dietary P levels and increased bone mineralization. This response would be expected because P in most diets was above the P levels evaluated by Vier et al. (2019b), and higher levels of dietary P

would not be expected to produce any further increases in bone mineralization. There was little to no improvement in bone mineralization when lame and unhealthy pigs were fed diets formulated with higher dietary P, with improvements in bone mineralization for healthy pigs depending on the bone measured and analysis used to measure bone mineralization.

The requirement for Ca in growing pigs has been heavily researched (Gonzalez-Vega et al., 2016a; Gonzalez-Vega et al., 2016b; Stein et al., 2016). In the current study, most of the significant correlations were observed between dietary Ca and different measurements of bone mineralization, with correlations observed for each of the different pig types. When P levels are fed above the pig requirement, increasing the diet Ca level increases bone mineralization (Williams et al., 2023d); whereas, when the P level fed is at or below the requirement, increased Ca can reduce growth performance and bone mineralization (Merriman et al., 2017; Lagos et al., 2019; Lautrou et al., 2020). This reduction in growth and bone mineralization is due to reduced absorption of P because of Ca:P complexes are formed in the small intestine. Due to the majority of production systems feeding adequate dietary P levels (136% of the NRC 2012 requirement estimates), increased dietary Ca was associated with increases in bone mineralization.

It is common in the swine industry to feed vitamin D levels that are well above NRC (2012) requirement estimates (Faccin et al., 2023). The NRC (2012) recommendation for vitamin D in growing pigs is 200 IU/kg. A recent survey was conducted to compare concentrations of added vitamins and trace minerals in diets in the United States (Faccin et al., 2023). The authors observed that vitamin D concentrations were approximately 5 to 10 times the NRC (2012) requirement estimates in growing pigs. Due to the role vitamin D has in Ca and P absorption and retention (O'Doherty et al., 2010), there is speculation that providing vitamin D in the diet well above NRC (2012) estimates may lead to increased bone mineralization. In the literature, there is

limited research that has observed an increase in bone mineralization when dietary vitamin D is increased above the NRC requirement estimates (Regassa et al., 2015; Zhang et al. 2021; Williams et al., 2023a,b). In the study herein, we observed limited correlations on bone mineralization when increased levels of vitamin D in the diet were fed. Interestingly, we observed a dietary Ca × Vitamin D interaction for bone mineralization. When feeding low levels of Ca and increased levels of vitamin D, bone mineralization was reduced. Similar responses have been observed in rats (Lee et al., 2014). In rats, feeding low levels of Ca and high levels of $25(OH)D_3$ increased serum $1,25(OH)_2D_3$ which stimulated bone resorption and inhibition of bone mineralization (Lee et al., 2014).

In summary, this diagnostic survey demonstrates differences between healthy, lame, and unhealthy pigs for serum Ca, P, and vitamin D, as well as differences across the various bones and analytical measurements to assess bone mineralization. By using the defatted bone ash method, the amount of variation across bones and different pig types was reduced, but no differences were observed between healthy, lame, and unhealthy pigs. For non-defatted bone ash, there is more variation, with unhealthy pigs having greater percentage bone ash compared to healthy pigs because of lipid mobilization from the bones of unhealthy pigs. When comparing all swine production systems included in this diagnostic survey, we observed considerable variation across different measurements, with serum vitamin D having a wide range between the different pig types. Although serum vitamin D had the highest mean serum concentration for healthy pigs, there were healthy pigs below the reference range threshold for clinically normal serum vitamin D analysis. Increasing the dietary Ca showed greater improvements in bone mineralization compared to changes in dietary P and vitamin D. This data can be used to help guide future

diagnostic investigations with baseline levels of different methodologies to determine bone mineralization in pigs.

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Table 4.1. Interactive effect of bone and pig type[1]

	Pig type						
	Healthy		Lame		Unhealthy		
Item	Mean	Range[2]	Mean	Range	Mean	Range	SEM
Defatted bone ash, %[3]							
Metacarpal	60.9[a]	51.0-69.3	59.8[b]	49.3-69.1	58.7[c]	47.3-63.7	0.37
Fibula	64.4	56.0-67.9	64.6	59.8-67.8	63.7	50.7-69.8	
2nd rib	62.2	56.5-65.3	62.4	59.2-65.0	62.4	56.8-66.4	
10th rib	62.3	55.5-65.0	62.7	59.5-67.0	62.3	51.4-66.3	
Defatted bone ash, g							
Metacarpal	5.19[a]	0.74-10.45	4.53[a]	0.58-10.34	3.46[b]	0.48-8.56	0.352
Fibula	2.94[a]	0.42-7.47	2.52[ab]	0.33-7.72	1.96[b]	0.19-6.06	
2nd rib	2.91[a]	0.37-6.92	2.53[ab]	0.20-6.25	1.99[b]	0.14-6.33	
10th rib	5.03[a]	0.79-11.54	4.62[a]	0.62-14.12	3.39[b]	0.34-10.86	
Defatted bone ash content							
Ca, g							
Metacarpal	2.53[a]	0.30-6.09	2.19[ab]	0.21-5.92	1.68[b]	0.20-4.62	0.220
10th rib	2.41[a]	0.36-6.62	2.25[a]	0.26-7.41	1.55[b]	0.18-4.50	
P, g							
Metacarpal	1.21[a]	0.14-2.96	1.01[ab]	0.10-2.83	0.79[b]	0.09-2.16	0.104
10th rib	1.16[a]	0.18-3.18	1.09[a]	0.12-3.47	0.74[b]	0.08-2.17	
Ca, %							
Metacarpal	48.50	35.04-66.84	47.24	33.54-64.17	46.56	31.33-62.07	0.916
10th rib	47.64	29.92-63.30	47.45	33.12-65.33	45.24	34.37-60.42	
P, %							
Metacarpal	23.25	16.75-33.62	22.51	15.62-30.44	22.22	15.23-30.32	0.476
10th rib	22.83	14.31-29.42	22.81	15.82-31.18	21.66	16.41-29.59	
Non-defatted bone ash, %[4]							
Metacarpal	43.2	35.1-54.0	43.3	34.8-56.4	44.4	36.2-58.2	0.57
Fibula	54.0	44.6-62.3	53.9	39.1-61.1	55.0	48.1-65.1	

2nd rib	54.3[b]	46.1-60.3	55.4[ab]	44.1-62.8	56.0[a]	46.9-62.1	
10th rib	55.4[b]	45.9-61.2	56.6[ab]	47.6-62.4	57.2[a]	48.8-62.3	
Non-defatted bone ash, g							
Metacarpal	5.14[a]	0.83-10.44	4.53[a]	0.64-10.32	3.51[b]	0.48-8.67	0.369
Fibula	3.43[a]	0.63-7.41	2.99[ab]	0.21-8.34	2.41[b]	0.25-7.60	
2nd rib	3.01	0.31-7.52	2.66	0.30-7.63	2.17	0.16-7.17	
10th rib	5.29[a]	0.38-13.16	4.71[a]	0.47-13.59	3.58[b]	0.19-12.18	
Bone density, g/mL[5]							
Metacarpal	1.30[a]	1.17-1.41	1.28[a]	1.12-1.38	1.26[b]	1.13-1.36	0.008
Fibula	1.38[a]	1.25-1.61	1.37[a]	1.26-1.54	1.34[b]	1.16-1.50	
2nd rib	1.32	1.24-1.74	1.32	1.24-1.47	1.30	1.20-1.44	
10th rib	1.34	1.30-1.55	1.34	1.28-1.50	1.33	1.22-1.51	
Breaking strength, kg							
Metacarpal	115.2[a]	3.12-328.3	99.6[b]	18.8-280.1	73.1[c]	7.7-196.8	6.00
Fibula	47.7	9.9-100.1	43.1	6.6-131.9	32.4	4.3-91.8	
2nd rib	39.3	7.0-97.9	35.0	4.5-93.8	26.8	0.9-103.3	
10th rib	74.8[a]	11.3-168.0	67.6[a]	11.0-206.7	47.5[b]	5.7-170.2	

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

[1]Bone × pig type interaction ($P < 0.001$) for defatted bone ash percentage, defatted bone ash grams, non-defatted bone ash, bone density, and bone breaking strength. SEM for the interaction is reported.

[2]The range is the minimum and maximum value for each bone within each pig type.

[3]All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105°C for 7 d, and then ashed in a muffle furnace at 600°C for 24 h.

[4]All bones were cleaned of tissue and then dried at 105°C for 7 days and then ashed in a muffle furnace at 600°C for 24 h.

[5] Bone density was measured on each bone based on Archimedes principle.

Table 4.2. The effect of pig type on serum and urine analysis

	Pig type							
	Healthy		Lame		Unhealthy			
Item	Mean	Range	Mean	Range	Mean	Range	SEM	*P* =
Serum analysis[1]								
$25OHD_3$, ng/mL	25.2[a]	5.6-78.9	21.0[b]	4.6-64.0	15.5[c]	2.8-71.8	1.88	< 0.001
Ca, mg/dL	10.4[a]	6.9-14.4	9.7[b]	6.7-13.4	9.2[c]	6.6-14.5	0.16	< 0.001
P, mg/dL	10.2[a]	6.4-18.0	9.4[b]	5.5-15.8	8.8[b]	4.5-14.7	0.26	< 0.001
Urine analysis[2]								
Ca, mg/dL	10.9	1.0-35.6	11.1	1.0-41.0	9.5	1.0-35.6	1.88	0.740
P, mg/dL	50.9[b]	5.5-142.9	75.2[ab]	5.5-903.0	96.9[a]	5.5-286.0	11.76	0.003
Creatinine, mg/dL	103.1[b]	9.1-255.9	135.1[ab]	3.2-378.3	151.3[a]	16.4-381.1	14.08	0.022
Calcium:creatinine	0.13	---	0.19	---	0.13	---	0.048	0.456
Phosphorus:creatinine	0.63	---	0.71	---	0.94	---	0.151	0.303

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

[1]Serum Ca and P were measured at Iowa state Veterinary Diagnostic Lab (Ames, IA). The vitamin D serum analysis was conducted at Heartland Assays (Ames, IA).

[2]Urine was collected from the bladder of each pig and analyzed for Ca, P, and creatinine at Iowa State Veterinary Diagnostic Lab (Ames, IA).

Table 4.3. The effect of pig type on bone analysis[1]

	Pig type							
	Healthy		Lame		Unhealthy			
Item	Mean	Range	Mean	Range	Mean	Range	SEM	*P* =
Defatted bone ash[2]								
Bone ash, g	4.02[a]	0.37-11.54	3.55[a]	0.20-14.12	2.71[b]	0.14-10.86	0.337	0.001
Bone ash, %	62.4	51.0-67.9	62.3	49.3-69.1	61.8	47.3-69.8	0.29	0.122
Non-defatted bone ash[3]								
Bone ash, g	4.23[a]	0.31-13.16	3.73[a]	0.21-13.59	2.92[b]	0.16-12.18	0.354	0.001
Bone ash, %	51.7[b]	38.1-62.3	52.3[ab]	34.8-65.8	53.1[a]	36.2-65.1	0.53	0.037
Bone density, g/mL[4]	1.33[a]	1.16-1.56	1.33[a]	1.16-1.52	1.31[b]	1.14-1.44	0.007	0.001
Breaking strength, kg	69.1[a]	3.1-328.3	61.1[a]	4.5-280.1	44.9[b]	0.9-196.8	5.61	0.001
Defatted bone ash content[5]								
Ca, g	2.47[a]	0.30-6.62	2.22[a]	0.21-7.41	1.62[b]	0.18-4.62	0.214	0.001
P, g	1.18[a]	0.14-3.18	1.05[a]	0.10-3.47	0.76[b]	0.08-2.17	0.100	0.001
Ca, %	48.0	29.9-66.8	47.4	33.1-65.3	45.9	31.3-62.1	0.648	0.064
P, %	23.0[a]	14.3-33.6	22.7[ab]	15.6-31.2	21.9[b]	15.2-30.3	0.354	0.050

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

[1]The mean of all 4 bones measured are combined for each pig type.

[2]All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105°C for 7 d, and then ashed in a muffle furnace at 600°C for 24 h.

[3]All bones were cleaned of tissue and then dried at 105°C for 7 d and then ashed in a muffle furnace at 600°C for 24 h.

[4]Bone density was measured on each bone based on Archimedes principle.

[5]After bone ash was completed, the ash from the metacarpal and 10th ribs were digested and analyzed for Ca and P using ICP by the KSU Soils lab (Manhattan, KS).

Table 4.4. Effect of different bones on bone analysis of pigs

	Metacarpal	Fibula	2nd rib	10th rib	SEM	*P* =
Defattted bone ash[1]						
Bone ash, g	4.39[a]	2.47[b]	2.48[b]	4.35[a]	0.287	0.001
Bone ash, %	59.7[c]	64.2[a]	62.3[b]	62.5[b]	0.25	0.001
Non-defatted bone ash[2]						
Bone ash, g	4.39[a]	2.94[b]	2.61[c]	4.53[a]	0.301	0.001
Bone ash, %	43.6[d]	54.3[c]	55.2[b]	56.4[a]	0.44	0.001
Bone density, g/mL[3]	1.28[d]	1.36[a]	1.31[c]	1.33[b]	0.006	0.001
Bone breaking strength, kg	96.7[a]	41.0[c]	33.6[d]	63.2[b]	4.63	0.001
Defatted bone ash content[4]						
Ca, g	2.14	---	---	2.07	0.177	0.281
P, g	1.00	---	---	0.99	0.084	0.799
Ca, %	47.4	---	---	46.8	0.532	0.381
P, %	22.7	---	---	22.4	0.304	0.537

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

[1]All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105°C for 7 d, and then ashed in a muffle furnace at 600 °C for 24 h.

[2]All bones were cleaned of tissue and then dried at 105°C for 7 d and then ashed in a muffle furnace at 600°C for 24 h.

[3]Bone density was measured on each bone based on Archimedes principle.

[4]After bone ash was completed, the ash from the metacarpal and 10th ribs were digested and analyzed for Ca and P using ICP by the KSU Soils lab (Manhattan, KS).

Table 4.5. Descriptive statistics of serum 25OHD$_3$ (ng/mL) in healthy, lame, and unhealthy pigs across swine production systems in the US[1]

	Healthy pig				Lame pig				Unhealthy pig			
System	Count, n	Average[2]	SD	Range[3]	Count, n	Average	SD	Range	Count, n	Average	SD	Range
1	6	24.4	8.2	17.3-40.1	6	17.8	8.0	9.4-32.8	6	16.3	14.9	6.4-48.5
2	4	12.4	1.9	9.8-15.1	4	11.3	2.3	9.7-15.2	4	7.1	3.1	3.1-10.4
3	2	18.6	1.3	17.3-19.9	2	11.8	0.6	11.2-12.4	2	7.9	1.2	6.7-9.0
4	3	22.0	3.1	17.8-25.4	3	15.7	5.1	9.9-22.3	3	9.2	2.0	6.3-10.8
5	6	16.9	3.3	12.2-21.3	6	10.2	5.2	5.5-19.9	6	11.5	5.7	6.4-22.1
6	7	36.7	16.9	20.4-73.4	7	36.6	14.0	21.6-64.0	7	24.7	11.4	15.6-51.8
7	4	47.6	19	28.3-78.9	4	47.3	11.8	27.2-57.1	4	45.2	19	27.0-71.8
8	4	30.4	19.2	13.3-61.1	4	30.7	16.5	11.1-48.8	4	11.6	4.6	5.1-16.9
9	6	16.0	4.3	9.5-23.1	6	11.9	5	6.1-19.3	6	10.3	4.9	3.9-17.9
10	6	22.1	24.7	5.6-74.9	6	21.4	20.9	4.6-60.5	6	8.9	6.7	2.8-21.6
11	4	34.2	9.2	19.0-42.2	4	20.6	3.2	16.7-25.7	4	18.0	3.5	12.0-20.8
12	1	41.4	---	---	1	24.1	---	---	---	---	---	---
13	3	19.8	8.7	12.8-32.0	2	12.2	0.6	11.6-12.7	3	11.3	3	7.1-13.7
14	6	21.5	9.3	7.9-36.7	6	15.7	6.8	4.8-26.8	6	12.5	4.5	6.8-17.9
Average[4]	62	25.2	16.0	5.6-78.9	61	20.9	15.1	4.6-64.0	61	15.3	12.8	2.8-71.8

[1]The vitamin D serum analysis was conducted at Heartland Assays (Ames, IA). Each system sampled a healthy, lame, and unhealthy pig from the same population as each site.

[2]The average represents the mean of all samples from the system within each pig type.

[3]The range is the minimum and maximum value for each blood sample within each pig type.

[4]Average is accounting for all samples from each system within the different pig types.

Table 4.6. Descriptive statistics of serum Ca (mg/dL) in healthy, lame, and unhealthy pigs across swine production systems in the US[1]

	Healthy pig				Lame pig				Unhealthy pig			
System	Count, n	Average[2]	SD	Range[3]	Count, n	Average	SD	Range	Count, n	Average	SD	Range
1	6	10.9	1.1	9.9-13.3	5	9.6	0.3	9.2-10.1	6	9.5	0.6	8.4-10.2
2	4	10.2	1.1	9.1-11.8	4	8.8	0.9	7.9-10.2	4	9.5	1.6	7.2-11.1
3	2	10.6	0.6	10.0-11.2	2	9.9	0.7	9.2-10.6	2	8.9	0.4	8.5-9.2
4	3	11.3	0.5	10.6-11.8	3	9.3	1.8	6.7-10.9	3	9.5	0.7	8.6-10.3
5	6	9.5	0.4	9.0-10.1	6	8.7	1.1	6.7-10.4	5	8.7	0.7	7.8-9.9
6	7	10.7	1.2	8.9-12.0	6	11.2	0.5	10.5-11.7	6	10.0	1.8	6.8-12.1
7	4	8.5	1.3	6.9-10.4	4	9.4	1.1	8.1-10.7	4	8.0	1.2	7.2-10.0
8	4	10.2	0.2	9.8-10.4	4	9.9	0.5	9.2-10.4	4	9.5	0.8	8.7-10.5
9	5	11.8	1.3	10.6-14.4	5	10.1	0.9	9.0-11.5	6	10.3	1.3	9.0-13.0
10	6	9.9	0.4	9.2-10.5	6	9.7	0.6	8.8-10.5	6	8.6	0.6	7.9-9.4
11	4	10.3	0.8	9.5-11.6	4	9.7	0.7	8.8-10.6	4	8.5	14.0	6.6-10.2
12	1	10.1	---	---	1	8.9	---	---	---	---	---	---
13	3	10.2	1.1	9.4-11.8	2	10.3	0.4	9.9-10.6	2	9.1	0.6	8.4-9.7
14	6	10.9	0.4	10.3-11.6	6	10.2	0.8	8.7-11.0	6	9.2	0.8	7.7-10.0
Average[4]	61	10.4	1.19	6.9-14.4	58	9.7	1.1	6.7-11.7	58	9.2	1.3	6.6-13.0

[1]Serum Ca was analyzed at Iowa state Veterinary Diagnostic Lab (Ames, IA) as part of the large animal complete profile. Each system sampled a healthy, lame, and unhealthy pig from the same population as each site and is represented by count in the table.

[2]The average represents the mean of all the sample from the system within each pig type.

[3]The range is the minimum and maximum value for each blood sample within each pig type.

[4]Average is accounting for all of the samples from each system within the different pig types.

Table 4.7. Descriptive statistics of serum P (mg/dL) in healthy, lame, and unhealthy pigs across swine production systems in the US[1]

	Healthy pig				Lame pig				Unhealthy pig			
System	Count, n	Average	SD	Range	Count, n	Average	SD	Range	Count, n	Average	SD	Range
1	6	10.4	3.5	7.7-18.0	5	7.9	1.0	6.6-9.3	6	8.7	1.0	7.2-9.9
2	4	10.1	1.2	9.1-12.2	4	8.2	0.9	6.9-9.3	4	8.3	2.2	5.1-11.1
3	2	9.5	0.1	9.4-9.5	2	8.3	0.8	7.4-9.1	2	6.8	0.3	6.5-7.0
4	3	11.7	0.8	10.6-12.6	3	8.5	2.1	5.5-10.3	3	9.9	1.7	8.5-12.3
5	6	9.0	0.8	8.0-10.5	6	8.1	1.2	6.9-10.4	5	7.6	0.8	6.9-9.0
6	7	11.3	1.6	8.6-13.6	6	11.2	0.8	10.0-12.2	6	11.0	2.4	7.1-14.1
7	4	9.0	1.6	6.4-10.7	4	9.3	1.1	8.3-10.9	4	8.2	1.2	6.6-9.4
8	4	9.4	0.4	8.7-9.8	4	8.9	0.5	8.3-9.4	4	8.5	0.4	8.0-9.0
9	5	13.4	2.1	10.5-16.2	5	11.0	2.0	8.3-12.8	6	10.4	2.1	7.9-14.1
10	6	9.7	1.3	7.4-11.4	6	10.2	1.5	8.3-12.2	6	8.6	1.1	6.6-9.8
11	4	10.4	1.9	8.2-12.8	4	9.5	0.7	8.9-10.6	4	9.0	3.7	4.5-14.7
12	1	8.6	---	---	1	7.6	---	---	---	---	---	---
13	3	8.3	0.4	7.8-8.7	2	9.0	0.6	8.4-9.6	2	7.4	1.4	6.0-8.7
14	6	10.2	0.7	9.4-11.0	6	10.1	1.0	8.7-11.7	6	8.3	0.9	7.0-9.5
Average[4]	61	10.2	2.1	6.4-18.0	58	9.4	1.7	5.5-12.8	58	8.8	2.1	4.5-14.7

[1]Serum P was analyzed at Iowa state Veterinary Diagnostic Lab (Ames, IA) as part of the large animal complete profile. Each system sampled a healthy, lame, and unhealthy pig from the same population as each site and is represented by count in the table.

[2]The average represents the mean of all the sample from the system within each pig type.

[3]The range is the minimum and maximum value for each blood sample within each pig type.

[4]Average is accounting for all of the samples from each system within the different pig types.

Table 4.8. Descriptive statistics of bone density (g/mL) in healthy, lame, and unhealthy pigs in swine production systems in the US[1]

	Metacarpal			Fibula			2nd rib			10th rib		
System	Healthy	Lame	Unhealthy	Healthy	Lame	Unhealthy	Healthy	Lame	Unhealthy	Healthy	Lame	Unhealthy
1	1.32	1.31	1.24	1.41	1.38	1.36	1.33	1.31	1.30	1.37	1.31	1.31
2	1.29	1.28	1.24	1.39	1.36	1.32	1.32	1.33	1.29	1.37	1.33	1.32
3	1.34	1.30	1.24	1.48	1.39	1.38	1.36	1.34	1.33	1.38	1.34	1.34
4	1.32	1.28	1.29	1.41	1.35	1.36	1.32	1.28	1.32	1.32	1.27	1.32
5	1.30	1.26	1.27	1.33	1.37	1.35	1.30	1.27	1.29	1.33	1.33	1.33
6	1.33	1.30	1.28	1.42	1.38	1.38	1.36	1.33	1.32	1.38	1.36	1.32
7	1.25	1.27	1.22	1.30	1.34	1.29	1.28	1.32	1.26	1.32	1.33	1.27
8	1.31	1.31	1.29	1.40	1.40	1.38	1.34	1.34	1.32	1.38	1.37	1.33
9	1.30	1.26	1.26	1.39	1.37	1.34	1.33	1.31	1.31	1.36	1.35	1.34
10	1.26	1.26	1.23	1.30	1.32	1.28	1.30	1.30	1.29	1.30	1.35	1.32
11	1.33	1.28	1.26	1.36	1.34	1.34	1.31	1.34	1.32	1.32	1.32	1.36
12	1.32	1.28	1.23	1.41	1.35	1.39	1.30	1.33	1.35	1.33	1.35	1.36
13	1.28	1.28	1.27	1.40	1.38	1.36	1.29	1.32	1.29	1.31	1.34	1.34
14	1.27	1.26	1.22	1.39	1.37	1.31	1.32	1.33	1.31	1.33	1.35	1.32

[1] Bone density was measured on each bone based on Archimedes principle. The value is the average of all the bones from the same system within each pig type.

Table 4.9. Descriptive statistics of non-defatted percent bone ash in healthy, lame, and unhealthy pigs across systems in the US swine industry[1]

	Metacarpal			Fibula			2nd rib			10th rib		
System	Healthy	Lame	Unhealthy	Healthy	Lame	Unhealthy	Healthy	Lame	Unhealthy	Healthy	Lame	Unhealthy
1	44.6	43.5	44.2	54.7	54.0	56.0	55.6	55.0	57.4	56.6	57.2	58.7
2	42.1	42.5	41.5	54.3	54.0	52.4	55.8	56.7	54.8	57.6	57.4	56.3
3	44.4	41.4	38.7	57.6	53.1	54.5	56.7	56.2	57.0	57.7	56.1	57.6
4	42.4	37.7	44.1	54.9	47.9	56.7	50.1	46.6	56.5	51.7	49.4	57.0
5	42.5	44.6	43.9	52.8	56.6	56.4	53.8	54.6	55.5	53.9	56.8	57.2
6	43.8	42.0	42.6	55.8	53.1	52.9	55.2	52.1	53.4	56.2	54.4	54.9
7	42.6	42.4	41.7	51.8	49.4	51.4	52.9	55.2	53.3	54.5	56.8	54.7
8	44.8	45.2	47.5	56.6	56.0	56.5	57.2	57.8	56.4	58.4	58.4	56.0
9	42.3	46.0	45.1	53.1	54.5	54.4	52.6	57.7	55.4	53.3	57.8	58.2
10	44.1	45.5	51.1	52.9	55.6	58.8	57.4	58.7	59.9	58.2	59.5	60.3
11	43.5	43.8	45.0	52.5	55.8	55.0	53.0	56.3	56.8	54.6	56.6	57.6
12	42.9	41.4	39.5	55.4	54.0	52.2	49.6	55.3	56.3	52.0	56.7	57.6
13	40.3	40.9	44.2	51.8	51.7	56.3	51.2	54.6	55.1	51.6	56.0	56.8
14	44.4	44.5	46.0	56.2	57.0	55.2	55.1	57.5	57.9	56.6	58.7	59.3

[1]All bones were cleaned of tissue and then dried at 105°C for 7 d and then ashed in a muffle furnace at 600°C for 24 h. The value is the average of all the bones from the same system within each pig type.

Table 4.10. Descriptive statistics of defatted bone ash percent in healthy, lame, and unhealthy pigs across systems in the US swine industry

	Metacarpal			Fibula			2nd rib			10th rib		
System	Healthy	Lame	Unhealthy	Healthy	Lame	Unhealthy	Healthy	Lame	Unhealthy	Healthy	Lame	Unhealthy
1	61.6	63.4	57.9	64.9	65.3	65.2	63.2	63.1	62.8	63.2	63.4	63.0
2	60.4	61.6	55.8	64.6	64.6	62.6	62.2	62.7	61.7	62.4	62.2	61.9
3	63.0	58.8	56.6	65.6	63.0	62.1	61.4	61.4	61.0	61.9	62.0	61.4
4	62.2	63.4	62.5	64.6	64.4	63.3	62.5	62.2	63.2	62.8	62.6	62.8
5	59.0	59.3	59.6	63.3	65.2	64.2	62.3	63.1	63.3	62.2	62.7	63.0
6	62.6	60.4	60.7	65.9	65.3	65.1	63.1	62.5	62.8	62.8	63.2	63.1
7	60.3	58.8	57.8	63.7	64.5	64.0	60.9	62.2	61.8	61.3	62.4	62.5
8	61.6	60.3	62.0	66.1	64.5	64.7	64.3	63.1	63.6	63.8	62.7	61.8
9	60.3	59.2	61.9	64.9	65.2	65.5	61.9	62.8	64.3	61.8	63.5	64.4
10	59.4	54.7	59.8	61.2	64.0	63.3	62.2	62.0	62.3	62.5	62.5	62.6
11	60.7	58.0	56.9	64.9	63.0	57.6	62.1	61.9	61.0	61.2	62.4	61.9
12	60.8	52.6	59.2	65.4	66.6	65.6	91.6	64.9	63.3	61.1	64.0	60.5
13	60.3	61.0	55.9	64.7	64.5	63.2	59.9	61.1	60.3	61.8	61.3	61.8
14	60.2	59.4	54.5	64.8	64.3	61.5	61.2	62.0	61.1	62.3	62.0	61.6

[1]All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105°C for 7 d, and then ashed in a muffle furnace at 600°C for 24 h. The value is the average of all the bones from the same system within each pig type.

Table 4.11. Correlation coefficients (*r)* between individual bone analysis of healthy, lame, and unhealthy pigs and dietary P and Ca[1]

	Healthy pig		Lame pig		Unhealthy pig	
Item	STTD P, %	Total Ca, %	STTD P, %	Total Ca, %	STTD P, %	Total Ca, %
Metacarpal						
Non-defatted bone ash, %	0.27**	0.11	0.11	-0.01	0.036	-0.19
Defatted bone ash, %	0.14	0.20	0.23*	0.38***	-0.058	0.026
Bone density, g/mL	0.11	0.44***	0.21	0.42***	-0.036	0.32**
Breaking strength, kg	0.052	0.30**	0.11	0.39***	0.057	0.45***
Bone ash, g	-0.024	0.37***	-0.061	0.27**	-0.13	0.29**
Bone ash P, %	-0.063	-0.15	0.13	0.12	0.095	-0.022
Bone ash P, g	-0.018	0.32**	-0.049	0.25*	-0.11	0.26*
Bone ash Ca, %	-0.066	-0.097	0.11	0.15	0.095	0.046
Bone ash Ca, g	-0.02	0.34**	-0.051	0.25*	-0.11	0.28**
Fibula						
Non-defatted bone ash, %	0.37***	0.21	0.26*	0.14	0.013	-0.18
Defatted bone ash, %	0.091	0.38***	0.13	0.16	-0.096	0.079
Bone density, g/mL	0.2	0.45***	0.12	0.40***	0.076	0.31**
Breaking strength, kg	0.095	0.43***	0.12	0.39***	-0.037	0.40***
Bone ash, g	0.051	0.34**	0.065	0.36***	-0.037	0.36***
2nd rib						
Non-defatted bone ash, %	0.40***	0.013	0.096	-0.057	0.055	-0.17
Defatted bone ash, %	0.30**	0.28**	0.18	0.2	0.074	0.16
Bone density, g/mL	0.2	0.34**	0.22	0.21	0.021	0.15
Breaking strength, kg	0.15	0.45***	0.16	0.41***	0.047	0.27**
Bone ash, g	-0.023	0.35***	0.15	0.36***	0.039	0.34***
10th rib						
Non-defatted bone ash, %	0.47***	0.11	0.18	-0.029	-0.081	-0.23*
Defatted bone ash, %	0.39***	0.29**	0.036	0.13	-0.11	0.17
Bone density, g/mL	0.26*	0.45***	0.2	0.048	-0.11	0.17
Breaking strength, kg	0.079	0.44***	0.22	0.42***	0.036	0.40***
Bone ash, g	0.019	0.34***	0.17	0.39***	0.029	0.39***
Bone ash P, %	0.11	0.067	-0.06	0.23*	0.054	-0.12

Bone ash P, g	0.031	0.32**	0.16	0.43***	0.028	0.36***
Bone ash Ca, %	0.12	0.094	-0.069	0.26*	0.044	-0.091
Bone ash Ca, g	0.027	0.32**	0.15	0.44***	0.023	0.37***

*$P < 0.10$; **$P < 0.05$; ***$P < 0.01$.

[1]The dietary weighted average of STTD P and total Ca was calculated for each pig and correlated with each bone measurement across the 4 different bones measured. For STTD P and total Ca the amount in the diet was compared as a percentage to the NRC (2012). The number of days on feed in each phase was calculated to determine the weighted average of each dietary nutrient compared to the NRC to determine the total amount of STTD P and total Ca up until the time of collection.

Table 4.12. Correlation coefficients (*r)* between individual bone analysis of healthy, lame, and unhealthy pigs and dietary vitamin D and serum vitamin D[1]

	Healthy pig		Lame pig		Unhealthy pig	
Item	Vitamin D3	Serum 25(OH)D_3	Vitamin D3	Serum 25(OH)D_3	Vitamin D3	Serum 25(OH)D_3
Serum analysis						
Ca, mg/dL	0.018	-0.061	0.071	0.29**	-0.016	0.034
P, mg/dL	-0.19	-0.025	-0.13	0.33**	-0.18	0.15
25(OH)D_3, ng/mL	-0.009	---	-0.054	---	-0.022	---
Metacarpal						
Non-defatted bone ash, %	-0.17	0.10	-0.13	-0.24*	-0.059	-0.34***
Defatted bone ash, %	0.016	0.33*	0.19	0.10	-0.26*	0.092
Bone density, g/mL	-0.03	0.33***	0.041	0.32**	0.04	0.11
Breaking strength, kg	0.015	0.19	0.027	0.064	-0.084	0.092
Bone ash, g	0.043	0.26*	0.059	0.11	-0.074	0.11
Bone ash P, %	-0.069	0.12	-0.039	0.039	-0.28**	0.14
Bone ash P, g	0.013	0.30**	0.046	0.12	-0.14	0.13
Bone ash Ca, %	-0.05	0.14	-0.027	0.019	-0.26*	0.11
Bone ash Ca, g	0.019	0.29**	0.049	0.11	-0.13	0.12
Fibula						
Non-defatted bone ash, %	-0.11	-0.003	-0.095	-0.22*	0.06	-0.38***
Defatted bone ash, %	0.11	0.036	-0.065	-0.003	-0.075	0.13
Bone density, g/mL	0.13	0.24*	0.031	0.14	0.19	0.11
Breaking strength, kg	0.05	0.23*	0.024	0.17	0.02	0.15
Bone ash, g	0.047	0.21*	0.025	0.17	0.021	0.093
2nd rib						
Non-defatted bone ash, %	-0.18	-0.10	0.001	-0.29**	-0.057	-0.37***
Defatted bone ash, %	-0.19	0.27**	-0.15	0.027	-0.32**	-0.007
Bone density, g/mL	-0.13	0.36***	0.11	0.32**	-0.063	0.094
Breaking strength, kg	0.036	0.27**	0.11	0.18	0.031	0.31**
Bone ash, g	-0.058	0.22*	-0.006	0.14	0.031	0.23*
10th rib						
Non-defatted bone ash, %	-0.21	-0.041	0.003	-0.21	-0.042	-0.39***

Defatted bone ash, %	-0.051	0.20	-0.19	0.10	-0.20	0.06
Bone density, g/mL	-0.087	0.27**	-0.007	0.24*	0.062	-0.25*
Breaking strength, kg	-0.041	0.17	0.045	0.22*	0.092	0.24*
Bone ash, g	0.035	0.25**	0.065	0.24*	0.019	0.20
Bone ash P, %	0.044	0.18	0.25*	0.15	0.19	0.068
Bone ash P, g	0.05	0.28**	0.085	0.25*	0.07	0.20
Bone ash Ca, %	0.023	0.15	0.24*	0.089	0.21	0.006
Bone ash Ca, g	0.044	0.27**	0.08	0.24*	0.069	0.19

*$P < 0.10$; **$P < 0.05$; ***$P < 0.01$.

[1]The dietary weighted average of vitamin D3 was calculated for each pig and correlated with each bone measurement across the 4 different bones measured. For vitamin D3, the amount in the diet was compared as a percentage to the NRC (2012). The number of days on feed in each phase was calculated to determine the weighted average of each dietary nutrient compared to the NRC to determine the total amount of vitamin D3 up until the time of collection. The serum $25OHD_3$ levels were collected from each pig at the time of sample collection.

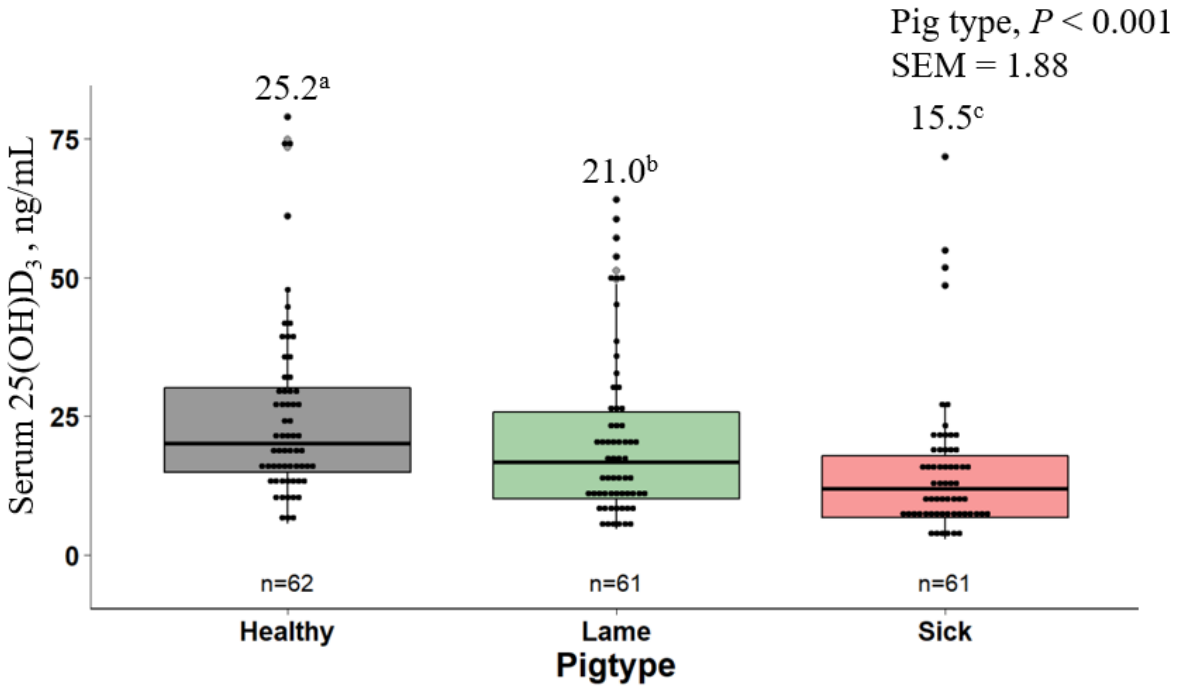

Figure 4.1. Serum vitamin D levels comparing the healthy, lame, and unhealthy pigs across all systems.

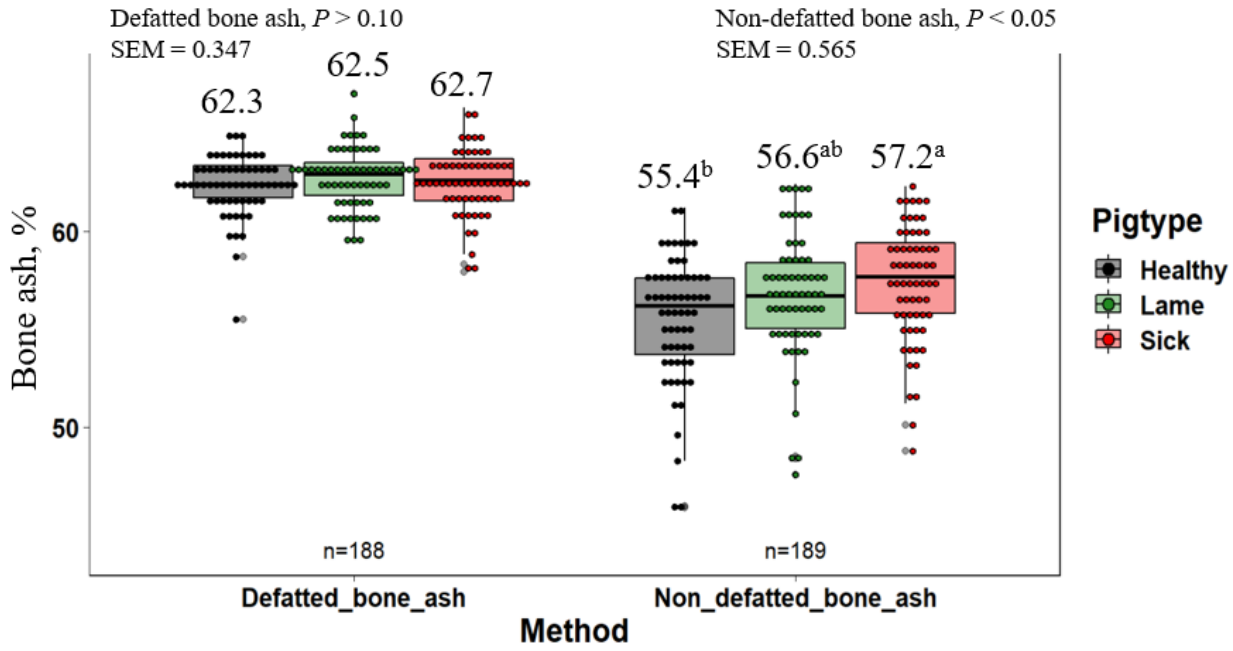

Figure 4.2. Bone ash percentage for 10th rib for healthy, lame, and unhealthy pigs using defatted and non-defatted bone ash procedures.

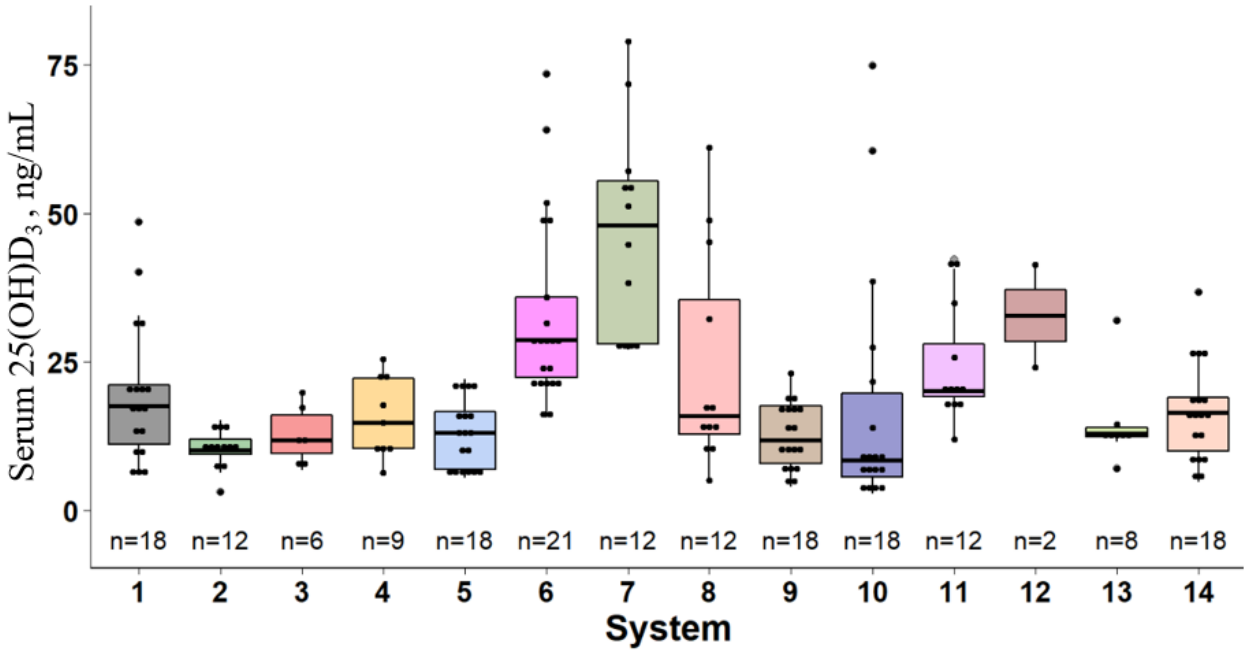

Figure 4.3. Serum vitamin D levels comparing all of the samples across the systems.

Chapter 5 - Comparing analytical methods used to measure bone mineralization across different bones of nursery and finishing pigs

Abstract

Data from 8 experiments were used to compare different bones and analytical procedures used to measure bone mineralization. The objective of the study was to determine whether the results of analytical methods used to measure bone mineralization vary across different bones and to characterize ranges of bone mineralization found in healthy nursery and finishing pigs. In each experiment (nursery pigs in experiments, 1, 2, 3, and 4, and finishing pigs in experiment 5, 6, 7, and 8), data was used from pigs fed diets with dietary P level above NRC (2012) requirement estimates with the goal to generate normal physiological levels of bone mineralization. At the completion of each study, a subset of pigs were euthanized, and either metacarpals, fibulas, 2nd ribs, and 10th ribs, were collected to determine bone mineralization using bone density or percentage bone ash There were positive correlations ($P < 0.05$) between bone density and bone ash procedures for each bone, with greater correlations observed in nursery pigs compared to finishing pigs. In nursery pigs, the highest correlation ($P < 0.05$) between non-defatted percentage bone ash and defatted percentage bone ash were observed in 2nd and 10th ribs ($r > 0.71$). When comparing each analytical measurement for different bones, there were higher positive correlations ($P < 0.05$) observed in nursery pigs than finishing pigs, with the greatest correlation observed between bone density and non-defatted bone ash. In nursery pigs, the correlation coefficients were $r > 0.64$, whereas in finishing pigs correlations coefficients were $r > 0.35$. Using the defatted bone ash procedure, variation was reduced across different bones when compared to the non-defatted bone ash, with lower variation observed for

finishing pig bones. In summary, bone density and bone ash can be used to determine bone mineralization in nursery and finishing pigs due to the increased correlation between methods, with defatted bone ash preferred compared to non-defatted ash in finishing pigs due to a greater reduction in variation. The study also suggests that procedures for bone density are simpler and allow for a more rapid assessment of bone mineralization compared to bone ash. These data provide a range of diagnostic results within healthy populations of nursery and finishing pigs for multiple diagnostic techniques and bones.

Introduction

Bone characteristics are the most sensitive indicators of the response to dietary Ca and P concentrations in swine diets (Cromwell, 2009). There are different measurements that can be used to determine bone mineralization, with bone ash the most common method (Lee et al., 2021; Williams et al., 2023a,c). There are two different procedures used to measure bone ash, one which removes the fat from the bone before ashing (defatted) and the other does not (non-defatted). The defatted procedure removes the lipid from the bone before the ashing takes place, which results in reduced variation between bones compared to the non-defatted procedure, and this variation is greater in finishing pigs compared to nursery pigs due to the increased fat accumulation in bones of mature pigs (Wensley et al., 2020; Williams et al., 2023c). In finishing pigs, the defatted percentage bone ash is 14 to 15 percentage units greater than the same bone measured using the non-defatted bone ash method (Williams et al., 2023c); whereas in nursery pigs, the defatted percentage bone ash is 11 to 12 percentage units greater than non-defatted bones (Williams et al., 2023a). This makes interpretation of diagnostic results challenging with a large magnitude of difference between the two methods if the actual procedure is not reported with the results. Another procedure that can be used to determine bone mineralization is using

the Archimedes Principle, which uses water displacement to determine bone density. Determining bone density is a simpler procedure than bone ash and can be more sensitive to detect differences in dietary P, specifically in fibulas and 2nd rib in nursery pigs (Williams et al., 2023a). Differences can also be observed between the different bones and analytical method used to measure bone mineralization. Metacarpals have the lowest bone mineralization in nursery and finishing pigs, with fibulas and ribs similar (Williams et al., 2023a,c).

Currently, reference values for bone mineralization for growing and finishing pigs used by diagnosticians, which have been adapted from research conducted the last 50 years (Field et al., 1974), do not always specify which procedures are used and typically show the range for a healthy pig. Recently, Williams et al. (2023b) evaluated different bones and various analytical measurements between healthy, lame, and sick pigs. The authors observed sick and lame pigs have lower bone density compared to healthy pigs, but no difference between the different pig types for percentage defatted bone ash. When samples are submitted to diagnostic laboratories for analysis, they are typically from unhealthy pigs or those that have been off feed, and results may not be representative of the larger population. Therefore, our objective was to characterize the relationship between different analytical methodologies and bones used to determine bone mineralization in healthy nursery and finishing pigs. Furthermore, these data can be used to quantify ranges in diagnostic results for bone density and percentage bone ash procedures for different bones from healthy nursery and finisher pigs.

Materials and Methods

All data used in the analysis were from experiments conducted in nursery and finishing pigs. A total of 4,887 bones (1,329 metacarpals; 1,190 fibulas; 1,178 2nd ribs; and 1,190 10th ribs) from 8 different experiments were used in the current analysis (Vier et al., 2019; Becker et al., 2023;

Cordoba et al., 2023; Reeb et al., 2023ab; Williams et al., 2023a,b,c). The studies were conducted between 2018 and 2022 and took place at the Kansas State University Swine Teaching and Research Center in Manhattan, KS or commercial research facilities in southwestern MN. All studies were conducted with appropriate approvals provided by the Kansas State University Institutional Animal Care and Use Committee.

Pigs from each study were fed a range of dietary P levels above the NRC (2012) requirement to determine the effects of bone mineralization in response to increasing dietary P level above the pigs' requirement, with pigs used in the analysis coming from diets fed above the NRC (2012) requirement. At the conclusion of each study, healthy pigs closest to the mean body weight in each pen was euthanized and either metacarpals, fibulas, 2nd ribs, and 10th ribs, were collected to determine bone mineralization using bone density, or percentage bone ash. Procedures for bone density and percentage bone ash can be found in Table 1 and 2. For bone density, a dry bone weight was collected, then bones were submerged in ultra-purified water under a vacuum with 1.06 kg per cubic centimeter for a minimum of 4 h. Bones were then weighed while suspended in a vessel of ultra-purified water, and the weight used to calculate bone density. For defatted bone ash, all bones were placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Following this step, bones were then dried at 105˚C for 7 d and then ashed in a muffle furnace at 600˚C for 24 h. For non-defatted bone ash, the procedure of ashing was the same as the defat procedure with the exception of skipping the step of fat extraction using petroleum ether. These methods were used to determine total bone ash weight and percentage ash relative to dried bone weight.

Correlation coefficients (*r*) between analytical measurements within each bone and across the different bones were determined using the lm function in R Stats Package [Version 4.1.1

(2021-08-10); R Core Team, 2021] using the RStudio environment (Version 1.4.1717; RStudio Team, 2021). Results were considered significant at $P \leq 0.05$ and marginally significant at $0.05 \leq P \leq 0.10$. For Table 6, the data were summarized by pig type and bone and data reported as the average, standard deviation, and range of observed values.

Results and Discussion

Metabolic bone disease occurs in growing and finishing pigs when there are imbalances in the levels of essential vitamins and minerals, resulting in lameness. Lameness poses significant economic and animal welfare challenges for swine producers. It is important for diagnosticians to accurately diagnose cases of lameness, considering the substantial financial repercussions it holds for both producers and nutritionists. Bone mineralization is often measured to determine the severity of lameness cases. It is also routinely measured for studies comparing Ca and P requirement estimates (Lagos et al., 2019; Vier et al., 2019; Lautrou et al., 2020). The two main procedures used to measure bone mineralization in pigs are bone density and bone ash. Misdiagnosis of bone mineralization can potentially lead to excessive dietary vitamin D, Ca, and P, which may prove unnecessary and result in diminished performance and impaired bone mineralization if not executed correctly (Williams et al., 2023b).

Currently, research that compares the different procedures used to measure bone mineralization has been conducted, but there is limited data that determined the relationship between results from different bones and analytical measurements used to determine bone mineralization. Correlations are important because not all labs or experiments use the same procedures on the same bones to measure bone mineralization. Thus, being able to compare different techniques and bones may be useful for diagnosticians, veterinarians, and nutritionists to integrate information from multiple sources. In the present study, there were significant

positive correlations ($P < 0.05$; Table 3, 4, and 5) between bone density and non-defatted bone ash for metacarpals, fibulas, 2nd ribs, and 10th ribs for nursery and finishing pigs. Correlation coefficients between bone density and non-defatted bone ash were greater for nursery pigs than finishing pigs, with the average correlation coefficients (*r*) of all bones being 0.77 for nursery pigs' and 0.55 for finishing pigs. The 2nd and 10th ribs had greater correlation coefficients between bone density and non-defatted bone ash than fibulas and metacarpals for both nursery and finishing pigs. The reason for ribs to have greater correlation between bone density and non-defatted bone ash is due to lower fat accumulation in ribs compared to metacarpals and fibulas.

Bone density using Archimedes Principle determines density of bone using water displacement. Recent work in nursery and finishing pigs have found bone density to be a good measurement to detect differences in dietary P (Williams et al., 2023a,c). When comparing different bones, the 2nd rib and fibulas were more sensitive to detect these differences compared to metacarpals and 10th ribs. Bone density can also detect differences in bone mineralization between healthy and unhealthy pigs. In the current analysis, the mean bone density in nursery pigs for the 4 bones is between 1.21 and 1.33 g/mL (Table 6), with metacarpals lowest and 10th ribs greatest. In finishing pigs, mean bone density for the 4 bones is between 1.32 and 1.45, with metacarpals lowest and fibulas greatest. When measuring bone mineralization at diagnostic labs, results are routinely compared to reference ranges that have been established from previous research. For bone density of nursery and finishing pigs, the reference range is between 1.4 and 1.5 g/mL regardless of the bone measured. The average bone density for metacarpals, fibulas, 2nd rib, and 10th ribs of clinically healthy pigs fed dietary P levels above the pigs' requirement were below reference ranges used by diagnosticians for both nursery and finishing pigs. The wide range demonstrates that variation can be observed for each bone in healthy pigs with some

values above and below the reference range of 1.4 – 1.5 g/mL “M. C. Rahe (Iowa State Veterinary Diagnostic Lab, Ames, Iowa, personal communication)” and the low end being well below the reference range.

Percentage bone ash is routinely measured to determine bone mineralization in growing pigs (Vier et al., 2019; Lee et al., 2021; Williams et al., 2023a,b,c). There are two main procedures used for bone ash, one which removes the fat from the bone before ashing (defat) and the other does not (non-defat). In the current analysis, nursery and finishing pigs have similar percentage non-defatted bone ash for 2nd and 10th ribs demonstrating reduced lipid accumulation in ribs compared to fibulas and metacarpal as the pig grows. Finishing pigs have greater non-defatted bone ash for fibulas and metacarpals compared to nursery pigs. Defatted bone ash is greater than non-defatted bone ash for both nursery and finishing pigs, with the magnitude of change between the two procedures greater in finishing pigs than nursery pigs. In nursery pigs, the defatted percentage bone ash is 11 to 12 percentage units greater than the same bone measured using the non-defatted bone ash method, whereas in finishing pigs, the defatted percentage bone ash is 14 to 15 percentage units greater than the non-defatted bone ash procedure. These results are consistent with observations of Wensley et al. (2020) who analyzed the two different bone ash methods nursery pig fibulas and finishing pig metacarpals. The authors found a 10 percentage unit difference between defatted and non-defatted bone ash in nursery pigs and a 16 percentage unit difference between methods for finishing pigs. For bone ash, the reference range used by most diagnosticians is 58 – 62%. If conducting the defatted bone ash procedure, overall averages for each bone in nursery and finishing pigs in the current analysis would be within the reference range. For non-defatted bone ash, the averages for each bone are all below the reference range. Interestingly, when comparing non-defatted bone ash in

healthy, lame, and unhealthy pigs, sick pigs have increased non-defatted percentage bone ash compared to healthy pigs (Williams et al., 2023b). Unhealthy pigs have reduced feed intake relative to healthy pigs, which results in mobilizing lipid from the skeletal system as a source of energy. This results in unhealthy pigs having a greater percentage bone ash than healthy pigs when the non-defatted bone ash technique is used. These differences between the bone ash procedures can make it hard for diagnosticians to properly diagnose lameness cases. To reduce misdiagnosis occurring in lameness cases, it is important to de-fat the bone prior to ashing due to a reduction in variation between bones (Williams et al., 2023a,c) and health statuses (Williams et al., 2023b).

With the two different procedures, there are different methods to clean and prepare the bones before ashing begins. The soft tissue can be removed prior to processing by autoclaving (Gonzalez-Vega et al., 2016), boiling (Gourley et al., 2018), or being manually defleshed (Williams et al., 2023a,b,c). If conducting bone density on the same bone that bone ash will be conducted, it is important to use the manual defleshing process because autoclaving and boiling change the nature and density of the bone by applying heat and steam, while manual defleshing does not incorporate heat, steam or water. By defatting bones and removing the lipid and water, the variation across the bones is reduced (Wensley et al.,2020; Williams et al., 2023abc). Due to mature animals having increased lipid accumulation in bones (Lian et al., 1999) compared to nursery pigs, the magnitude of difference between the non-defatted and defatted methods is greater in finishing pigs.

The greatest correlation between the two bone ashing methods was within the 2nd and 10th ribs (r = 0.71 and r = 0.76, respectively) and the lowest correlation between methods for metacarpals (r = 0.15). The metacarpals have increased lipid accumulation compared to ribs,

which explains the reduced correlation. Increased lipid accumulation in the metacarpals can be observed when comparing the difference between the two ashing methods, which means more fat was removed from the metacarpals compared to fibulas, 2nd ribs, and 10th ribs. Wensley et el. (2020) observed a positive correlation between non-defatted bone ash and defatted bone ash for fibulas from nursery pigs (r = 0.61). In contrast, they observed a negative correlation coefficient (r = - 0.20) in finishing pigs when comparing non-defatted and defatted bone ash for metacarpals. The authors determined the negative correlation in finishing pigs is due to the increased variation in mature pig bones when the non-defatted bone ash procedure is conducted, compared to the defatted procedure. In the current analysis, we observed similar positive correlations as Wensley et al. (2020) in nursery pigs comparing non-defatted and defatted for the different bones. In finishing pigs, we observed inverse results comparing non-defatted and defatted bone ash as compared to Wensley et al. (2020). In the current analysis, we observed positive correlations between the two techniques for all bones; however, similar to Wensley et al (2020) correlations were lower within finishing pigs than in nursery pigs. The results demonstrate the increased variation in bones of finishing pigs compared to nursery pigs. In the current analysis, we used bones from pigs fed dietary P above their requirements, whereas Wensley et al. (2020) used bones from studies that had dietary treatments with P levels below the requirement of the pig which could have resulted in finishing pigs having a negative correlation for the two different bone ashing methods.

Similar to the correlations between non-defatted and defatted bone ash, there are positive correlations between bone density and the two bone ash techniques. In nursery pigs, bone density is highly correlated with both ash procedures, with a greater correlation between bone density and non-defatted bone ash. The increased correlation between bone density and non-defatted

bone ash is due to the lipid remaining during the bone density procedure. Similar to correlation coefficients comparing non-defatted and defatted bone ash, ribs have the greatest correlation coefficients between bone density and both bone ash techniques in nursery and finishing pigs. Nursery pigs have greater correlations between the different methods compared to finishing pigs, which continues to demonstrate the increased variation in finishing pigs, which is consistent with previous research (Wensley et al., 2020; Williams et al., 2023abc).

Based on the results comparing the different procedures across the different bones in nursery and finishing pigs, we propose that different procedures should be used for the different ages. In nursery pigs, bone density, non-defatted bone ash, and defatted bone ash appear to be reliable and consistent methods to determine accuracy, with ribs having the greatest correlations ($64 < r < 81$) compared to fibulas and metacarpals ($15 < r < 64$). When measuring bone mineralization in finishing pigs, defatting bones prior to ashing should be conducted as a standard procedure due to the increased variation compared to nursery pigs and between the different bones. Finishing pigs have greater lipid accumulation in bones compared to nursery pigs, which leads to greater variation between bone ashing methods.

In summary, bone ash is the primary method used for measuring bone mineralization, which involves two techniques: one which removes the fat from the bone (defatted) and one which does not (non-defatted). Non-defatted and defatted bone ash have a positive correlation, which is greater in nursery pigs than finishing pigs. Also, bone density is highly correlated with both non-defatted and defatted bone ash. In nursery pigs, bone density, non-defatted and defatted percentage bone ash can be used to accurately assess bone mineralization, with 2nd and 10th ribs having the lowest variation compared to fibulas and metacarpals. In the current analysis, bone density and non-defatted bone ash are below the reference ranges used by diagnosticians, which

may lead to a clinical misdiagnosis. For nursery pigs, rib analysis was more consistent and thus could be proposed as the preferred bone to measure compared to the fibulas and metacarpals. In finishing pigs, defatting bones prior to ashing should be conducted due to the increased lipid content which leads to greater variation across bones being measured. It is also important to compare results to reference values using the same bone and procedure due to the differences between methods, particularly bone ash.

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Table 5.1. Procedures for determination of bone mineralization using bone ash[1]

1.	Clean all bones of extraneous soft tissue. Recommend to use a scalpel and necropsy knife to remove tissue. Bones should be completely cleaned of all soft tissue.
2.	Leave bones out overnight to dry at room temperature if removed from the freezer. During this time, place clean, prelabeled, empty crucibles in the drying oven (105 ˚C) for 24 h.
3.	Wrap bones in cheesecloth with permanent identification.
4.	Place bones in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. The number of bones that can fit in each extraction chamber will vary depending on the size of the bones being processed and the size of the extraction chamber.
5.	After 7 d, remove bones from the Soxhlet extractor and leave under a fume hood overnight to dry to allow any remaining ether to evaporate.
6.	Remove the empty crucibles from the drying oven and place them in glass desiccators until cooling is complete. The desiccators are used to aid in further removal of moisture while protecting the bones from water vapor in the air. Once cooled, weight the empty crucibles.
7.	Transfer full bones to prelabeled crucibles to maintain identification, collect weight of crucible and bone to obtain weight of bone, and then dry in a drying oven at 105 ˚C for 7 d. If full bones do not fit in crucible, cut them in half to fit them more easily inside the crucibles.
8.	After 7 d, remove the crucibles with bones from drying oven and place in glass desiccators until cooling is complete.
9.	Weigh the crucibles with bones to obtain a dry bone weight.
10.	Transfer crucibles with bones to a muffle furnace and ash at 600 ˚C for 24-h.
11.	Remove the crucibles with bones from the drying oven and place them in glass desiccators until cooling is complete.
12.	Weigh the crucibles with bones to obtain a bone ash weight.
13.	Bone ash percentage = (bone ash weight, g / dry bone weight, g) × 100

[1]If conducting defatted bone ash, conduct steps 1-13. If conducting non-defatted bone ash, skip steps 3-5.

Table 5.2. Procedures for determination of bone mineralization using Archimedes Principle to calculate bone density

1.	Clean all bones of extraneous soft tissue. Recommended to use a scalpel and necropsy knife to remove tissue. Bones should be completely cleaned of all soft tissue.
2.	Weigh all bones to get initial bone weight.
3.	Tag bones with individual identifications and place in Erlenmeyer flask. It is best to use 17-gauge wire to wrap a waterproof tag around each bone.
4.	Fill flask with ultra-pure water until all bones are fully submerged.
5.	Place stopper on the top of the Erlenmeyer flask.
6.	Connect Erlenmeyer flask to vacuum with 1.06 kg per cubic centimeter and run for a minimum of 4 h. Due to the bone being porous, the vacuum removes all ancillary products, such as blood, and ensures the density reading is just bone itself.
7.	Drain water and remove bones from Erlenmeyer flask. Rinse bones with tab water and pat dry.
8.	Collect weight of bone fully submerged under water. Bone should be free floating in water and make no contact with container it is being weighed in.
9.	Bone density, g/mL = (Weight of the bone prior to vacuum – weight of bone fully submerged in water) / weight of bone fully submerged in water × P. With P being the density of distilled water.

Table 5.3. Correlation coefficients (*r*) between analytical method within bone and phase of production for healthy nursery and finisher pigs[1]

	Bone density[2]		Non-defatted bone ash[3]	
Item	Nursery pig[4]	Finisher pig[5]	Nursery pig	Finisher pig
Metacarpal				
Non-defatted bone ash, %	0.64*	0.35*	---	---
Defatted bone ash, %[6]	0.31*	0.28*	0.15	0.16*
Fibula				
Non-defatted bone ash, %	0.64*	0.36*	---	---
Defatted bone ash, %	0.54*	0.38*	0.34*	0.21*
2nd rib				
Non-defatted bone ash, %	0.80*	0.48*	---	---
Defatted bone ash, %	0.64*	0.47*	0.71*	0.23*
10th rib				
Non-defatted bone ash, %	0.81*	0.52*	---	---
Defatted bone ash, %	0.70*	0.46*	0.76*	0.18*
All bones				
Non-defatted bone ash, %	0.77*	0.55*	---	
Defatted bone ash, %	0.56*	0.58*	0.43*	0.47*

*$P < 0.05$

[1]Bones from nursery and finishing pigs were measured for both bone density and either non-defatted or defatted bone ash to determine the correlation between the different analytical measurements for each bone.

[2]Bone density was measured on each bone based on Archimedes principle.

[3]All bones were cleaned of tissue and then dried at 105°C for 7 days and then ashed in a muffle furnace at 600°C for 24 h.

[4]Bones came from nursery pigs that were approximately 30 kg body weight.

[5]Bones came from growing and finishing pigs, with the approximate weights ranging between 70 and 130 kg.

[6]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 105°C for 7 days, and then ashed in a muffle furnace at 600°C for 24 h.

Table 5.4. Correlation (*r*) between analytical results across bone and method for healthy nursery pigs[1]

Bone	Bone density[2]	Non-defatted bone ash[3]	Defatted bone ash[4]
Metacarpal			
Bone density			
Metacarpal	---	0.64***	0.31***
Fibula	0.62***	0.61***	0.48***
2nd rib	0.66***	0.49***	0.30***
10th rib	0.66***	0.63***	0.25***
Non-defatted bone ash			
Metacarpal	---	---	0.15
Fibula	0.58***	0.84***	0.11
2nd rib	0.68***	0.78***	0.19*
10th rib	0.65***	0.78***	0.14
Defatted bone ash			
Metacarpal	---	---	---
Fibula	0.41***	0.33***	0.44***
2nd rib	0.58***	0.48***	0.22***
10th rib	0.57***	0.61***	0.28***
Fibula			
Bone density			
Fibula	---	0.64***	0.54***
2nd rib	0.57***	0.51***	0.55***
10th rib	0.76***	0.66***	0.54***
Non-defatted bone ash			
Fibula	---	---	0.34***
2nd rib	0.57***	0.76***	0.56***
10th rib	0.71***	0.80***	0.46***
Defatted bone ash			
Fibula	---	---	---
2nd rib	0.48***	0.56***	0.46***

10th rib	0.53***	0.59***	0.56***
2nd rib			
Bone density			
2nd rib	---	0.80***	0.64***
10th rib	0.82***	0.78***	0.62***
Non-defatted bone ash			
2nd rib	---	---	0.71***
10th rib	0.72***	0.95***	0.70***
Defatted bone ash			
2nd rib	---	---	---
10th rib	0.69***	0.80***	0.72***
10th rib			
Bone density			
10th rib	---	0.81***	0.70***
Non-defatted bone ash			
10th rib	---	---	0.76***
Defatted bone ash			
10th rib	---	---	---

*$P < 0.10$; **$P < 0.05$; ***$P < 0.01$.

[1]Bones came from nursery pigs that were approximately 30 kg body weight.

[2]Bone density was measured on each bone based on Archimedes principle.

[3]All bones were cleaned of tissue and then dried at 105°C for 7 days and then ashed in a muffle furnace at 600°C for 24 h.

[4]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 105°C for 7 days, and then ashed in a muffle furnace at 600°C for 24 h.

Table 5.5. Correlation (*r*) between analytical results across bone and method for healthy finishing pigs[1]

Bone	Bone density[2]	Non-defatted bone ash[3]	Defatted bone ash[4]
Metacarpal			
Bone density			
Metacarpal	---	0.35***	0.29***
Fibula	0.78***	0.12	0.34***
2nd rib	0.52***	0.39***	0.26***
10th rib	0.51***	0.29***	0.31***
Non-defatted bone ash			
Metacarpal	---	---	0.16**
Fibula	0.24***	0.74***	0.18**
2nd rib	-0.11	0.55***	-0.067
10th rib	-0.085	0.55***	-0.024
Defatted bone ash			
Metacarpal	---	---	---
Fibula	0.43***	0.21***	0.43***
2nd rib	0.38***	0.29***	0.38***
10th rib	0.33***	0.23***	0.37***
Fibula			
Bone density			
Fibula	---	0.36***	0.38***
2nd rib	0.51***	0.50***	0.097
10th rib	0.52***	0.40***	0.19***
Non-defatted bone ash			
Fibula	---	---	0.21***
2nd rib	-0.075	0.63***	-0.14
10th rib	-0.061	0.65***	-0.13*
Defatted bone ash			
Fibula	---	---	---
2nd rib	0.33***	0.31***	0.35***

10th rib	0.37***	0.29***	0.40***
2nd rib			
Bone density			
2nd rib	---	0.48***	0.47***
10th rib	0.79***	0.37***	0.45***
Non-defatted bone ash			
2nd rib	---	---	0.23***
10th rib	0.51***	0.84***	0.22***
Defatted bone ash			
2nd rib	---	---	---
10th rib	0.37***	0.16**	0.66***
10th rib			
Bone density			
10th rib	---	0.52***	0.46***
Non-defatted bone ash			
10th rib	---	---	0.18**
Defatted bone ash			
10th rib	---	---	---

*$P < 0.10$; **$P < 0.05$; ***$P < 0.01$.

[1]Bones came from growing and finishing pigs, with the approximate weights ranging between 70 and 130 kg.

[2]Bone density was measured on each bone based on Archimedes principle.

[3]All bones were cleaned of tissue and then dried at 105°C for 7 days and then ashed in a muffle furnace at 600°C for 24 h.

[4]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 105°C for 7 days, and then ashed in a muffle furnace at 600°C for 24 h.

Table 5.6. Range of analytical measurements for select methodologies used to assess bone mineralization for metacarpal, fibula, 2nd and 10th ribs in healthy nursery and finishing pigs[1]

	Nursery pig[2]			Finishing pigs[3]			Reference range[4]
	Mean	Standard deviation	Range	Mean	Standard deviation	Range	Mean
Metacarpal							
Bone density, g/mL[5]	1.21	0.03	1.15 - 1.29	1.32	0.04	1.21 - 1.40	1.4 – 1.5
Non-defatted bone ash, %[6]	40.9	3.58	34.5 - 54.0	44.1	2.53	38.1 - 50.5	---
Defatted bone ash, %[7]	59.2	2.39	51.0 - 63.4	60.3	3.27	46.3 - 64.0	58 – 62
Fibula							
Bone density, g/mL	1.31	0.05	1.19 - 1.55	1.45	0.07	1.22 - 1.60	1.4 – 1.5
Non-defatted bone ash, %	50.9	4.01	37.4 - 62.3	54.3	3.14	47.9 - 61.9	---
Defatted bone ash, %	62.9	1.72	57.1 - 65.9	65.6	1.50	61.4 - 68.1	58 – 62
2nd rib							
Bone density, g/mL	1.28	0.04	1.18 - 1.42	1.34	0.05	1.16 - 1.47	1.4 – 1.5
Non-defatted bone ash, %	53.7	2.62	49.8 - 60.3	53.0	3.33	46.1 - 59.8	---
Defatted bone ash, %	59.2	2.19	54.3 - 64.0	62.4	1.63	56.5 - 65.3	58 – 62
10th rib							
Bone density, g/mL	1.33	0.08	1.23 - 1.72	1.39	0.05	1.26 - 1.51	1.4 – 1.5
Non-defatted bone ash, %	54.5	2.71	45.9 - 61.2	54.3	4.00	33.3 - 60.9	---
Defatted bone ash, %	60.2	1.92	55.9 - 63.8	62.6	1.52	55.5 - 65.0	58 – 62

[1]All bones used in the analysis are from pigs with relatively high health. All pigs were fed diets at or above the NRC (2012) requirement for P and reasonable Ca:P ratios.

[2]A total 484 bones (124 metacarpals, 124 fibulas, 112 2nd ribs, and 124 10th ribs) from 83 nursery pigs (approximately 30 kg) were included in the analysis.

[3]A total of 661 bones (277 metacarpals, 128 fibulas, 128 2nd ribs, and 128 10th ribs) from 172 growing and finishing pigs (approximately 70 to 130 kg) were included in the analysis.

[4]References ranges are adapted from veterinary diagnostic labs. Currently, no reference range is used for non-defatted bone ash, although it is typically the preferred method used by diagnosticians.

[5]Bone density was measured on each bone based on Archimedes principle.

[6]All bones were cleaned of tissue and then dried at 105°C for 7 d and then ashed in a muffle furnace at 600°C for 24 h.

[7]All bones were cleaned of tissue and then placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Bones were then dried at 105°C for 7 days, and then ashed in a muffle furnace at 600°C for 24 h.