Bone biochemical markers for assessment of bone responses to differentiated phosphorus supply in growing-finishing pigs

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ABSTRACT: Phosphorus (**P**) is essential for building and maintaining a healthy and strong skeleton. Moreover, dietary P supply may play a role for bone turnover, and the excretion of bone turnover metabolites may be useful as markers for sufficient dietary P supply. The objective was to study the long-term effects of low, medium, and high dietary P supply on bone metabolism in terms of serum concentration and urinary excretion of bone turnover components and metabolites in healthy growing-finishing pigs compared with bone mineral content (**BMC**) and bone mineral density (**BMD**) of humerus and femur. Pigs were fed diets containing low [**LP**; 4.1 g/kg dry matter (**DM**)], medium (**MP**; 6.2 g/kg DM), or high dietary P (**HP**; 8.9 g/kg DM) from 39.7 kg body weight (**BW**) until slaughter at 110 kg BW. Urine and blood were collected at 40, 70, and 110 kg BW while bones were collected at slaughter. Serum was analyzed for osteocalcin (**OC**), bone alkaline phosphatase (**BAP**), and C-terminal telopeptides of type I collagen (**CTX-I**), whereas urine was analyzed for pyridinoline (**PYD**), deoxypyridinoline (**DPD**), CTX-I, hydroxylysine (**HYL**), galactosyl-hydroxylysine (**GAL-HYL**), glycosyl-galactosyl-hydroxylysine (**GLC-GAL-HYL**), and hydroxyproline (**HYP**). Humerus and femur were analyzed for BMC and BMD. The LP diet caused reduced OC and increased BAP and CTX-I concentrations in serum. Furthermore, BAP was increased in response to the HP diet. Urine metabolites of bone resorption were all increased in pigs fed the LP diet, but only a few responses were obtained in response to the HP diet. Furthermore, age-related decreases were identified for BAP, HYL, GAL-HYL, and GLC-GAL-HYL. Bone mineral content and BMD were markedly lowered in pigs fed the LP diet but were not affected in pigs fed the HP diet. In conclusion, OC, BAP, and CTX-I in serum have proved useful for P adequacy in growing-finishing pigs. In addition, urine bone resorption metabolites have also proved useful for P adequacy and analysis of PYD, DPD, and CTX-I was considered to be the most relevant markers due to their specificity for bone and their negative correlation with BMD, BMC, ash, calcium (**Ca**), and P contents. Finally, DPD may be the preferred marker in long-term P feeding assessments.

Key words: bone biochemical markers, bone mineral content, bone mineral density, phosphorus, pigs

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INTRODUCTION

Phosphorus (**P**) is essential for developing and maintaining a healthy and strong skeleton (Eklou-Kalonji et al., 1999) and dietary P deficiency alters the body mineral balance and decreases bone mineral density (**BMD**) in the pig (Liesegang et al., 2002; Veum and Ellersieck, 2008). Bone is a metabolically active tissue with continuous turnover (Seibel, 2005). The components of formation are by-products of collagen synthesis, matrix proteins, or osteoblast enzymes,

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whereas the resorption metabolites arise from osteoclast degradation of type I collagen (summary in Table 1) (Seibel, 2002). Under normal conditions, bone formation and bone resorption are adjusted to each other and to the physiological status, but nutritional disturbances may result in more or less pronounced imbalances in the modeling and remodeling processes (Seibel, 2002; van Coeverden et al., 2002). An increase in bone formation components and resorption metabolites was seen in growing pigs when dietary P was reduced (Liesegang et al., 2002). Furthermore, increased bone metabolite levels in serum and urine caused by fasting were found to decrease with resumed calcium (**Ca**) intake (Aloia et al., 2010). These changes in bone metabolite releases induced by changes in mineral supply indicate that bone markers may be useful to assess changes in bone integrity when dietary P is changed. Traditionally, bone dynamics in pigs have been evaluated by measuring breaking strength, ash, and mineral contents. However, this requires invasive procedures (Koch and Mahan, 1986) and only supplies a static depiction of the bone condition (Jurimae, 2010). Oppositely, bone turnover measurements have the advantage that they can provide a dynamic picture of the bone tissue status by repetition of measurements (Jurimae, 2010). The working hypothesis is that biochemical components

Table 1. Products of bone turnover in serum and urine, tissue of origin, and remarks

	Tissue	Comments
Bone formation		
Bone-specific alkaline phosphatase (BAP)	Bone[1,2]	Membrane-bound enzyme on the osteoblast[1,2] In children BAP predominates (over alkaline phosphatase) due to skeletal growth[1]
Osteocalcin (OC)	Bone[1,2], dentin[2]	Synthesized by the osteoblast[1,2] Incorporated into bone matrix and a fraction is released to the circulation[2]
Bone resorption		
Deoxypyridinoline (DPD)	Bone, dentin[1,5]	Cross-link in collagen[1] Degradation product of mature collagen[1] Not metabolized in liver[4,5] Not influenced by food intake[1,5]
Pyridinoline (PYD)	Bone, cartilage (tendon, blood vessels)[1,5]	Cross-link in collagen[1] Degradation product of mature collagen[1] Not metabolized in liver[4,5] Not influenced by food intake[1,5]
Carboxyterminal cross-linked telopeptide of type I collagen (CTX-I)	Bone, skin[1,2]	Degradation product of collagen[1] Influenced by food intake[1]
Hydroxylysine (HYL)	Collagen[6]	Posttranslational modification of collagen[6] May be glycosylated[6] Not re-utilized for collagen biosynthesis[3,6]
Galactosyl-hydroxylysine (GAL-HYL)	Skeletal collagens[1,7] (also in cartilage and skin but to a much lower extent because partly processed to GLC-GAL-HYL[2,7])	Posttranslational modification of collagen[6,7] Degradation product of mature collagen[7] Not metabolized in liver[1,2,4,6] Not influenced by food intake[1,7]
Glucosyl-galactosyl-hydroxylysine (GLC-GAL-HYL)	Collagens of soft tissue[1] (derives to a minor extent from bone)	Posttranslational modification of collagen[6] Degradation product of mature collagen Not metabolized in liver[1,2,4,6] Not influenced by food intake[1]
Hydroxyproline (HYP)	Bone, cartilage, soft tissue, skin[1]	Degradation product of mature collagen[6] Product of posttranslational hydroxylation of proline in the procollagen chain[6] Extensive metabolism in liver[3,6] Re-utilized for collagen synthesis[1] Reflects both formation and resorption[1] Influenced by food intake[1]

[1]Seibel (2005).
[2]Garnero and Delmas (2004).
[3]Krane et al. (1977).
[4]Bettica et al. (1992).
[5]Branca et al. (1992).
[6]Szulc et al. (2000).
[7]Rauch et al. (1995).

and metabolites arising from the bone turnover process may be useful as indicators of changes in dietary P supply in the pig and thereby indicate to which extent lowering the dietary P level will affect bone integrity.

The objective of the present study was to examine if a long-term reduction of dietary P supply from above to below the pig's requirement affects the excretion of serum bone formation components and urinary bone resorption metabolites in growing-finishing pigs. The obtained results are compared to bone mineral content (**BMC**) and BMD in bone. Overall, the results may indicate if bone markers can be useful to assess dietary P adequacy for maintenance of bone integrity.

MATERIALS AND METHODS

All animal experimental procedures were carried out in accordance with the Danish Ministry of Justice, Law no. 253 of March 8, 2013 concerning experiments with animals and care of experimental animals, and license issued by the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food Administration.

Animals, Diets, and Housing

Eighteen female crossbred pigs (Landrace * Yorkshire * Duroc), from 6 litters of 3 pigs were included in the experiment. Littermates, with an initial body weight (**BW**) of 39.7 kg, were randomized to 3 treatment groups: low P (**LP**), medium P (**MP**), and high P (**HP**) (Table 2). A basic diet was formulated to fulfill the current Danish recommendations for growing-finishing pigs (45 to 105 kg BW) (Tybirk et al., 2014) except for the content of P and Ca (Table 2). The diet was steam-pelleted at 90 °C to deactivate the plant phytase, and then divided into 3 equal parts. Each part was supplemented with different amounts of calcium carbonate and/or monocalcium phosphate to define the 3 experimental diets (Table 2). The diets did not contain any components of collagenous origin that could influence the concentrations of bone resorption metabolites. The pigs had free access to water and all pigs were fed daily approx. 1.6 kg feed at the beginning of the experiment increasing to 2.7 kg feed at the end of the experiment. Throughout the experiment, the pigs were housed individually. Samples of the diet were collected and stored at −20 °C until analysis.

Table 2. Calculated and analyzed composition of the experimental diet

	Diet		
	LP	MP	HP
Planned			
Dry matter (DM), %	86	86	86
Total P, g/kg DM	4.2	6.5	8.6
Total Ca, g/kg DM	8.7	8.7	8.7
Total Ca:total P	2.1	1.3	1.0
Analyzed			
Dry matter (DM), %	91	91	91
Total P, g/kg DM	4.1	6.2	8.9
Total Ca, g/kg DM	7.4	8.1	9.0
Total Ca:total P	1.8	1.3	1.0
Phytate P, g/kg DM	2.7	2.7	2.7
Phytase, FTU/kg	ND	ND	ND
Digestible P, g/kg DM[1]	1.5	3.1	4.9
Total Ca:digestible P	4.9	2.6	1.8

Basic diet (%): barley, 55; wheat, 22.1; soybean meal, 18.1; animal fat, 2.0; molasses, 1.0; calcium carbonate, 0.9; sodium chloride, 0.5; vitamin/mineral premix, 0.2 (providing per kg diet: 84 mg iron; 100 mg zinc; 42 mg manganese; 15 mg copper; 0.21 mg iodine; 0.03 mg selenium; 76.4 mg DL-α-tocopherol; 2.1 mg vitamin K_3; 2.1 mg vitamin B_1; 2.1 mg vitamin B_2; 3.15 mg vitamin B_6; 10.5 mg D-pantotenic acid; 21 mg niacin; 0.05 mg biotin; 0.02 mg vitamin B_{12}; 4,200 IU vitamin A; 420 IU vitamin D_3); L-lysine HCl 80%, 0.13; L-threonine, 0.04; DL-methionine, 0.03. LP = low phosphorus diet; MP = medium phosphorus diet; HP = high phosphorus diet; ND = not detectable (below 50 FTU/kg DM).

[1]Experimentally determined apparent total tract digestibility of P (Sørensen et al., 2018).

Urine, Blood, and Bone Sampling

For separate collection of urine, each pig was fitted with a catheter (Silkolatex Rüsch Gold, size 16, 5.3 mm; Teleflex Medical, Germany) in the urinary bladder during each of the 3 intensive samplings [Balance A (40 kg BW = 14 wk of age), B (70 kg BW = 19 wk of age), C (100 kg BW = 24 wk of age)]. The catheter was connected to a closed bucket which was replaced every 12 h and placed at 4 °C. Each morning the 24-h urine was weighed, thoroughly mixed, and a representative sample was drawn and kept at 4 °C. This was done for each of the 7 d in trial. After termination of each specific balance period the 7 subsamples were pooled and kept at −20 °C until analysis. A blood sample (9 mL in 10 mL blood collection tubes, VENOSAFE, TERUMO) was collected between 1000 and 1100 h from each pig immediately after closing the balances. Blood was allowed to clot for 1 h and serum was isolated by centrifugation at 3,000 × *g* at 4 °C for 10 min and kept at −20 °C until analysis. At the end of the experiment, all pigs were slaughtered. From the left front leg and left rear leg, the humerus

and femur were removed and stored at −20 °C until analysis. Before analysis, the bones were thawed at 4 °C, and all tissues were manually removed.

Chemical Analysis of the Feed

The experimental diets were analyzed for total P, phytate P, and phytase according to Poulsen et al. (2012). Additionally, diets were analyzed for Ca by atomic absorption spectrometry (Model S2AA System, Thermo Electron Corporation Ltd, Cambridge, United Kingdom) after hydrochloric acid/nitric acid treatment of the ash fraction. Dry matter was assessed in diets by oven-drying at 103 °C for 20 h. All analyses were performed in duplicate.

Serum Analysis

The concentrations of osteocalcin (**OC**), bone alkaline phosphatase (**BAP**), and carboxyterminal cross-linked telopeptide of type I collagen (**CTX-I**) in serum were quantified by use of commercial kits from Immunodiagnostic Systems Ltd, United Kingdom, and all samples were assayed in duplicate after the protocols given by Immunodiagnostic Systems Ltd. The osteocalcin kit (N-MID Osteocalcin ELISA) was based on 2 highly specific murine monoclonal antibodies recognizing OC. The detection limit for OC was 0.5 ng/mL; in this range the intra- and interassay coefficients of variance were <2.2% and <5.1%, respectively. A test for parallelism was performed showing cross-reactivity with porcine OC. The C-terminal telopeptides of type I collagen kit (Serum Crosslaps ELISA) was based on 2 highly specific murine monoclonal antibodies recognizing the 8-amino-acid sequence of EKAHD-β-GGR, where the aspartic acid residue (D) was β-isomeric. The detection limit for CTX-I was 0.020 ng/mL; in this range the intra- and interassay coefficients of variance were <3.0% and <10.9%, respectively. A test for parallelism was performed showing cross-reactivity with porcine CTX-I. The bone-specific alkaline phosphatase kit (Ostase BAP) was based on a murine monoclonal antibody. The detection limit was 0.7 μg/mL; in this range the intra- and interassay coefficients of variance were <6.5% and <6.4%, respectively. A test for parallelism was performed showing cross-reactivity with porcine BAP.

Urine Markers of Bone Turnover

For determination of pyridinoline (**PYD**), deoxypyridinoline (**DPD**), CTX-I, hydroxylysine (**HYL**), galactosyl-hydroxylysine (**GAL-HYL**), glycosyl-galactosyl-hydroxylysine (**GLC-GAL-HYL**), and hydroxyproline (**HYP**) in urine, a Quattro LC from Micromass equipped with an electrospray source was used (Micromass Ltd, Manchester, United Kingdom). The MassLynx software version 4.0 was used for control of the HPLC and collection and analysis of data (Micromass Ltd). The settings for the electrospray were: capillary voltage 0.50 kV, extractor 3 V, RF lens 0.8 V, LM and HM resolution of MS1 and MS2 at 13.5, multiplier 650 V, and source temperature 120 °C. Desolvation gas (N_2) 550 liter/h was kept at a temperature of 350 °C. For the multiple reaction monitoring (**MRM**) method, argon at a pressure of 2.5×10^{-4} mbar was used for collision. An Agilent 1100 series HPLC equipped with a well-plate sampler, a thermo-stated column compartment, and a high-pressure binary gradient pump (flow rate 0.4 mL/min) was used (Agilent Technologies Inc., Palo Alto, CA), and a 25 μL sample was injected. The markers were separated on a Synergi MAX-RP column (2.5 μm, 3 × 100 mm, Phenomenex, Torrance, CA) fitted with a SecurityGuard MAX-RP pre-column (2 × 4 mm, Phenomenex) using a gradient between A: 20 mmol/liter ammonium carbonate and B: methanol. The Oasis MCX solid-phase extraction columns [96-well plates 30 μm (30 mg) from Waters, Milford, MA] were conditioned with 2 × 1 mL methanol and 2 × 1 mL water using a VacMaster-96 vacuum device (Biotage, Uppsala, Sweden). Urine samples (100 μL) were mixed with 100 μL 20 mmol/liter norleucin dissolved in 4% phosphoric acid (v/v). The mixture was passed slowly through a column by applying a mild vacuum. The column was washed with 1 mL formic acid/methanol (2:98, v/v) and 1 mL methanol and eluted with 3 × 500 μL ammonia solution 25%/methanol/water (3:20:77, v/v/v). The extracts were evaporated to dryness in vacuum at 30 °C using Maxi Dry Plus (Heto, Hillerød, Denmark). The residue was dissolved in 50 μL 0.2 mol/liter sodium borate buffer pH 8.8 and the markers derivatized by adding 20 μL of a solution of 6-aminoquinolyl-*N*-hydroxylsuccinimidyl carbamate (**AQC**) (10 mmol/liter) in dry acetone (9 mg/liter). The mixture was heated to 54 °C, kept for 10 min, and diluted with 50 μL of water. The samples were assayed in duplicate.

Creatinine

For quantitative determination of urinary creatinine (**crea**) concentration a commercial kit (CREA plus, Roche Diagnostics GmbH, Mannheim, Germany) was used. Samples were assayed in duplicate

after the protocol given by Roche Diagnostics. The detection limit was 27 µmol/liter.

Dual-Energy X-Ray Absorption

The BMC and BMD were measured in the diaphysis of both humerus and femur in cross-sections at 50% of the full bone length using a Hologic QDR Discovery A bone densitometer (Bedford, MA). For scanning and analyses, subregion high-resolution small animal software was used (version 13.0). The machine was calibrated daily with in vivo reproducibility of coefficient of variation 0.45% to 0.54%.

Statistical Analyses

The effect of dietary P on bone resorption metabolites and bone formation components were statistically analyzed using the MIXED procedure of SAS version 9.2 (Littell et al., 2006). Diet (LP, MP, HP), balance (A, B, C), diet and balance interaction were included in the analysis as fixed effects. Because the measurements were carried out on the same animal throughout the experiment, the repeated statement of animal within period was used and the unstructured (**UN**) covariance was fitted. Bone mineral content and BMD were analyzed according to the same model leaving out the fixed effect of period and the interaction between treatment and period as well as the repeated statement.

Data were tested for normal distribution and variance homogeneity. Data for urine biochemical metabolites showed not to be normal distributed, and consequently, data were log-transformed and normal distribution was obtained. The results and confidence intervals were back-transformed. The results are presented as least square means (**LSmeans**). For serum OC, BAP, and CTX-I, the variance is presented as standard errors of LSmeans (**SEM**), whereas the variance is presented as the confidence interval for the urine metabolites. Pairwise comparisons of LSmeans were made using the PDIFF option in SAS. Differences were considered significant when $P < 0.05$. The correlation between the urinary metabolites of bone resorption was analyzed by the CORR procedure in SAS.

RESULTS

Chemical Composition of the Diet

In general, the analysis of the 3 experimental diets met the expected levels for P, though the Ca content of the diets differed between 3% and 15% from the planned content. No phytase activity was detected (Table 2). The experimentally determined amounts of digestible P/kg DM increased as planned which was reflected in the ratio Ca:digestible P.

Serum Components

Osteocalcin was low when dietary P was low compared to the MP and HP diets ($P < 0.001$) (Table 3). In contrast, both the LP and the HP diets resulted in increased BAP concentrations in serum, though only the LP diet caused increased BAP concentrations in Balance C ($P < 0.001$). Furthermore, CTX-I was high in the LP group ($P < 0.01$), though the increase was only numerical in Balance C. Similar results were seen for the OC/CTX. The concentration of BAP, but not OC

Table 3. Mean values of average daily feed intake (ADFI) and CTX-I, OC, and BAP in serum of growing-finishing pigs

	Balance A			Balance B			Balance C				*P*-value		
	LP	MP	HP	LP	MP	HP	LP	MP	HP	SEM	Diet	Balance	D × B
ADFI (kg/d)	1.5	1.6	1.6	2.3	2.6	2.6	2.4	2.7	2.7	–	–	–	–
P, ingested (g/d)	5.72	9.05	13.25	8.68	14.30	20.98	9.12	14.89	21.65	0.25	–	–	–
OC, ng/mL	11.38^{a}	15.10^{b}	16.50^{b}	11.56^{a}	15.42^{b}	16.13^{b}	11.03^{a}	13.76^{ab}	15.74^{b}	1.14	***	NS	NS
BAP, µg/liter	36.93^{a}	23.28^{b}	33.69^{a}	30.00^{a}	13.80^{b}	24.01^{a}	25.77^{a}	16.38^{b}	18.86^{b}	3.17	***	***	NS
CTX-I, ng/mL	0.180^{a}	0.121^{b}	0.115^{b}	0.180^{a}	0.125^{ab}	0.120^{b}	0.206	0.163	0.148	0.02	**	NS	NS
OC/CTX-I	62.11^{a}	130.24^{b}	151.68^{b}	65.97^{a}	128.15^{b}	137.09^{b}	76.32	88.88	112.73	15.98	***	NS	NS

Balance A = 40 kg BW; Balance B = 70 kg BW; Balance C = 100 kg BW; LP = low phosphorus diet; MP = medium phosphorus diet; HP = high phosphorus diet; NS = not significant.

^{a,b}Within a row, means without a common superscript differ.

$^{**}P < 0.01$; $^{***}P < 0.001$.

and CTX-I, declined with growth independent of dietary P supply.

Urinary Metabolites

Urinary metabolites were measured as the concentration standardized to crea. The urinary crea concentration was low when the dietary P supply was low, and the concentration increased for all treatment groups with increasing BW ($P < 0.001$) (Table 4). In general, the concentrations of all measured urinary metabolites were greater in pigs fed the LP diet than in pigs fed the MP and HP diets. Even though, the effect was apparent already after 12 d of LP feeding (Balance A), the excretion of PYD, DPD, CTX-I, and HYP increased the longer the pigs were exposed to the LP diet (Balance A through C), whereas HYL, GAL-HYL, and GLC-GAL-HYL decreased. No clear effect of the HP compared to the MP diet was detected. The correlation between urinary metabolites showed that PYD, DPD, and CTX-I were highly and positively correlated ($0.655 < r < 0.810$) (Table 5). Furthermore, GAL-HYL and GLC-GAL-HYL were found to be strongly correlated ($r = 0.821$). Hydroxyproline, which is nonspecific for bone tissue, reflects both formation and resorption, and showed strong significant interaction between dietary P and the age of the pigs (Table 4). Thus, the HYP excretion remained fairly constant with age in pigs fed the LP diet, but decreased with age in the MP and HP fed pigs.

Bone Mineral Content and Density

Bone mineral content and BMD were low in both humerus and femur for pigs fed the LP diet compared to the other groups ($P < 0.05$) (Table 6), and the decrease in BMC and BMD was shown to be greater in the femur (43% to 46%) than in the humerus (25% to 28%). No significant long-term differences were detected between the MP and HP fed pigs.

DISCUSSION

Bone Formation Components in Serum

Osteocalcin is synthesized by osteoblasts and incorporated into the bone matrix, though a small

Table 4. Mean values of bone resorption metabolites in urine standardized to creatinine (crea) in growing-finishing pigs

	Balance A			Balance B			Balance C				*P*-value		
	LP	MP	HP	LP	MP	HP	LP	MP	HP	SEM	Diet	Balance	D × B
Crea, mmol/liter	4.21[a]	6.12[b]	5.68[ab]	5.69[a]	7.65[b]	8.08[b]	4.87[a]	7.37[b]	7.22[b]	0.53	***	***	NS
PYD, µmol/mmol crea	0.078[a] (0.063; 0.098)	0.042[b] (0.033; 0.054)	0.049[b] (0.039; 0.061)	0.081[a] (0.064; 0.102)	0.056[b] (0.044; 0.072)	0.057[b] (0.045; 0.072)	0.095[a] (0.074; 0.122)	0.063[b] (0.050; 0.079)	0.067[b] (0.053; 0.084)	–	**	**	NS
DPD, µmol/mmol crea	0.056 (0.048; 0.066)	0.048 (0.040; 0.057)	0.065 (0.056; 0.076)	0.068 (0.058; 0.080)	0.054 (0.045; 0.064)	0.054 (0.046; 0.063)	0.110 (0.094; 0.129)	0.059 (0.051; 0.070)	0.069 (0.059; 0.081)	–	–	–	**
CTX-I, µmol/mmol crea	0.039[a] (0.033; 0.045)	0.026[b] (0.022; 0.031)	0.032[ab] (0.027; 0.037)	0.030[a] (0.026; 0.035)	0.022[b] (0.019; 0.026)	0.025[ab] (0.022; 0.029)	0.074[a] (0.064; 0.086)	0.042[b] (0.036; 0.049)	0.045[b] (0.039; 0.053)	–	***	***	NS
GAL-HYL, µmol/mmol crea	1.83[a] (1.466; 2.285)	1.31[b] (1.053; 1.642)	1.29[b] (1.034; 1.611)	1.03[a] (0.828; 1.291)	0.74[b] (0.580; 0.944)	0.77[b] (0.619; 0.965)	0.90[a] (0.722; 1.125)	0.49[b] (0.394; 0.614)	0.53[b] (0.424; 0.661)	–	**	***	NS
HYL, µmol/mmol crea	2.67[a] (1.999; 3.575)	2.17[a] (1.624; 2.904)	1.27[b] (0.951; 1.701)	3.10[a] (2.320; 4.148)	1.93[b] (1.412; 2.644)	1.40[b] (1.048; 1.873)	2.34[a] (1.746; 3.123)	1.15[b] (0.863; 1.543)	1.04[b] (0.778; 1.392)	–	***	***	NS
GLC-GAL-HYL, µmol/mmol crea	1.68[a] (1.304; 2.165)	0.94[b] (0.714; 1.243)	1.30[ab] (1.009; 1.676)	0.69 (0.537; 0.892)	0.59 (0.450; 0.784)	0.73 (0.563; 0.935)	0.56[a] (0.435; 0.721)	0.38[b] (0.299; 0.496)	0.44[ab] (0.339; 0.563)	–	*	***	NS
HYP[1], µmol/mmol crea	7.59 (5.142; 11.21)	4.00 (2.709; 5.908)	1.54 (1.046; 2.281)	9.79 (6.628; 14.46)	2.44 (1.619; 3.678)	2.11 (1.427; 3.113)	9.51 (6.441; 14.05)	1.39 (0.940; 2.050)	1.03 (0.696; 1.519)	–	–	–	***

Numbers in parenthesis are the confidence interval. Balance A = 40 kg BW; Balance B = 70 kg BW; Balance C = 100 kg BW; LP = low phosphorus diet; MP = medium phosphorus diet; HP = high phosphorus diet; NS = not significant.

[a,b]Within a row, means without a common superscript differ.

[1]It should be noticed that HYP represents both formation and resorption.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 5. Correlation between bone resorption metabolites standardized to crea in urine of growing-finishing pigs

	DPD	CTX-I	HYP	HYL	GAL-HYL	GLC-GAL-HYL
PYD	0.714	0.655	0.433	0.543	0.546	0.404
DPD		0.810	0.344	0.400	0.352	0.247
CTX-I			0.295	0.252	0.315	0.255
HYP				0.709	0.462	0.203
HYL					0.435	0.177
GAL-HYL						0.821

Table 6. Mean values of area, BMC, and BMD (measured by DEXA) in humerus and femur in growing-finishing pigs

	LP	MP	HP	SEM	*P*-value
Humerus					
Area, cm^2	6.04	6.08	6.23	0.30	NS
BMC, g	3.00[a]	3.82[b]	4.14[b]	0.28	*
BMD, cm^2	0.50[a]	0.63[b]	0.67[b]	0.04	**
Femur					
Area, cm^2	6.08	6.20	6.04	0.39	NS
BMC, g	2.35[a]	4.37[b]	4.02[b]	0.25	***
BMD, cm^2	0.40[a]	0.70[b]	0.67[b]	0.036	***

DEXA = dual-energy X-ray absorption; LP = low phosphorus diet; MP = medium phosphorus diet; HP = high phosphorus diet; NS = not significant.

[a,b]Values within a row with unlike lowercase superscript letters were significantly different.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

part enters the blood (Lee et al., 2000). Long-term feeding of the low P led to decreased levels of OC in serum which were reflected in a parallel decrease in BMC and BMD and a positive correlation between serum OC and BMC and BMD, especially in the femur. Carter et al. (1996) reported that young pigs (20 to 40 kg BW) had decreased levels of OC in response to increased dietary levels of both Ca and P and that OC was negatively correlated to bone strength and bone ash. The apparent discrepancy between results is probably due to the higher dietary levels of P and Ca in the study by Carter et al. (1996). Growth-related changes in the OC concentration was not found in the present experiment. The OC concentration in relation to growth is not described in pigs raised for production; however, the OC was found to be strongly and negatively correlated to age in the Göttingen minipig (Tsutsumi et al., 2004). The observed discrepancy may be attributed to breed and physiological age differences between the minipig and the modern production pig.

The serum concentration of BAP was high when the dietary P supply was low, which is in agreement with previous results reporting a decline in the concentration of BAP (Liesegang et al., 2002) and the general alkaline phosphatase (**AP**) with increasing dietary P in young pigs (Boyd et al., 1983). Similar findings for AP are reported in finishing pigs, although the magnitude of the decline was less than in growing pigs (Koch and Mahan, 1986). In addition, a high concentration of AP correlated negatively with bone strength in pigs of 16 and 31 kg BW (Boyd et al., 1983), but not in pigs of 65 to 95 kg BW (Koch and Mahan, 1986). The present results confirm a negative correlation between BAP and BMD in the LP fed pigs. In contrast, the serum BAP concentration was greater in pigs fed the HP diet than the MP diet in the present study. This indicates increased osteoblast activity, which may be caused by the increased dietary P availability, associated with an increased overall body P retention observed when dietary P was high (Sørensen et al., 2018). Generally, the difference in OC and BAP excretion may be due to that they are formed differently by differentiated osteoblasts (Scott et al., 1997). The concentration of serum BAP declined when growth progressed, which is consistent with findings by Tsutsumi et al. (2004). The decline in the BAP concentration with age can be explained by the maturation of the skeleton, and thereby a lower level of osteoblast activity (Koch and Mahan, 1986).

Creatinine Standardization of Urine Measurements

Overall, the low or high dietary P supply resulted in few responses in the 24-h none-standardized crea metabolites (data not shown). However, when the concentrations were standardized to crea, the LP diet caused increased concentrations for all resorption metabolites. Creatinine standardization is commonly applied when interpreting differences in metabolite concentrations in urine samples (Husain et al., 1999; Tsutsumi et al., 2004) although previous results in human studies show inconsistency in the correlation between measurements made on 24-h urine collections and samples standardized to crea (Beardsworth et al., 1990; Fujimoto et al., 1995). Deguchi (1997) reported a significant positive correlation between BW and crea excretion in growing-finishing pigs, caused by the increase in muscle mass, because crea is produced endogenously from creatine during muscle metabolic processes (Borsook and Dubnoff, 1947). The crea excretion rate was found to remain constant, and is therefore a reliable index of the daily excretion in urine of other metabolites (Deguchi, 1997).

In agreement with Deguchi (1997), the observed lower urinary crea in the LP group can be explained by the lower BW, and by a lower N retention reflected in the muscle mass when dietary P supply was low (Sørensen et al., 2018). Overall, the standardization to crea can constitute a basis for direct comparisons in pigs of different size and weight. The observed effects of dietary P on bone markers, BMD, BMC, P and Ca retention, and femur ash and P content is summarized in Table 7.

Effect of Dietary P Supply on Bone Resorption Metabolites

Firstly, PYD, DPD, CTX-I, and GAL-HYL are reported to be relatively more specific for bone and reliable markers for bone resorption compared to HYL, GLC-GAL-HYL, and HYP (Bettica et al., 1992; Seibel, 2002). The concentration of serum CTX-I and all 7 urinary bone resorption metabolites increased in the LP fed pigs and the increases in bone metabolite concentrations were in general measurable already at the end of Balance A which is only 12 d of LP intake. Previous results indicated that a LP diet (3.7 g/kg DM vs. 6.5 g/kg DM) increased the concentration of CTX-I in serum of weaned pigs, which is in line with the present results (Liesegang et al., 2002). In contrast, low Ca (3.5 g/DM vs. 9.4 g/kg DM) fed to growing pigs showed no effect on HYP concentration in plasma (Larsen et al., 2000), although the present results showed that the urinary HYP concentration was markedly affected by the LP diet. This may point toward possible differences in deficient supplies of P and Ca and their effect on bone cells. Hydroxyproline is derived from all types of collagen, and besides its role as bone resorption metabolite, it also reflects collagen synthesis, and is therefore not recognized as a reliable marker exclusively for bone tissue turnover (Fujimoto et al., 1995; Seibel, 2005). However, the marked excretion in LP fed pigs may testify a negative influence on both processes and on HYP-rich collagen in general.

Previous reports indicate differences in bone metabolite concentrations between serum and urine. Serum and urine CTX-I were found to correlate in women (Kawana et al., 2002) and vaguely in dogs (Allen et al., 2000). Other studies found that no correlation occurs between serum and urine levels of DPD (Rauch et al., 1996). Both serum and urine CTX-I concentration increased in the present study indicating increased bone resorption. Pyridinoline, DPD, and CTX-I are reported to be relatively specific for bone collagen (Szulc et al., 2000; Seibel, 2005). They are favored metabolites as markers of bone resorption (Bettica et al., 1992; Allen et al., 2000; Tsutsumi et al., 2004), though few studies have examined these in relation to nutritional interventions (P and Ca) in pigs. Interestingly, the present results on PYD and CTX-I correlate inversely with the BMC and BMD results, which indicate that these metabolites may prove useful as markers for insufficient P supply to sustain bone mineralization in pigs. This is supported by previous results showing that decreased P supply to pigs reduced body P retention (Sørensen et al., 2018) and reduced Ca, P, and ash contents of bone (K. U. Sørensen, unpublished data) (overview in Table 7). Moreover, DPD showed almost the same pattern as PYD and CTX-I, yet DPD increased by 62% in the LP fed pigs despite that no increase in PYD and CTX-I was seen in the pigs weighing above 100 kg. Interestingly, Scott et al. (1997) also found an increase in DPD in growing lambs fed diets low in P compared with normal P for 3 mo. These observations may indicate that DPD seems to be especially valuable as long-time marker of too low dietary P supply. No clear explanation for this specific increase in DPD can be offered but may be an artifact or it may be speculated to be due to the amplified mobilization of P (and Ca) with age in pigs fed deficient P for a long time.

No increased excretion of bone resorption metabolites in urine were found in pigs fed the HP diet which is in line with the BMC and BMD results showing no increase compared to pigs fed the MP diet. High P has been shown to cause increased mineral retention in bone tissue of rats (Huttunen et al., 2007). This is in agreement with Fernandez (1995) who did not find an increased net Ca and P bone retention in growing pigs fed high Ca and P (Ca: 15 g/d; P: 13 g/d), but a reduced bone resorption compared with medium Ca and P supply (Ca: 10.2 g/d; P: 9.4 g/d). Fernandez (1995) suggested that the reduced bone resorption most likely affects normal bone development. A mechanism underlying the reduced resorption may be that the HP diet may induce osteoclast apoptosis and inhibit formation of new osteoclasts as shown in in vitro studies (Kanatani et al., 2003). It may be speculated that both osteoblasts and osteoclasts are less active in presence of high P whereby an imbalance in the turnover and modeling will occur. If so, no renewal and repair of the everyday abrasions will occur resulting in inflexible bones. This may also explain that no additional bone metabolites were excreted in the HP fed pigs. On the other hand, the present results show that the urinary DPD and HYL

Table 7. Overview of bone metabolite responses and classical traits associated with bone metabolism in growing-finishing pigs

	LP diet vs. MP diet			HP diet vs. MP diet		
	Serum	Urine	Mineralization and other classical traits	Serum	Urine	Mineralization and other classical traits
OC, ng/mL	(↓)			–		
BAP, µg/liter	↑			(↑)		
CTX-I, ng/mL	(↑)			–		
PYD, µmol/mmol crea		↑			–	
DPD, µmol/mmol crea		↑			(↑)	
CTX-I, µmol/mmol crea		↑			–	
GAL-HYL, µmol/mmol crea		↑			–	
HYL, µmol/mmol crea		↑			(↓)	
GLC-GAL-HYL, µmol/mmol crea		↑			–	
HYP, µmol/mmol crea		↑			–	
BMD, cm^2			↓			–
BMC, g			↓			–
P retention, g/d[1]			↓			(↑)
Ca retention, g/d[1]			↓			(↑)
Ash, femur, g/kg DM[2]			↓			–
P, femur, g/kg DM[2]			↓			–

LP = low phosphorus diet; MP = medium phosphorus diet; HP = high phosphorus diet; ↑ = indicates significantly increased effect compared to MP diet; ↓ = indicates significantly decreased effect compared to MP diet; (↑) = indicates increased effect compared to MP diet; (↓) = indicates decreased effect compared to MP diet.

[1]Sørensen et al. (2018).

[2]K. U. Sørensen, unpublished data.

concentration was reduced by the HP supply, but only in the youngest pigs. As such, the HP level in the present study did not have crucial influence on bone resorption, although it cannot be excluded that higher levels of dietary P may influence the excretion of bone resorption metabolites.

The Effect of Growth/Age

The excretion of bone turnover metabolites has been reported to be high in healthy young individuals of different species, followed by a decline with age (Husain et al., 1999; Allen et al., 2000; Tsutsumi et al., 2004). The high excretion of the metabolites is caused by a combination of skeletal modeling and remodeling (Seibel, 2005). In the present study, age-related decreases were identified for HYL, GAL-HYL, and GLC-GAL-HYL, whereas the other metabolites generally increased or remained constant with age. It might be speculated that the age-related decline occurs at different stages for the different metabolites, and that some metabolites in the present study already have reached a relatively constant level at the growing-finishing stage. However, the time frame of the present study may be too short to conclude whether age affects the concentration of the metabolites in growing-finishing pigs. Despite the advantages of being a noninvasive and dynamic method, bone metabolites as markers of bone metabolism are not site (bone)-specific, and therefore do not provide information about the metabolism in specific bones (Jurimae, 2010). The results of BMD and BMC show that femur tends to be more affected by LP supply than humerus because the difference between LP and the other groups is greater. Therefore, invasive measures such as BMD, and Ca, P, and ash determinations of bone tissue can provide site-specific effects of P supply.

Correlation Between Bone Metabolites and Other Measurements

The observed positive correlation between PYD and DPD (Table 5) is not surprising, because these cross-links share the same function in keeping the collagen tightly connected, and strictly reflect the degradation of mature collagen (Seibel, 2005). Furthermore, these 2 metabolites positively correlate to urine CTX-I. This finding is supported by results in dogs showing a positive correlation between urinary PYD, DPD, and CTX-I ($0.87 < r < 0.98$) (Allen et al., 2000). Moreover, GAL-HYL and GLC-GAL-HYL are strongly correlated with each other, as explained by a rather constant ratio between the 2 metabolites in collagen (Krane et al., 1977)

though neither of them was correlated with HYL. Even though the correlations are not strong between all metabolites, it is suggested that measuring PYD, DPD, and CTX-I would provide information about bone resorption in relation to dietary P supply. The rationale behind the proposed use of these 3 metabolites is primarily their specificity for bone tissue. Further, the response (increased secretion) is supported and validated by the correlated reduction in BMC, BMD, femur ash and P, and overall body P retention when pigs are fed a diet low in P, summarized in Table 7. Overall, the obtained results may also be valuable for determination of a proper dietary Ca:P ratio in diet formulation when P supply is increasingly restricted. Sørensen et al. (2018) suggest to shift from "total Ca:total P" to a Ca:P ratio based on digestible amounts to fulfill the requirement of both minerals without feeding Ca in surplus (or shortage). These authors suggest a dietary ratio of 1.4 (digestible Ca:digestible P) corresponding to around 2.5:1 (total Ca:digestible P) for growing-finishing pigs.

CONCLUSION

Bone formation components and resorption metabolites proved useful as markers for the evaluation of dietary P adequacy in pigs. The secretion of all bone turnover components and metabolites increased, except OC that decreased, in response to the LP diet already after 2 wk of deficient P supply and was not brought back to the values seen in pigs fed the MP or HP diets throughout the experiment. Osteocalcin, BAP, and CTX-I in serum appear to be useful as markers for dietary P deficiency. Furthermore, BAP seems suitable to identify dietary P overload. Moreover, the increased excretion of urine resorption metabolites in response to the LP diet was accompanied by significant inverse correlations with BMD, ash, Ca, and P contents in the bones of P-deficient pigs. Classical measures as BMC and BMD supported the results of bone markers and based on the present results, DPD, PYD, and CTX-I were considered to be the most valuable urinary bone resorption markers due to their high specificity to bone and correlation with other bone traits. In long-term assessments, DPD may seem to be the preferred marker.

IMPLICATIONS

The development of a noninvasive assay based on the measurement of bone turnover markers will be of great value in the pig production. This tool can be used to assess sufficient dietary P supply, and may become useful to determine more precise and differentiated P recommendations adjusted to the BW and physiological state of pigs. Moreover, bone markers may be useful in practical pig production when proper validations against classical bone measurements requiring slaughtering of the pigs have been accomplished experimentally under modern production levels.

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