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Non Ruminant Nutrition

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Technical Note: Assessment of two methods for estimating bone ash in pigs

Madie R. Wensley, Carine M. Vier, Jordan T. Gebhardt, Mike D. Tokach, Jason C. Woodworth, Robert D. Goodband,[1] and Joel M. DeRouchey

Department of Animal Sciences and Industry, College of Agriculture, Kansas State University, Manhattan, KS 66506-0201

[1]Corresponding author: goodband@ksu.edu

ORCiD number: 0000-0002-6144-6714 (J. T. Gebhardt).

Abstract

Data from three experiments conducted to evaluate the effects of increasing available P in swine diets were used to compare two different bone processing methods. Our objective was to determine if the procedures influenced treatment differences and the ability to detect changes in the percentage bone ash. In each experiment, pigs (nursery pigs in experiments 1 and 2, and finishing pigs in experiment 3) were fed a wide range of available phosphorus levels provided from either increasing monocalcium P or added phytase. At the completion of each experiment, a subset of pigs was euthanized, and either fibulas (experiments 1 and 2) or metacarpals (experiment 3) were collected to determine the percentage bone ash. Bones were processed by cleaning away all soft tissues followed by ether extraction for 7 d (defatted), or no lipid extraction (non-defatted), and then ashed. In nursery and finishing pigs, defatted bones had increased ($P < 0.001$) percentage bone ash compared with non-defatted bones. No evidence of a method × treatment interaction or linear and quadratic interactions were observed in bone ash weight and percentage bone ash ($P > 0.10$) for nursery pigs; however, a linear interaction was detected ($P < 0.05$) in percentage bone ash for grow-finish pigs. This response was minimal and likely due to increased variation observed in grow-finish pigs when bones were not defatted. The processing method did not affect the ability to detect differences among treatments as a result of changing dietary P concentrations in the nursery or grow-finish pigs. In summary, either non-defatted or defatted bone processing methods can be used to determine bone ash weight and percentage bone ash as a way to assess bone mineralization and dietary treatment differences in nursery pigs; however, the increased variation observed in mature pigs suggests that defatted bone processing is the preferred method for grow-finish pigs.

Key words: bone ash, ether extraction, phosphorus, pigs

Introduction

Extensive research has been conducted evaluating the impact of diet on different bone characteristics such as bone mineralization and bone-breaking strength. Bone characteristics are the most sensitive indicator of dietary calcium and phosphorus concentrations in swine diets (Koch and Mahan, 1985; Cromwell, 2009). Therefore, providing sufficient levels of these nutrients in the diet is crucial to optimizing percentage bone ash in order to maintain structural integrity and growth performance. Throughout the literature, many different methods for determining bone ash exist, which has led to increased variability in the responses observed. Bones may be

Abbreviations

aP	available phosphorous
BIC	Bayesian information criterion
BW	body weight
STTD P	standardized total tract digestible phosphorus

boiled (Gourley et al., 2018), autoclaved (González-Vega et al., 2016), or manually defleshed (Jendza et al., 2006) as a means to clean soft tissue prior to further processing. After cleaning, bone ash weight and percentage ash are determined by either drying and ashing bones (Gourley et al., 2018; Wu et al., 2018) or defatting the bones using solvent extraction prior to drying and ashing (Jendza et al., 2006; González-Vega et al., 2016). Often these steps vary in the solvent used, duration of solvent extraction, temperature and duration of drying and ashing, as well as the age of pig used in the experiment. Studies assessing the influence of processing methods on various bone response criteria have been conducted in poultry (Orban et al., 1993; Hall et al., 2003); however, no single processing method to estimate bone ash values in swine has been reported. Therefore, the objective of this study was to determine if differences exist between defatted and non-defatted processing methods and to determine their effects on the ability to detect treatment differences.

Materials and Methods

Experiments 1 and 2—nursery pigs

Growth performance data have been previously published for the described experiments (Wensley et al., 2020). Briefly, these studies involved nursery pigs (initially 10 kg) that were fed a range of increasing available phosphorus (**aP**) levels provided from either monocalcium P or added phytase to develop an aP release curve. In each experiment, diets were formulated below the P requirement as to effectively predict the P released from the phytase. Available P in the monocalcium P diets ranged from 0.11% to 0.27%, with increasing (150, 250, 500, 750, 1,000, or 1,500 FTU/kg) phytase (Smizyme TS G5 2,500) added to the 0.11% P diet.

At the conclusion of the 21-d study, the pig closest to the pen mean body weight (**BW**) was euthanized via penetrating captive bolt, and the right and left fibulas were collected to determine the percentage bone ash. After collection, bones were individually placed in plastic bags with permanent identification and stored at –20 °C until the analysis of bone mineral content. On the day of analysis, after thawing overnight, bones were wrapped in cheesecloth with permanent identification and autoclaved for 1 h at 121 °C. Once cooled for a period of 30 min, any leftover extraneous soft tissue including cartilage caps was cleaned from the fibulas. Bones were then dried at an ambient temperature for 24 h, cut in half to fit into crucibles, and weighed. After weighing, fibulas were processed either with or without lipid extraction, and left and right fibulas were alternated between procedures.

Using the non-defatted method (Table 1), a total of 64 (experiment 1) or 56 (experiment 2) fibulas were dried at 105 °C for 7 d in a drying oven. Once removed from the drying oven, bones were placed in desiccators while cooling to aid in further removal of moisture while protecting the bones from water vapor in the air. After approximately 2 h, bones were weighed and then ashed in a muffle furnace at 600 °C for 24 h.

For the defatted method (Table 2), a total of 64 (experiment 1) or 55 (experiment 2) fibulas were wrapped in cheesecloth to keep their tag identification and placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Defatted fibulas were then dried at 105 °C for 24 h and ashed at 600 °C for 24 h following the same procedures mentioned previously. Both processing methods were used to determine the percentage ash relative to dry bone weight. This was done by dividing the ash weight by dried, defatted bone weight (extracted weight), or by dividing the ash weight by dried, non-defatted bone weight (non-extracted weight).

Experiment 3—grow-finish pigs

Growth performance data have been previously published for the described experiment (Vier et al., 2019). Briefly, this study involved grow-finish pigs (initially 24 kg) that were fed diets containing 80%, 90%, 100%, 115%, 130%, or 150% of the NRC (2012) requirement estimates for standardized total tract digestible P (**STTD P**), with no added phytase. This was done by increasing the amount of monocalcium P at the expense of corn.

At the conclusion of the 111-d study (approximately 130 kg), one barrow and one gilt of intermediate BW were selected from each pen and transported to a commercial abattoir in northwest Iowa (Natural Food Holdings Inc., Sioux Center, IA) for the processing and collection of metacarpal bones. Following processing, the left front feet were separated at the junction

Table 1. Non-defatting bone processing procedures

1.	Wrap bones in cheesecloth with permanent identification and autoclave for 1 h at 121 °C.
2.	After removed from the autoclave, allow a ~30-min cooling period prior to cleaning bones of any leftover exogenous soft tissue and cartilage caps. We recommend using your hands, a scalpel, and a toothbrush to remove all tissues.
3.	Leave bones out overnight or for a 24-h period to dry at room temperature. During this time, place clean, prelabeled, empty crucibles in the drying oven.
4.	Remove crucibles from the drying oven and place them in glass desiccators for 2+ h or until cooling is complete. Once cooled, weigh the empty crucibles.
5.	Transfer bones to prelabeled crucibles to maintain necessary identification and then dry in a drying oven at 105 °C for 7 d. You can cut bones in half if needed in order to more easily fit them inside the crucibles.
6.	Remove the crucibles with bones from the drying oven and place them in glass desiccators for at least 2 h or until cooling is complete. Desiccators are used to aid in further removal of moisture while protecting the bones from water vapor in the air.
7.	Weigh the crucibles with bones to obtain a dry bone weight.
8.	Transfer crucibles with bones to a muffle furnace and ash at 600 °C for a 24-h period.
9.	Remove the crucibles with bones from the drying oven and place them in glass desiccators for at least 2 h or until cooling is complete.
10.	Weigh the crucibles with bones to obtain a bone ash weight.

Table 2. Defatting bone processing procedures

1.	Wrap bones in cheesecloth with permanent identification and autoclave for 1 h at 121 °C.
2.	After removed from the autoclave, allow a ~30-min cooling period prior to cleaning bones of any leftover exogenous soft tissue and cartilage caps. We recommend using your hands, a scalpel, and a toothbrush to remove all tissues.
3.	Leave bones out overnight or for a 24-h period to dry at room temperature.
4.	Cut each bone in half and wrap the halves together in the same piece of cheesecloth with permanent identification.
5.	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 bone being processed and the size of the extraction chamber.
6.	Remove bones from the Soxhlet extractor and leave under a fume hood overnight or for a 24-h period to dry. During this time, place clean, prelabeled, empty crucibles in the drying oven.
7.	Remove crucibles from the drying oven and place them in glass desiccators for 2+ h or until cooling is complete. Once cooled, weigh the empty crucibles.
8.	Transfer bones to the prelabeled crucibles to maintain necessary identification and then dry in a drying oven at 105 °C for a 24-h period.
9.	Remove the crucibles with bones from the drying oven and place them in glass desiccators for at least 2 h or until cooling is complete. Desiccators are used to aid in further removal of moisture while protecting the bones from water vapor in the air.
10.	Weigh the crucibles with bones to obtain a dry bone weight.
11.	Transfer crucibles with bones to a muffle furnace and ash at 600 °C for a 24-h period.
12.	Remove the crucibles with bones from the drying oven and place them in glass desiccators for at least 2 h or until cooling is complete.
13.	Weigh the crucibles with bones to obtain a bone ash weight.

of carpal bones and radius and ulna and individually placed in a zip-lock plastic bag with permanent identification. Feet were then transferred on dry ice to the Kansas State University Swine Laboratory and stored at –20 °C until the analysis of bone mineral content. On the day of analysis, after thawing overnight, the feet were autoclaved for 1 h at 121 °C. Once cooled for a period of 30 min, the third and fourth metacarpals of each foot were removed and cleaned of any leftover extraneous soft tissue, including cartilage caps. Bones were then dried at an ambient temperature for 24 h, cut in half, and weighed. After weighing, metacarpals were processed either with or without defatting. A subset of six bone samples was used for the development of the analytical assays and, therefore, was not able to be analyzed in the final dataset. Using the non-defatted method, a total of 77 fourth metacarpals (one from each pig; 35 gilts and 42 barrows) were dried at 105 °C for 7 d in a drying oven and ashed in a muffle furnace at 600 °C for 24 h using the same procedures as experiments 1 and 2. For the defatted method, a total of 83 third metacarpals (one from each pig; 39 gilts and 44 barrows) were wrapped in cheesecloth to keep their tag identification and placed in Soxhlet extractors containing petroleum ether for 7 d as a means of removing water and fat. Defatted metacarpals were dried at 105 °C for 24 h to determine the dry fat-free weight and then ashed at 600 °C for 24 h to determine ash weight. Similar to experiments 1 and 2, ash was expressed as a percentage of dried fat-free bone weight.

Statistical analysis

Data were plotted for visualization using the ggplot and ggscatter functions from the tidyverse and ggpubr packages in R (version 3.5.1 [07/02/2018]).

Data were analyzed as a randomized complete block design using a linear mixed model fit using the PROC GLIMMIX procedure of SAS version 9.4 (SAS Institute, Inc., Cary, NC). Data from experiments 1 and 2 were combined into a single dataset. For experiments 1 and 2, experiment × treatment × weight block was included in the model as a random effect, which served as the blocking factor for the fixed effect of bone processing method with individual fibula serving as the experimental unit. For experiment 3, gender × treatment × weight block was included in the model as a random effect, which served as the blocking factor for the fixed effect of processing method with individual metacarpal bone serving as the experimental unit. For every response, visual assessment of studentized residuals was performed. If visual indication of heterogeneous residual variance was observed, a second statistical model was fit explicitly modeling unique residual variance estimates for each analytical method. The Bayesian Information Criterion (**BIC**) was then compared between the two competing models, with the model having the lowest BIC used as the best-fitting model. After the selection of the best-fitting model for each outcome, the LSMEANS procedure was used to output mean values for each analytical method and an *F*-test was used to evaluate if evidence of a difference in outcome was present between analytical methods. Results were considered significant at $P \leq 0.05$ and marginally significant at $0.05 \leq P \leq 0.10$.

To determine if processing method affects the ability to detect differences in bone ash percent between dietary treatments,

Table 3. Bone ash weight and percent bone ash of non-defatted and defatted bones in nursery and grow-finish pigs

Item	Non-defatted	Defatted	SEM	*P*-value
Experiments 1 and 2 (nursery pigs)[1]				
Bone ash, g	0.76	0.74	0.012	0.054
Bone ash, %	40.5	50.0	0.303	<0.001
Experiment 3 (grow-finish pigs)[2]				
Bone ash, g	8.80	9.15	0.103	<0.001
Bone ash, %	44.9	61.3	—[3]	<0.001

[1]A total of 120 or 119 fibulas were used for the non-defatted or defatted bone processing methods, respectively.
[2]A subset of six bone samples was used for the development of the analytical assays and, therefore, was not able to be analyzed in the final dataset. A total of 77 fourth metacarpals or 83 third metacarpals were used for the non-defatted or defatted bone processing methods, respectively.
[3]Heterogenous residual variance resulted in a non-defatted SEM of 0.26 and a defatted SEM of 0.10, indicating increased variance in non-defatted bones.

predetermined orthogonal contrasts were used to evaluate the interactive effects of processing method × treatment, with weight block as the random effect. Models accounted for heterogeneous residual variance when appropriate as previously described. Data from experiments 1 and 2 were analyzed separately due to different dietary treatment structures. Linear and quadratic contrasts were evaluated within increasing inorganic P or phytase treatments, according to the bone processing method. For experiment 3, linear and quadratic contrasts were evaluated within increasing the STTD P level. Contrast coefficients for each experiment were adjusted to account for unequal spacing. Results were considered significant at $P \leq 0.05$ and marginally significant at $0.05 \leq P \leq 0.10$.

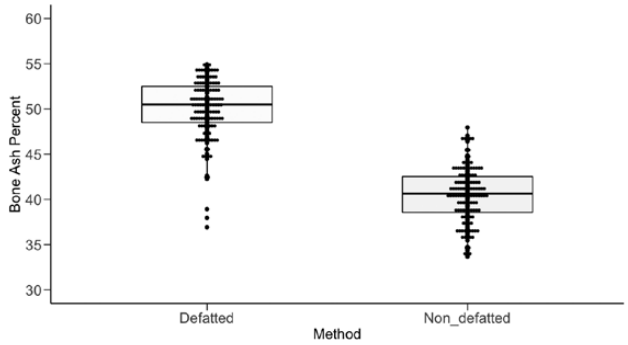

Figure 1. Percentage bone ash of defatted and non-defatted bones from experiments 1 and 2 (nursery pigs). A total of 119 or 120 fibulas were used for the defatted or non-defatted bone processing methods, respectively.

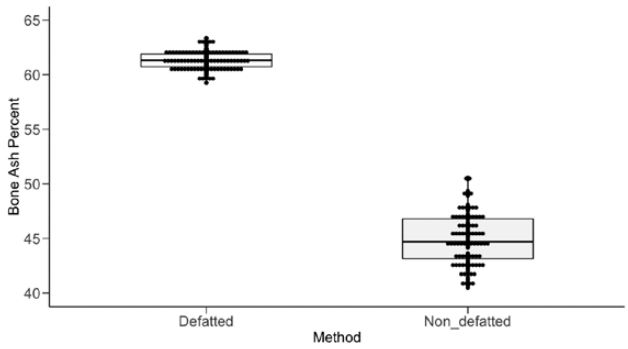

Figure 2. Percentage bone ash of defatted and non-defatted bones from experiment 3. A total of 83 third metacarpals or 77 fourth metacarpals were used for the defatted or non-defatted bone processing methods, respectively.

Results and Discussion

In nursery pigs, defatted bones tended to have decreased ($P = 0.054$) ash weight compared with non-defatted bones (Table 3). As anticipated, defatted bones had increased ($P < 0.001$) percentage bone ash compared with non-defatted bones. A similar response was observed in grow-finish pigs with defatted bones having increased ($P < 0.001$) percentage bone ash compared with non-defatted bones. Different from the nursery data, defatted bones also had increased ($P < 0.001$) bone ash weight in grow-finish pigs.

There was more variation in grow-finish pigs for non-defatted percent bone ash values compared with nursery pigs. Heterogenous residual variance was also observed between treatment groups, indicating increased variance in mature pigs when water and fat were not extracted from bones prior to determining the percentage bone ash (Figures 1 and 2). Previously, Lian et al. (1999) reported that bones from mature animals contain approximately 45% water, 25% ash, 20% protein, and 10% fat. As a result of age and nutritional status, fat and water content of bones will change (Crenshaw, 2001). The results herein further support these observations. When plotting bone ash values by the two processing methods (Figures 3 and 4), a positive correlation ($R = 0.61$) was observed in nursery pigs, indicating a linear relationship, in which values determined by one processing method can be related to the other. In contrast, a negative correlation ($R = -0.20$) was observed in grow-finish pigs. The smaller correlation coefficient in grow-finish pigs further demonstrates the increased variation in mature pig bones when electing to use non-defatted vs. defatted bones for experimental analysis. Hall et al. (2003) observed a similar response in poultry, with increased variance as birds aged and was further exacerbated when only autoclaving bones compared with solvent extraction.

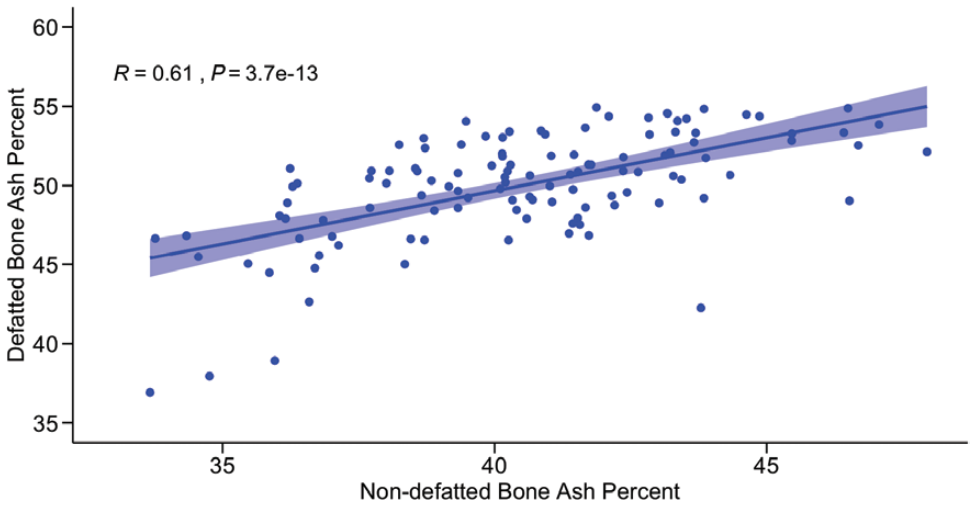

Figure 3. The relationship between percentage bone ash values for non-defatted and defatted bones from experiments 1 and 2. Individual points represent a pig, with one pig randomly selected from each pen.

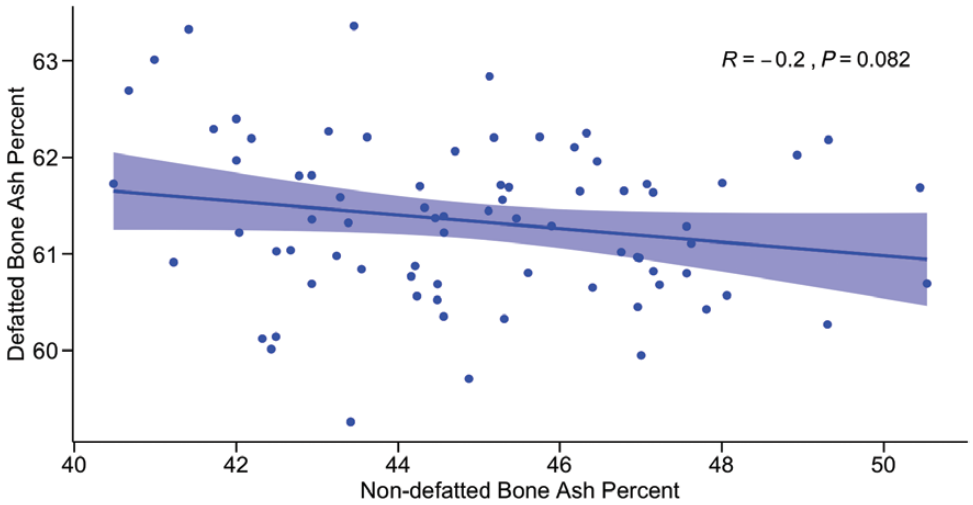

Figure 4. The relationship between percentage bone ash values for non-defatted and defatted bones from experiment 3. Individual points represent a pig, with one pig randomly selected from each pen.

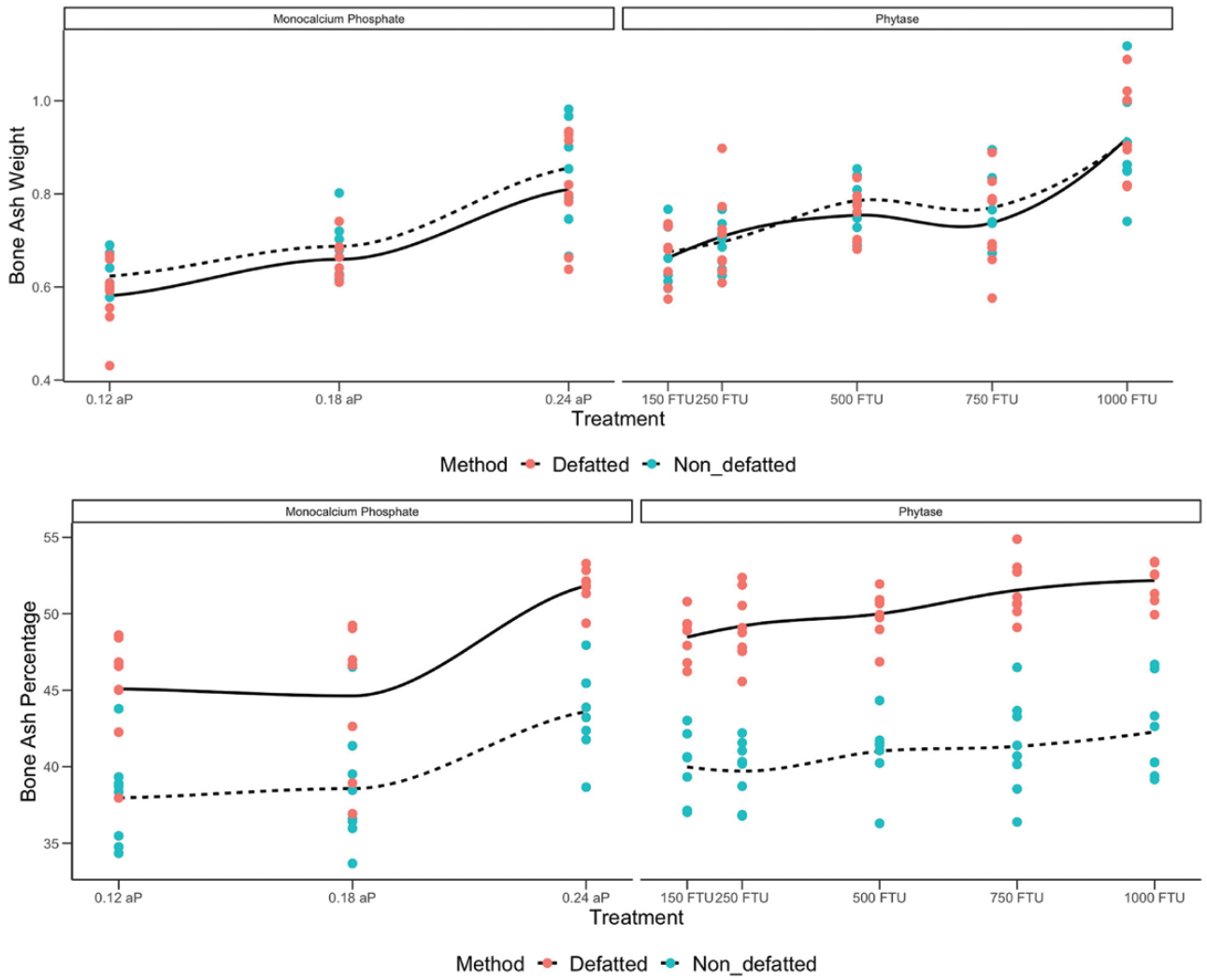

Figure 5. Treatment means and 95% confidence intervals for bone ash weight (panel A) and percentage bone ash (panel B) from experiment 1. Pigs were fed a range of increasing aP levels provided from either monocalcium P or added phytase. Diets were formulated below the P requirement with the aP in the monocalcium P diets ranged from 0.12% to 0.24%, with increasing (150, 250, 500, 750, or 1,000 FTU/kg) phytase added to the lowest inorganic P diet.

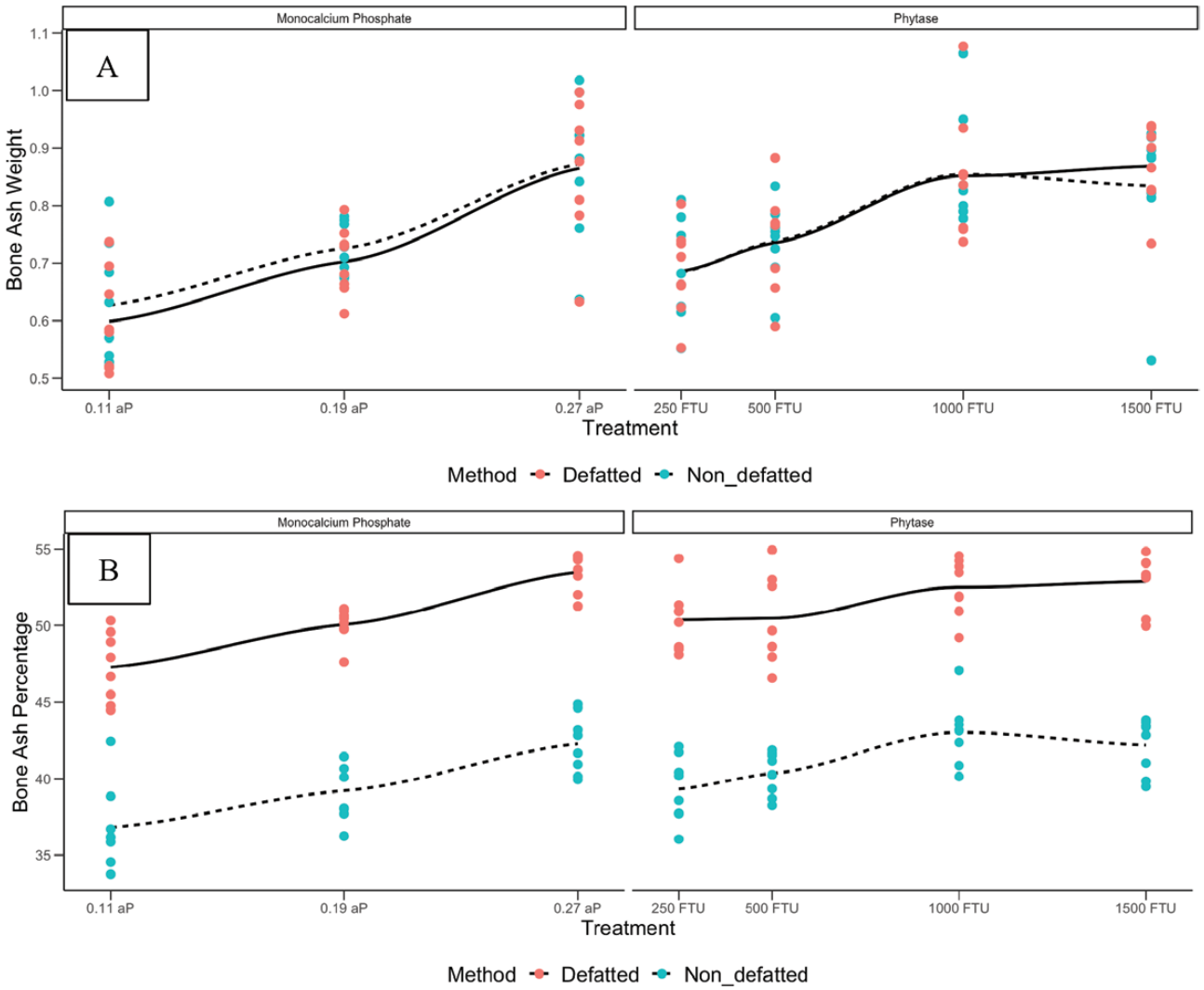

Figure 6. Treatment means and 95% confidence intervals for bone ash weight (panel A) and percentage bone ash (panel B) for experiment 2. Pigs were fed a range of increasing aP levels provided from either monocalcium P or added phytase. Diets were formulated below the P requirement with the aP in the monocalcium P diets ranged from 0.11% to 0.27%, with increasing (250, 500, 1,000, or 1,500 FTU/kg) phytase added to the lowest inorganic P diet.

The influence of processing method on the ability to detect treatment differences in bone ash percent can be found in Figures 5—7. In experiments 1 and 2, no evidence for a method × treatment interaction or linear and quadratic interactions was observed in bone ash weight and percentage bone ash ($P > 0.10$). Similarly, in experiment 3, no evidence for a method × treatment interaction or linear and quadratic interactions was observed in bone ash weight ($P > 0.10$); however, for percentage bone ash, a linear interaction ($P < 0.05$) was detected indicating that the slope of the defatted and non-defatted bone lines differed. For all practical implications, differences in the ability to detect treatment responses due to methods appear to be minimal. Therefore, the processing method does not impact the ability to detect treatment differences in nursey or grow-finish pigs; however, it is important to note that bone processing method will influence reference values for veterinary diagnostics and should consequently be taken into consideration in order to prevent inaccurate interpretations of diagnostic test results.

Lack of standardized test procedures led Crenshaw et al. (1981) to evaluate different techniques used to determine bone strength as a way to assess bone mineralization in swine. More recently, Crenshaw and Rortvedt-Amundson (2014) reviewed the histology, gravimetric, and mechanical procedures used to quantify bone integrity in clinical and research settings to understand their ability to detect nutrient deficiencies in production systems. Conversely, throughout the literature, there continues to be inconsistent processing methods to estimate bone ash values. While there are limited data on the difference between the two processing methods in nursery pigs, research shows that defatting bones processing method is more useful in determining percentage bone ash in mature pigs (Crenshaw, 2001). The results observed in experiment 3 confirm that in mature pigs it is important to defat bones when assessing bone mineralization to reduce variation due to increased water and fat content of the bone compared with younger pigs.

It is important to consider how different bones respond to dietary Ca and P levels. While this was not considered herein, major load-bearing bones were analyzed to ensure partitioning of mineral reserves. Crenshaw (1986, 2001) provides useful guidance on bone development, and consequently, the mechanism of skeletal storage and mobilization are beyond the scope of this paper and will not be discussed.

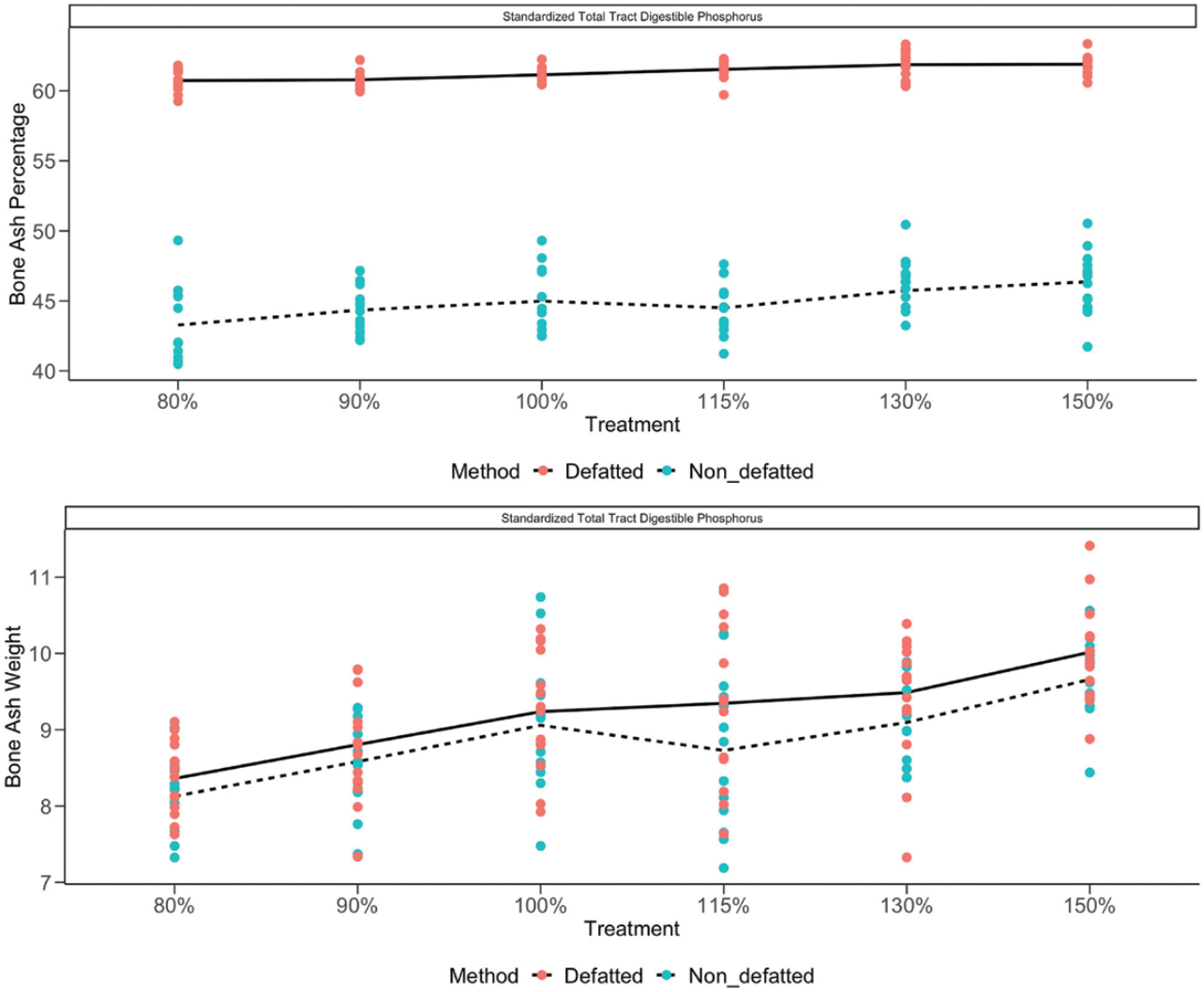

Figure 7. Treatment means and 95% confidence intervals for bone ash weight (panel A) and percentage bone ash (panel B) for experiment 3. Pigs were fed diets containing 80%, 90%, 100%, 115%, 130%, or 150% of the NRC (2012) requirement estimates for STTD P, with no added phytase.

Although the bone processing method influenced variation in percent bone ash in grow-finish pigs, these experiments demonstrate that the processing method did not alter the ability to detect treatment differences in experimental settings, regardless of maturity of pig. In summary, either method can be used to determine bone ash weight and percentage bone ash as a way to assess bone mineralization and dietary treatment differences in nursery pigs; however, the increased variation observed in mature pigs suggests that defatting bones is the preferred processing method for grow-finish pigs.

Acknowledgment

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Conflict of interest statement

The authors declare no conflict of interest.

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