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MARKERS OF BONE TURNOVER AND BIOMECHANICAL PROPERTIES OF THE THIRD METACARPAL BONE OF GROWING PIGS SUBJECTED TO THE DIFFERENT DIETARY PHOSPHORUS AND CALCIUM CONTENT*

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Abstract

Eighteen weaned piglets were subjected to deficiency, adequate or excess of dietary phosphorus and calcium (group C_{-50}, C and C_{+50}, respectively) during growth from 33 to 110 day of age. However, the ratio of calcium/digestible phosphorus in all feeds was the same and consistent with the nutritional recommendation. Performance, bone growth, concentration of osteocalcin (OC) and carboxyterminal telopeptide of type I collagen (CTX-I), densitometry, geometry and mechanical properties of the 3rd metacarpal bone were measured. Growth rate and final body weight did not differ between C and C_{+50} animals, however, they grew approximately 5.9% faster and were heavier by about 4.0% than C_{-50} pigs. In turn, mass and length of bones were similar in C_{-50} and C_{+50} groups, however, they were approximately 11% lighter and 4% shorter compared to C pigs. Serum content of OC took the following order: $C_{-50} < C < C_{+50}$ group (P<0.01). Content of CTX-I did not differ between C and C_{+50} animals, however they were lower by 36% (P<0.01) than in the C_{-50} pigs. All measured geometry parameters (cortical wall thickness, cross section area and cortical index) did not differ between C and C_{+50} pigs and were higher than in C_{-50} pigs (P<0.01). Bones of C_{-50} pigs contained less minerals than bones of the C and C_{+50} pigs (5.24 g vs mean 6.14 g, P<0.01). The 3rd metacarpal bone mineral density (g/cm²) was the lowest in pigs in group C_{-50}, higher in pigs in group C, and the highest in animals in group C_{+50} (0.398, 0.431 and 0.475, respectively, P<0.01). Maximum strength of bones was similar in the C and C_{+50} pigs, however it was 13% greater (P<0.01) compared to C_{-50} animals. In turn, elastic strength and elastic deflection did not differ between the C and C_{-50} animals and were over 20% lower (P<0.01) than in the C_{+50} pigs. Whereas, there was no significant difference between groups in bone stiffness (mean 27.13 mm). The results of the present study proved that even a strong excess of phosphorus and calcium content in the diet for growing pigs is inadvisable and does not improve performance and bones parameters. While excessively lowering the dietary phosphorus and calcium concentration (e.g. to reduce environmental pollution) may adversely affect growth rate and bone characteristics.

Key words: pigs, dietary phosphorus and calcium, bone metabolism, dual-energy X-ray absorptiometry

The phosphorus is an essential nutrient component influencing health and functioning of the organism. Approximately 85% of this element is found in the bones as hydroxyapatite. Remaining 15% is distributed in the soft tissues as a component of cell membranes, intracellular organelles and the sugar-phosphate backbone of the nucleic acids (Lall, 2002). Additionally, phosphorus as a component of cells energy carriers (e.g. ATP, ADP) plays an important role in energy metabolism, acid/base balance and in intracellular cell signalling (Serna and Bergwitz, 2020). In turn, the calcium is the most abundant mineral in the body (Suttle, 2010) and is indispensable for bone mineralization, muscle contraction, and nerve impulse propagation. Absorption and utilization of the phosphorus is related to that of the calcium, thus phosphorus and calcium requirements cannot be dissociated and must be estimated together. According to nutritional recommendation (NRC, 2012) the ratio of total calcium to digestible phosphorus for growing pigs should amount to 2.1. When they are unbalanced, insoluble and indigestible calcium-phosphorus complexes can form in the intestines (Masuyama et al., 2003, Selle et al., 2009) influencing body mineral balance, achievement of peak bone mass (Heaney et al., 2000), bone mineral

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density (Sørensen et al., 2018 b, 2019; Grela et al., 2020), metabolites of bone turnover (Liesegang et al., 2002; Drews et al., 2016; Sørensen et al., 2018 b; Gerlinger et al., 2019), and consequently bone material properties and strength (Huttunen et al., 2007; Grela et al., 2020).

Mechanic, mineralization and density measurements are extremely accurate indicators of bone health and quality. However, some authors (Rieger, 2021) indicate that dietary phosphorus has greater, than calcium, impact on bone morphology and mechanic properties. The availability of phosphorus from plant feeds can be increased by the addition of microbial phytase to the animals' diet, which also has a positive effect on the physicochemical status of bones (Grela et al., 2020).

Osteoblasts (bone creating cells) and osteoclasts (bone resorbing cells) with commitment of hormones, receptors, signalling molecules, and vitamins are involved in the regulation of calcium and phosphorus homeostasis (Berndt and Kumar, 2009). Under normal conditions, bone formation and bone resorption are adjusted to each other and to the animal physiological status. Osteocalcin (OC) and carboxyterminal telopeptide of type I collagen (CTX-I) are markers of bone metabolism (Greenblatt et al., 2017). Osteocalcin is synthesized by osteoblast and its activity in the blood is usually elevated when bone formation rate increases (Gerstenfeld et al., 1987). In turn, during osteoclastic resorption of bone, type I collagen is degraded to small peptide fragments (among others CTX-I) and secreted into the bloodstream at a rate proportional to bone resorption intensity (Szulc et al., 2017). Nutritional disturbances (e.g. mineral supply) may result in more or less pronounced imbalances between both processes (Rizzoli and Bonjour, 2006). Thus, changes in OC and CTX-I released into blood during bone turnover process provide an important information on degree of intensity of both bone formation and bone resorption processes (Sørensen et al., 2018 b).

Studies on the supply of phosphorus and calcium in the diet on bone metabolism and bone biomechanical properties are rare and usually concerned changes (excess or deficiency) of one of the elements (calcium or phosphorus). Previous experiments were usually carried out during a short growth period and usually on older animals. It is known that the duration of use of the experimental agent and the age of the animal in this type of research are of key importance. The supply of calcium and phosphorus as elements that determine the growth rate and properties of bones is particularly important in animals in the initial period of their growth, when the animal's bones are developing very intensively and animals are more sensitive to changes in the supply of nutrients. Moreover, in previous studies, the model animals were usually rodents, while there are only a few studies that have used larger animal models (e.g. pig). Thus, the aim of the present study was to determine responses of growing pigs (aged from 33 to 110 days) to varied supply of dietary phosphorus and calcium (deficiency, adequate and excess) in relation to growth performance and concentration of bone turnover markers (OC and CTX-I), and the 3rd metacarpal bone properties: growth (mass and length), geometry (cortical wall thickness, cross section area, cortical index), densitometry (mineral content and mineral density), strength (maximum and elastic) and stiffness, when dietary ratio of calcium/digestible phosphorus is constant and consistent with nutrient requirements (NRC, 2012). It was assumed that markers of the bone turnover process may be useful as indicators of changes in dietary phosphorus and calcium supply for the growing pigs and indicate to which extent disturbances in their dietary supply will affect bone integrity.

Material and methods

Ethics

The design of the study, animal care, and all procedures applied during the experiment were performed in accordance with the relevant principles of the European Union and the Polish Animal Protection Act and were approved by the III Local Ethics Committee on Animal Experimentation of Warsaw University of Life Sciences, Warsaw, Poland (resolution No. 1/2013). The experiment was designed to minimize the number of animals whilst maintaining high statistical power according to the principles of the 3 R's (replacement, reduction and refinement).

Animals, diets, housing, and treatment

The experiment was carried out on 18 female piglets (♀ Polish Large White × ♂ Danish Landrace). The study was conducted in the period of the rapid growth of the animals (including bones/skeleton mineralization) when animals are very sensitive to experimental factors. From day 28 to 33 of age piglets were subjected to acclimatization. During all experimental period pigs were fed one type of diet, which fulfilled the nutritional recommendations of NRC (2012) for this age group of animals. Such kind of feeding method was used to avoid undesirable effects of feed changing, which could additionally affect the tested parameters. During the experiment conducted from 33 to 110 days of age animals were assigned to three groups (six animals each) and fed diets differing in calcium and phosphorus content, but the proportion of calcium/digestible phosphorus was constant in all feeds. Pigs received a dry granulated feed offered *ad libitum*. Diets were formulated based on cereals (barley, 20.5%; wheat, 20.5%; maize, 28%), protein (soybean meal, 17%; rapeseed meal, 5%) and energetic (rapeseed oil, 5%) compounds and mineral-amino acid-vitamin mix (4%). Each mineral-amino acid-vitamin mix provided per kg of complete diet the same amount of: Fe, 62.5 mg; Zn, 62.5 mg; Mn, 18 mg; Cu, 4 mg; I, 0.2 mg; Se, 0.2 mg; vitamin A, 2000 IU; vitamin D_3, 245 IU; vitamin E, 40 mg; vitamin K_3, 2 mg; vitamin B_1, 2 mg; vitamin B_2, 2.5 mg; vitamin B_6, 3.1 mg; vitamin B_{12}, 0.02 mg; lysine, 5.27 g; methionine, 1.55 g; threonine, 2.37 g; tryptophan, 0.53 g; NaCl,

1.31 g. However, mineral-amino acid-vitamin mixtures provided per kg of complete diet different amounts of calcium (mixture C – 5.68 g; mixture C_{-50} – 2.18 g; mixture C_{+50} – 9.18 g) and phosphorus (mixture C – 1.92 g; mixture C_{-50} – 0.32 g; mixture C_{+50} – 3.52 g). Despite differences in the content of phosphorus and calcium in the diets, the ratio of calcium to digestible phosphorus was the same (2.1) in all diets and corresponded to nutritional recommendations (NRC, 2012). The dry matter, nitrogen, ash, crude fibre, ether extract, phosphorus and calcium contents in the feeds were analysed using the AOAC (2011) methods (934.01; 984.13 and 942.05, 978.10, 920.39, 965.17, 927.02, respectively). Crude protein was calculated as N × 6.25. Nitrogen free extract was calculated according to the following formula, based on the analysed chemical components:

Nitrogen free extract = Organic matter – (Crude protein + Fat (ether extract) + Crude fibre).

Content of protein/amino acids in each diet was balanced according to NRC (2012). Metabolisable energy content was calculated based on chemical components according to formula given by Beyer et al. (2003). Lysine content (standardized ileal digestible) amounted to 13.38 g per kg dry matter of feed, whereas methionine, threonine and tryptophan were balanced in proportion to the lysine content (100: 33: 63:18). The mono- and di-calcium-phosphate were the source of inorganic calcium and phosphorus. Changes in content of these elements in particular feeds were achieved by increasing/decreasing the amount of mono-calcium phosphate at the expense of maize content in the diet. Although, other sources of these minerals can be used (Ahlstrøm and Skrede, 2020). Design of the experiment is presented in Table 1 and nutritive and energetic values of diets are presented in Table 2.

Table 1. Design of the experiment

Group	Protein/ amino acids	Vitamins	Ca	Digestible P	Ca/ digestible P
C_{-50}	100	100	50	50	2.19
C	100	100	100	100	2.19
C_{+50}	100	100	150	150	2.19

Group C_{-50} – fed a diet with lower content of calcium and phosphorus, both –50%; Group C – fed a diet with recommended content of calcium and phosphorus (NRC, 2012); Group C_{+50} – fed a diet with higher content of calcium and phosphorus, both +50%; Ca – calcium; P – phosphorus; Values marked as "100" and those for Ca/digestible P correspond to nutritional recommendation (NRC, 2012).

The animals were kept individually in pens (3.2 m^2), equipped with nipple drinkers, on a concrete floor without straw under thermo-neutral conditions (EC Regulation No 1924/2006) maintained by a central heating and air conditioning system (Fancom, model ISM0.12; Fancom BV). Body weight of animals was recorded at the beginning (33 days of age, average 8.86 kg ± 0.265) and at the end of the study (110 days of age, average 73.62 kg). Moreover, daily feed intake and daily body gain were recorded. Based on the feed intake the intake of the phosphorus was also calculated. In turn, based on feed intake and body gain the feed conversion ratio was also calculated.

Table 2. Chemical composition, nutritive and energetic values of the diets offered to pigs between 33 and 110 days of age

	Group		
	C	C_{-50}	C_{+50}
Analysed chemical composition (g/kg dry matter)			
Organic matter	967.8	971.5	965.8
Nitrogen free extract	650.8	653.1	648.8
Crude protein	187.1	187.9	187.1
Fat (ether extract)	85	85.4	85
Crude fibre	44.9	45.1	44.9
Crude ash	32.2	28.5	34.2
Calculated nutritive value (g/kg dry matter)			
Lysine	13.38	13.38	13.38
Methionine	4.42	4.42	4.42
Threonine	8.39	8.39	8.39
Tryptophan	2.38	2.38	2.38
Sodium	1.70	1.70	1.70
Total phosphorus*	6.59 (6.53)	4.79 (4.85)	10.53 (10.57)
Digestible phosphorus	3.62	1.81	5.55
Calcium**	7.94 (7.96)	3.99 (3.96)	12.06 (11.95)
Calcium/digestible phosphorus***	2.193 (2.198)	2.204 (2.187)	2.17 (2.193)
Metabolisable energy (MJ/kg)****	15.99	16.01	15.99
Lysine/ metabolisable energy	0.84	0.84	0.84

*Values given in parentheses are based on chemical analysis; **Values given in parentheses are based on chemical analysis; ***Values given in parentheses were calculated based on chemical analysis of Ca; ****Calculated according to Beyer et al. (2003); Group C_{-50} – fed a diet with lower content of calcium and phosphorus, both –50%; Group C (control) – fed a diet with recommended content of calcium and phosphorus (NRC 2012); Group C_{+50} – fed a diet with higher content of calcium and phosphorus, both +50%.

Slaughter of the animals

At 110 days of age the pigs were slaughtered in the experimental slaughterhouse of The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences after a fasting period of 16 h. Before slaughter the pigs were electrically stunned (STZ 3 apparatus; P.P.H. MASTER Sp. J., Solec Kujawski, Poland) and then exsanguinated.

Blood sample collection and determination of OC and CTX-I

During slaughter the blood samples were collected for analysis of the concentration of OC and CTX-I.

Blood samples were allowed to clot at –4°C and serum was collected after centrifugation (1500 × g for 15 minutes at –4°C). Serum samples were stored at –80°C until analysis. Concentration of both OC and CTX-I was determined using ready-to-use ELISA kits dedicated for pigs (MyBioSource, Inc., San Diego, CA, USA, products number: MBS2019048 and MBS706746) according to the manufacturer's instructions.

Bones collection

After exsanguination the 3rd metacarpal bones (left and right) were dissected from the carcass, cleaned of any remaining flesh, weighed and next frozen (–20°C) for subsequent dual-energy X-ray absorptiometry scanning, three-point bending test and geometry measurements. Densitometry scanning and three-point bending test were performed on the left, whereas, geometry measurements on the right metacarpal bones.

Densitometry scanning

Dual-energy X-ray absorptiometry scans (densitometer XR-800™, Norland Medical Systems, Cooper Surgical, Fort Atkinson, WI, USA) were obtained according to the manufacturer's protocol of scanning and analysis (research-scan type). As recommended by the device producer a test verifying the stability of the calibration system, was performed on a daily basis using a Quality Control Phantom and Quality Assurance Calibration standard (Norland, A Cooper Surgical Company). Specimens for scanning were thawed at 23°C for 12 h prior to use. During scanning, the bone was positioned horizontally, with the head facing upwards and the condyles facing downwards, and then scanned from the distal to proximal end. All scans were performed in three replicates, the mean value was used for statistical analysis. Bone mineral content (BMC), bone mineral density (BMD), and bone area were recorded.

Three-point bending test

After dual-energy X-ray absorptiometry scanning the three-point bending test using TAHDi Texture Analyser (Stable Micro Systems LTD., Godalming, UK) was applied to determine the mechanical properties of the bones. The test was performed according to the protocol described in our previous work (Skiba et al., 2018) The distance between the supports of the bone was set at 40% of the bone length, and the measuring head loaded the bone samples at the mid shaft. However, due to small size of the tested bones a constant speed of 10 mm/min was applied (Skiba et al., 2016). The following measurements were performed: maximum and elastic strength (kG), maximum and elastic deflection (mm). Based on the obtained measurements the stiffness (kG/mm) of the bones was calculated according to the following formula:

$$\textit{Stiffness (kG/mm)} = \textit{elastic strength (kG)/elastic deflection (mm)}$$

Geometry measurements

Each bone was cut in the middle part of the bone shaft to measure, using a calliper, its horizontal and vertical diameter (both external and internal). Obtained values were used to calculate the geometric properties: cortical wall thickness (mm), cross section area (mm^2) and cortical index (%) according to the following formulas (Ferretti et al., 1993):

$$\textit{Cortical wall thickness} = [(B + H) - (b + h)] / 4$$
$$\textit{Cross sectional area} = \Pi / 4 \times [(B \times H) - (b \times h)]$$
$$\textit{Cortical index} = \{[((H - h) / H) + ((B - b) / B)]/2\}*100$$

where: B – vertical external diameter, H – horizontal external diameter, b – vertical internal diameter, h – horizontal internal diameter.

Statistical analyses

Statistical analyses were performed using Statistica software (version 13.3, StatSoft, Tulsa, OK, USA). The examined bone characteristics in different groups are presented as mean values. The results were analysed using one way ANOVA. When the F ratio was significant, the Student test was used to determine differences between the groups. Statistical significance was set at $P<0.05$. A borderline significant trend was set at $P<0.10$. Correlation between analysed bone properties and phosphorus intake was presented as Pearson's correlation.

Results

Performance

All pigs completed the study and remained healthy (without evident disorders of the musculoskeletal system). Pigs of the C and C_{+50} groups grew at a similar rate (on average 856 g/day), however, faster ($P = 0.0171$) than the C_{-50} pigs (809 g/day) (Table 3). Consequently the final body mass of the C_{+50} and C pigs was similar (on average 74.55 kg) and heavier ($P = 0.0171$) compared to the C_{-50} animals (71.70 kg).

Table 3. Body mass of animals at 33 days of age (initial) and at 110 days of age (final), and daily feed intake, daily body gain and feed conversion ratio during growth from 33 to 110 days of age

Item	C_{-50}	C	C_{+50}	S.E. pooled	P-value
Initial body mass (kg)	9.42	8.05	9.23	0.31	0.1389
Final body mass (kg)	71.72 a	73.81 b	75.33 b	0.604	0.0171
Daily feed intake (kg)	1.58	1.55	1.58	0.018	0.7932
Daily body gain (g)	809 a	855 b	858 b	9.07	0.0171
Feed conversion ratio (kg/kg body gain)	1.95	1.81	1.83	0.03	0.1437

Group C_{-50} – fed a diet with lower content of calcium and phosphorus, both –50%; Group C (control) – fed a diet with recommended content of calcium and phosphorus (NRC, 2012); Group C_{+50} – fed a diet with higher content of calcium and phosphorus, both +50%.

a, b – means with different letters are significantly different ($P<0.05$).

Concentration of bone turnover markers

Osteocalcin (OC) content differed (P = 0.0001) between groups of pigs and was the lowest in the C_{-50}, higher in the C and the highest in the C_{+50} animals (Table 4). Content of CTX-I was similar in the C and C_{+50} pigs (on average 133 ng/mL) and was lower (P = 0.0001) compared to C_{-50} pigs (0.207 ng/mL).

Table 4. Concentration of the osteocalcin (OC) and carboxyterminal telopeptide of type I collagen (CTX-I) in the serum of pigs slaughtered at the end of the study

Item	C_{-50}	C	C_{+50}	S.E. pooled	P-value
OC (ng/mL)	11.57 A	16.80 B	19.05 C	0.757	0.0001
CTX-I (ng/mL)	0.207 B	0.139 A	0.127 A	0.0068	0.0001

Group C_{-50} – fed a diet with lower content of calcium and phosphorus, both –50%; Group C (control) – fed a diet with recommended content of calcium and phosphorus (NRC, 2012); Group C_{+50} – fed a diet with higher content of calcium and phosphorus, both +50%.

A, B, C – means with different letters are significantly different (P<0.01).

Correlation between phosphorus intake and markers of bone turnover

Intake of the phosphorus was strongly correlated to both OC (P = 0.0001) and CTX-I (P = 0.0001) content in the blood serum (Table 5). However, a positive correlation was detected for OC, whereas negative for CTX-I. Moreover, digestible phosphorus intake was stronger than total phosphorus, correlated to both of bone turnover markers. Depending on the form of dietary phosphorus the Pearson correlations amounted to 0.8739 and 0.9270 for OC and –0.7927 and –0.8712 for CTX-I, respectively.

Table 5. Pearson correlations between phosphorus intake and content of osteocalcin (OC) and carboxyterminal telopeptide of type I collagen (CTX-I) in the serum of pigs slaughtered at the end of the study

Item	OC	CTX-I
Intake total phosphorus	0.8739 (P = 0.0001)	–0.7927 (P = 0.0001)
Intake of digestible phosphorus	0.9270 (P = 0.0001)	–0.8712 (P = 0.0001)

Parameters of the bones

The mass of the 3rd metacarpal bone did not differ between groups C_{-50} and C_{+50} (on average 21.88 g), however it was lower (P = 0.0032) than in the C animals (24.58 g, Table 6). Similar relation was observed for length of the bone (on average 7.11 vs. 7.40 cm, respectively, P = 0.0488). Minerals content in the bones of the C and C_{+50} animals was similar (on average 6.14 g), however higher (P = 0.0001) than the C_{-50} animals (5.24 g). Bone mineral density took the following order: $C_{-50} < C < C_{+50}$ (P = 0.0001). Bone area was similar in groups C_{-50} and C_{+50} (on average 13.08 cm^2), but was lower (P = 0.0001) than in C group (14.17 cm^2). All measured geometry parameters did not differ between C and C_{+50} animals and were higher than in C_{-50} pigs. The values for individual features were as follows: cortical wall thickness (on average 3.75 vs 3.03 mm, respectively, P = 0.0002), cross section area (on average 145.05 vs 125.3 mm^2, respectively, P = 0.0007), cortical index (45.34 vs 36.46%, respectively, P = 0.0005).

Table 6. Physical, densitometry, geometry and mechanical parameters of the 3rd metacarpal bone of animals slaughtered at the end of the study

Item	C_{-50}	C	C_{+50}	S.E. pooled	P-value
Physical parameters					
Mass (g)	21.53 A	24.58 B	22.23 A	0.5406	0.0032
Length (cm)	7.13 a	7.40 b	7.08 a	0.0883	0.0488
Densitometry parameters					
Bone mineral content (g)	5.24 A	6.07 B	6.20 B	0.0747	0.0001
Bone mineral density (g/cm^2)	0.398 A	0.431 B	0.475 C	0.0040	0.0001
Bone area (cm^2)	13.14 A	14.17 B	13.01 A	0.1256	0.0001
Geometry parameters					
Cortical wall thickness (mm)	3.03 A	3.80 B	3.70 B	0.1052	0.0002
Cross section area (mm^2)	125.3 A	147.5 B	142.6 B	3.3436	0.0007
Cortical index (%)	36.46 A	45.39 B	45.30 B	1.4040	0.0005
Mechanical parameters					
Maximum strength (kG)	89.74 A	100.30 B	102.87 B	3.721	0.0002
Elastic strength (kG)	62.87 A	70.42 A	86.47 B	2.774	0.0001
Maximum deflection (mm)	5.81	6.45	5.70	0.2652	0.1325
Elastic deflection (mm)	2.41 A	2.52 A	3.16 B	0.1067	0.0003
Stiffness (kG/mm)	26.08	27.94	27.36	1.8856	0.7551

Group C_{-50} – fed a diet with lower content of calcium and phosphorus, both –50%; Group C (control) – fed a diet with recommended content of calcium and phosphorus (NRC, 2012); Group C_{+50} – fed a diet with higher content of calcium and phosphorus, both +50%.

A, B, C – means with different letters are significantly different (P<0.01).

a, b – means with different letters are significantly different (P<0.05).

Bone maximum strength was similar in the C and C_{+50} pigs (on average 101.59 kG), however greater (P = 0.0001) compared to C_{-50} animals (89.74 kG). In turn, elastic strength and elastic deflection did not differ between the C and C_{-50} animals and was lower compared to C_{+50} pigs (on average 66.65 kG vs. 86.47 kG, P = 0.0001 and on average 2.47 mm vs. 3.16 mm, P = 0.0003, respectively). Whereas no significant difference between groups was found for the bone stiffness.

Correlation between phosphorus intake and bone parameters

Densitometry measurements (both mineral content and density) were strongly correlated to phosphorus in-

take (P = 0.0002 and P = 0.0001, respectively, Table 7). Depending on consumption of a dietary form of this element (total or digestible), the Pearson correlation ranged from 0.6954 to 0.9051. Mechanical parameters (elastic and maximum strength, and elastic deflection) were also strongly (P = 0.0001) correlated to phosphorus intake (correlation coefficients ranged from 0.7798 to 0.8351). However, the phosphorus intake was slightly weaker correlated to geometry parameters. The significance of the correlation (P value) depending on the measured geometry feature and form of the consumed phosphorus ranged from 0.0281 to 0.0024 (correlation coefficient correlation ranged from 0.5169 to 0.6685).

Table 7. Pearson correlations between phosphorus intake and physical, densitometry, mechanical and geometry parameters of the 3rd metacarpal bone of animals slaughtered at the end of the study

Bone parameters	Intake	
	total phosphorus	digestible phosphorus
Physical parameters		
Mass	0.0053 (P = 0.9835)	0.1444 (P = 0.5675)
Length	0.2016 (P = 0.4224)	0.0950 (P = 0.7077)
Densitometry parameters		
Bone mineral content	0.6954 (P = 0.0014)	0.7695 (P = 0.0002)
Bone mineral density	0.9038 (P = 0.0001)	0.9051 (P = 0.0001)
Bone area	–0.2366 (P = 0.3445)	–0.0986 (P = 0.6972)
Geometry parameters		
Cortical wall thickness	0.5169 (P = 0.0281)	0.6323 (P = 0.0049)
Cross section area	0.4211 (P = 0.0818)	0.5465 (P = 0.0189)
Cortical index	0.5669 (P = 0.0141)	0.6685 (P = 0.0024)
Mechanical parameters		
Maximum strength	0.7978 (P = 0.0001)	0.7798 (P = 0.0001)
Elastic strength	0.8208 (P = 0.0001)	0.7991 (P = 0.0001)
Maximum deflection	–0.1933 (P = 0.4423)	–0.0831 (P = 0.7431)
Elastic deflection	0.8351 (P 0.0001)	0.7963 (P = 0.0001)
Stiffness	–0.0239 (P = 0.9249)	0.0077 (P = 0.9759)

Discussion

As the only source of phosphorus and calcium, for animals, are feed and their amount deposited in the skeleton (bones), thus disorders in supply of these elements forces the organism to change the intensity of physiological processes regulating the homeostasis of these elements in the body. Especially long-term changes in supply will cause an imbalance between the processes of bone formation and resorption. Degree of the intensification of these processes can influence the growth performance as well as the content and density of minerals conditioning biomechanical features of the bone.

Performance

In the presented study feeds offered to particular groups of pigs differed in phosphorus and calcium content (deficiency, adequate, excess), however, in all feeds the ratio of calcium/digestible phosphorus was stable. All remaining nutrients were balanced according to pigs' requirements. In such conditions feed intake by both groups of pigs fed a diet with strong phosphorus and calcium deficiency and excess did not differ from the intake of feed by animals fed a diet with the appropriate content of this ingredient. Similarly, other authors also found no effect of the dietary phosphorus content and feed intake in weaned piglets (Liesegang et al., 2002), growing pigs (Nieto et al., 2016), and poultry (Valable et al., 2018). However, according to some authors phosphorus and calcium deficiency resulted in limiting feed intake (Vier et al., 2019). In turn, Gerlinger et al. (2019) found that feeding weaned piglets a diet with phosphorus excess (thus with changed Ca : P ratio) resulted in lower feed intake (albeit only after two weeks of experiment), whereas solely phosphorus deficiency did not affect feed intake. Discrepancies between present and discussed results were probably due to the fact that requirement for protein and energy as the main factor regulating feed intake (Symeou et al., 2014) in the present study was fully covered in all groups of pigs, thus feed intake could be independent from phosphorus content in the diets.

Results of the present study indicated that despite the lack of difference between groups in feed intake, the dietary phosphorus and calcium deficiency reduced growth rate and thus final mass of pigs compared to those consuming a diet with adequate (recommended) and excess of this element. Similarly, Liesegang et al. (2002) and Vier et al. (2019) showed that low dietary phosphorus and both phosphorus and calcium concentrations can negatively affect animals' daily gain. On the contrary, Huttunen et al. (2007) reported that solely phosphorus excess, changing the ratio of Ca : P, reduced growth rate and body mass in rats. In turn, Gerlinger et al. (2019) reported significantly decreased body weight gain in weaned piglets offered a diet with surplus of phosphorus, whereas phosphorus deficiency did not affect body weight gain. Other authors (Hastad et al., 2004; Nieto et al., 2016) found that daily gain and final mass of pigs were not affected by increasing available phosphorus in the diet, regardless of the proportion of Ca : P. Others reported no influence of diets deficient in both calcium and phosphorus (Nicodemo et al., 1998) on animals' growth rate. The reason for lower growth of pigs consuming a diet with low content of phosphorus and calcium was that they are essential for proper muscle and bone growth. During the first growth phase pigs prefer to develop both of these tissues instead of forming fat. Thus, in pigs fed diet with deficiency of phosphorus and calcium a reduced growth of bone (especially long bone) is usually observed, consequently growth of the whole body is also slower. However, feeding pigs a diet with increased content of both bone forming minerals did not result in their faster growth probably because their excess was removed from the body during metabolism processes. In turn, the difference in feed intake between our and cited results could probably result from the fact

that requirement for protein and energy is the main factor regulating feed intake. In the present study needs of animals for protein and energy were fully covered in all groups of pigs. Thus, in this case feed intake was independent from dietary phosphorus.

Bone parameters

Results of the present study showed that both deficiency and excess of the phosphorus and calcium influenced negatively the mass and length of the 3rd metacarpal bone. However, the negative impact of these phosphorus and calcium disturbances was much more visible in the case of bone mass than in bone length. Bone mass of pigs subjected to disturbances in phosphorus and calcium supply was on average 11% lower, while bone length by 4% lower as compared to adequately fed animals. Similar reaction of animals, concerning mass of the bone was presented by Sobol et al. (2022), albeit for the femur when pigs were fed a diet with lower calcium and phosphorus content. Thus, it seems that in the case of bone mass the short (3rd metacarpal) and long (femur) bones respond similarly to disturbances in phosphorus supply in the diet. The same authors indicate that length of the femur was negatively influenced only by phosphorus and calcium deficiency. This may indicate that sensitivity of the lengthening of long (femur) and short (metacarpal) bones for excess of the phosphorus and calcium could be different. Other authors (Swelum et al., 2017) showed that lower phosphorus intake diminished femur length in lambs. However, in this case, the negative impact of phosphorus intake was closely related to the lower intake of other nutrients (calcium, protein/amino acids and energy) significantly affecting animal growth (including skeleton). Huttunen et al. (2007) showed that growing rats responded to a decrease of the bone length when fed the diet with increased phosphorus content and changed ratio of Ca : P. In turn, Nicodemo et al. (1998), Liesegang et al. (2002), and Sørensen et al. (2018 a) demonstrated that dietary phosphorus deficiency did not affect the length and mass of the femur in growing pigs compared to a sufficient supply, regardless of the Ca : P ratio. These authors reported also that if mass of the defatted bone was considered the phosphorus deficiency significantly lowered bone mass. Most likely, this was due to the fact that osteoblasts (whose role is to synthesize and mineralize the bone matrix) and fat cells originated from mesenchymal stem cells (Pierre, 2008). Thus, it seems that disturbances in phosphorus supply and in the case of present study also calcium (deficiency and possibly excess), can promote the formation of fat cells, but not osteoblasts.

Results of the presented study showed that phosphorus and calcium excess does not improve, but deficiency results in deterioration of bone mineralization. Whereas, phosphorus and calcium excess influences bone mineral density, as this indicator improves along with increasing content of these elements in the feed. Contrary to present results, Ren et al. (2023) revealed that an extra supplementation of diet for hens only with phosphorus (inorganic phosphate) increased minerals (ash) content. On the contrary, Gutiérrez et al. (2015) found that long-term feeding of mice with high phosphorus diet with changed Ca : P ratio reduced spine and femur bone mineral density relative to the control group, due to an increase in the porosity of the examined bones. Intensification of these changes increased along with increasing the length of the experiment. Contrary to present results Huttunen et al. (2007) found that high-phosphate intake reduced femur mineral properties (both mineral content and mineral density) in growing rats, even when calcium intake was sufficient (but ratio of Ca : P was lowered). Moreover authors revealed that reduction in mineral density was observed mainly in midshaft of the bone and was probably caused by the different amount of trabecular bone which is metabolically more active. Less content of the trabecular bone in the midshaft did not allow to compensate (through changes in body mineral metabolism) disturbances in supply of bone creating minerals. However, it cannot be ruled out that different results obtained by cited authors could result from disturbed calcium/phosphorus ratio in the used diets. In some studies phosphorus was supplemented solely, which disturbed the proportion between these elements. It is well known that both minerals should be balanced together (Selle et al., 2009). This theory is confirmed by the results obtained by Katsumata et al. (2015) as these authors found that supplementation of a high phosphorus diet with additional calcium blunted the negative effect of solely increased phosphorus on bone mineral content and density.

Concerning reduction of dietary phosphorus Gerlinger et al. (2021) showed a lower trabecular bone mineral density and trabecular number in pigs fed low phosphorus and calcium diet compared to pigs fed diet with adequate content of these elements, regardless of the dietary ratio of Ca : P. However, values for cortical tissue mineral density were unaffected. Similarly Liu et al. (2017) found that broilers responded to the deficiency of only dietary phosphorus with a reduced content and density of minerals in the bones. In turn, in the study by Gerlinger et al. (2019) weaned piglets fed diet with phosphorus deficiency, showed significantly lower trabecular bone mineral density when compared to animals of control and high phosphorus group, however, the last two groups had similar value of this indicator. However, these authors did not find any differences in bone cortical mineral density. Liesegang et al. (2002), based on computed tomography of the midshaft and metaphyses of the femur, revealed lower both cortical and trabecular bone mineral content and density in the piglets fed low phosphorus diet compared to those fed a phosphorus adequate diet. Li et al. (2020) in a study with broilers found that solely decreasing dietary phosphorus, or both calcium and phosphorus decreased the bone content of minerals and mineral density. Varley et al. (2011) also noted that growing gilts respond to lower content of minerals in bones (ash, calcium and phosphorus) when offered a diet with reduced amount of both phosphorus

and calcium. Interestingly, Li et al. (2020) found that solely reduction of phosphorus had a greater effect on these characteristics than the simultaneous reduction in calcium and phosphorus. Similarly, Shapiro and Heaney (2003) also pointed out that influence of phosphorus deficiency on femur mineral content in rats was six-fold larger than that of calcium deficiency. Thus, results of two last cited authors can indicate that bone mineralization is much more sensitive to dietary deficiency of the phosphorus than calcium. Moreover, insufficient intake of one or both of these minerals interferes with homeostasis of the second one, which consequently usually results in retarded growth rate and deterioration in properties of bone mineralization.

The results of the cited studies indicate that the effect of calcium and phosphorus deficiency is the most rapidly observed in the trabecular bone tissue. This reaction results from the fact that this tissue is metabolically more active and its total surface is larger than that of the cortical tissue, which is why changes are usually observed just after 3 to 5 weeks, as demonstrated by the cited researchers. However, in our studies, the mineral content and density are the average for both types of bone tissues (cortical and trabecular). Moreover, bone geometric parameters (crucial for bone strength) in pigs subjected to calcium and phosphorus deficiency were worsened, which indicated that prolongation of deficiency till 10 weeks must also cause changes in the cortical bone layer.

Results of the presented study showed that measured geometry parameters (cortical wall thickness, cross section area, and cortical index) were uninfluenced by excess of phosphorus and calcium content in the diet, but deficiency of these elements lowered them. Literature data concerning influence of dietary phosphorus on geometry parameters of the bones are very few and inconsistent. Huttunen et al. (2007) using computed tomography revealed that high-phosphate diets did not affect trabecular bone but decreased, dose dependently, cortical cross section area and cortical thickness of the femur midshaft. Gutiérrez et al. (2015) in a long-term study with mice found that animals fed the high phosphorus diet reduced cortical wall thickness, cross section area, and bone area relative to the control group. These changes were already observed after the fifth week of the study. Authors also reported that longer feeding with phosphorus excess resulted in lower percentage of bone volume/trabecular volume as well as trabecular thickness and number, however, increased trabecular spacing. Thus, it means that only during short lasting disturbances in minerals supply animals are able to change mineral metabolism to protect bone growth and properties.

Results of the present study showed that phosphorus and calcium deficiency decreased maximum bone strength, however excess of this element not at all increased the value of this parameter. Interestingly, results of the present study revealed that dietary phosphorus and calcium content (both deficiency and excess) did not influence bone stiffness. Similarly to results of the present study Gerlinger et al. (2019) found a lowered bone fracture load and a higher maximum deflection in piglets offered low phosphorus diet compared to control and high phosphorus diet. Moreover, authors did not observe differences between the animals of the control and high phosphorus groups. Li et al. (2020) found that solely decreasing dietary phosphorus, or simultaneously reducing dietary calcium and phosphorus decreased strength of tibia in broilers. However, the authors found that as in the case of densitometry measurements solely reduction of phosphorus had a greater effect on bone strength than the simultaneous reduction in calcium and phosphorus. Similarly, Liu et al. (2017) found that broilers fed phosphorus deficient diet had lower strength of tibia than the control birds. On the contrary, Venäläinen et al. (2006) in the study with broilers and Konakov et al. (2005) in the study with growing/fattening pigs reported that lowering phosphorus content in the diet had no effect on animals' bone strength. Concerning influence of elevated phosphorus content Ren et al. (2023), contrary to our results, revealed that supplementation of diet for hens only with extra phosphorus increased bone breaking strength. In turn Huttunen et al. (2007) found that rats fed the high phosphorus diet had 40% lower bone maximum strength and stiffness.

Bone strength is a combination of bone structural, geometric, and material properties (van der Meulen et al., 2001). The most important material property of bone is the mineral content; generally the more mineral in the bone resulted in the greater stiffness and strength (Seeman, 2003). Therefore, it is obvious that animals fed a diet deficient in ingredients that determine bone mineral content and density have reduced both of these indicators. However, in the case of an excess supply of calcium and phosphorus, some authors suggest that it causes an increase in the parathyroid hormone (PTH) content in the blood, which may inhibit the synthesis of collagen, one of the determinants of bone strength (Canalis et al., 1990), probably via blocking osteoblast synthesis (Wang et al., 2005).

Relation between the phosphorus intake and the concentration of bone turnover markers in the blood serum

Osteocalcin (OC) and carboxyterminal telopeptide of type I collagen (CTX-I) are markers of bone metabolism (Greenblatt et al., 2017). Osteocalcin is a hydroxyapatite-binding protein exclusively synthesized by osteoblast or derived from procollagen metabolism. Its activity is usually elevated when bone formation rate is increased (Gerstenfeld et al., 1987), thus reflecting the activity of bone osteoblast. Bone organic matrix contains more than 90% of type I collagen which is mainly synthesized in bones. During osteoclastic resorption of bone, type I collagen is degraded and its small peptide fragments (among others CTX-I) are released and secreted into the bloodstream at a rate proportional to bone resorption intensity (Szulc et al., 2017).

Results of the present study indicated that dietary phosphorus (especially its digestible form) strongly influenced serum concentration of both OC and CTX-I. It has been found a strongly diminished content of OC in the blood serum along with decreasing content of the dietary phosphorus. In turn, increased content of the phosphorus did not change content of CTX-I, however, low dietary concentration of this element increased serum content of this bone resorption marker. The increased serum levels of CTX-I indicate that dietary phosphorus deficiency forced animals to mobilize calcium and phosphorus from bones to support growth and balance (homeostasis) of these elements. Similar results were presented by Sørensen et al. (2018 b) who found that serum OC content was lower when pigs were subjected to phosphorus deficiency compared to animals fed the diet with adequate and high phosphorus content. However, diet applied in this study differed in ratio of Ca : P. Authors also found that value of this marker also did not differ between last two groups. On the contrary, studies with growing pigs (Nicodemo et al., 1998; Varley et al., 2011) and weaned piglets (Liesegang et al., 2002) revealed that varying phosphorus intakes did not change serum OC concentrations despite the dietary Ca : P ratio which was stable in the study by Nicodemo et al. (1998) and Varley et al. (2011), however, different in the study by Liesegang et al. (2002). Gutiérrez et al. (2015) in long-term study found that dietary phosphorus deficiency significantly decreased serum OC content in mice compared to control group. Along with increasing the length of the experiment the observed differences increased from 20% after 5 weeks of study to 50% after 15 weeks. Similarly, Carter et al. (1996) reported that growing pigs had decreased levels of OC in response to decreased dietary levels of both Ca and P. Concerning influence of phosphorus excess results of the study by Ren et al. (2023) showed that supplementation a diet with this element (inorganic phosphate) had no effect on serum level of OC in hens. Conversely, Katsumata et al. (2015) found that high phosphorus diets caused an increase of the serum OC in rats and that supplementation of extra calcium to such diet prevented this change. Literature data concerning the influence of dietary phosphorus as well as calcium content on activity of bone resorption markers in the blood serum are ambiguous. Similarly to results of the present study other experiments with pigs (Sørensen et al., 2018 b) and cows (Cohrs et al., 2022) also found that the concentration of serum CTX-I increased in animals fed low phosphorus diet (Ca concentration was stable and covered animals' requirements) compared to adequate fed. In both experiment such a response of animals was observed after just two weeks of feeding and in the study by Cohrs et al. (2022) even increased when the duration of the experiment was prolonged. In turn, Gerlinger et al. (2021) did not find differences for CTX-I concentration between pigs fed low, adequate and high level of dietary phosphorus regardless of whether the Ca : P ratio in the diets was constant or different. Whereas, in the study by Gutiérrez et al. (2015) mice fed a diet with low phosphorus content had significantly lower CTX-I content in the blood serum. However, along with increasing the length of the experiment the differences disappeared and at the end of the study authors did not observe any differences. In the study by Ren et al. (2023) hens consuming a diet supplemented with phosphorus (inorganic phosphate) had lower serum content of CTX-I than birds fed control (proper balanced) diet.

The discrepancies in the concentration of the tested markers of bone turnover (OC, CTX-I) under the influence of excess/deficiency of calcium and phosphorus observed in the cited studies may result from the duration of treatment action (ranging from 3 to 15 weeks). This seems to be confirmed by Gutiérrez et al. (2015), who proved that prolonging the duration of the treatment action for 10 weeks significantly affected the difference between the concentration of the markers of bone turnover. In addition, analyses of the results of the cited studies indicated that varying degrees of excess/deficiency of one or both minerals (i.e. calcium and/or phosphorus) could also significantly affect values of the tested parameters. Therefore, the simultaneous effect of the above mentioned factors undoubtedly affect the intensity of bone turnover, resulting in different levels of markers reflecting synthesis and resorption processes. It also seems that the ratio of Ca : P in the diets, which also differed between studies, could have an important meaning for all examined features. As long as literature data prove that deficiency of the phosphorus has a negative impact on bone and that increasing a share of calcium in such diet does not improve bone properties. However, in the case of solely excess of the phosphorus in the diet some authors (Katsumata et al., 2015) showed that increasing the Ca : P ratio in such diets (by extra supply of Ca) can reduce the negative individual effect of the phosphorus excess on bone metabolism and bone characteristics. This was also visible in the results of the present work. Thus, due to the fact that in the present study the same ratio of Ca : P was maintained in all diets, higher phosphorus intake was also closely related to higher calcium intake. Probably for this reason, positive correlations were obtained between the intake of phosphorus (as well as calcium) and the measured bone characteristics.

Possible mechanisms of actions of the applied experimental factors, in the present study, on the examined bone properties

The action of the multi-tissue axis involved in maintaining phosphorus-calcium homeostasis is usually related to a moderate deficiency/excess of one mineral (phosphorus or calcium). However, in the present study the ratio of Ca : P was kept constant in all diets. This caused that the high supply of phosphorus was closely connected with high supply of calcium and, thus, probably increased levels of both phosphate anions and calcium cations in the blood serum. Thus, usually observed increase in release of PTH due to increased level of se-

rum phosphate (Katsumata et al., 2015) may have been blocked by the high calcium levels. Thus, PTH secretion and its action (release of calcium from the bones and increase of its reabsorption) was unjustified. These suggestions seem to be confirmed by the last cited authors who revealed that increased release of PTH due to consumption of high phosphorus diets is blunted when such diet is supplemented with additional calcium. Thus, it is most likely that in the present study the dietary excess of both elements (calcium and phosphorus) in animals was excreted by the kidneys. This seems to be confirmed by the same BMC as in control pigs. As densitometry measurement presents the BMD as ratio of amount of minerals (g) : bone area (cm^2), the higher bone density in animals fed high phosphorus and calcium diet resulted mainly from lower mass and length of their bone. In other words the same amount of minerals was "located" in a smaller bone space. As PTH action was unjustified in this group of pigs the bone formation was dominated over bone resorption as evidenced by lower content/activity of CTX-I, and even higher activity of OC as well as by positive correlation between intake of the phosphorus and serum content of this marker.

Furthermore, in the present study the low supply of phosphorus (and thus calcium), has favored the process of bone resorption. The low phosphorus supply must have diminished both serum phosphate and calcium content. In turn, the low serum calcium probably must have induced the secretion of PTH by parathyroid glands to increase calcium reabsorption by kidneys, and increased activity of osteoclasts to dissolution and degradation of hydroxyapatite from the bone (Teitelbaum, 2000). Calcium reabsorption is also supported by the secretion of calcitriol (active form of vitamin D_3) usually observed at phosphorus deficiency (Penido and Alon, 2012). Although, an increased absorption of calcium and phosphorus in the intestines and decreased reabsorption of phosphates in the kidneys, have been hindered by the low supply of calcium with the feed (as a result of balanced ratio of calcium/phosphorus), the same level of vitamin D_3 in all diets as well as equal breeding condition. Thus, the only source of calcium (and also phosphorus), necessary for the functioning of the body, was calcium released from the bones. This probably caused that bone resorption was dominated over the bone formation in the pigs fed low phosphorus and calcium diet. This is indicated by worse physical, densitometry, geometry and strength parameters of bones, as well as a higher concentration of CTX-I in the blood serum. This was also evidenced by negative correlation between phosphorus intake and activity of the CTX-I found in the present study.

Conclusions

Based on the results of the present study it could be concluded that strong differentiation of the phosphorus and calcium level in the pigs' diet, while maintaining stable ratio of Ca : digestible P, do not affect feed intake. Despite the lack of a difference in feed consumption the low dietary phosphorus and calcium content deteriorate, however excess of these elements did not improve growth rate of pigs. Markers of bone formation are positively while markers of bone resorption are negatively correlated with phosphorus intake. Feed with strong phosphorus and calcium deficiency decreases both bone formation and bone resorption processes, however strong excess of these elements increases bone formation and does not change bone resorption. Measured parameters of bones are positively correlated with phosphorus intake. However, strong deficiency of dietary phosphorus and calcium deteriorates, whereas its excess does not improve physical parameters, minerals content, geometry parameters and strength of the bones. Thus, from the viewpoint of growth performance and parameters of the bones increasing the phosphorus and in the case of present study also calcium content in the diet for growing pigs is inadvisable. While excessively lowering the dietary phosphorus concentration (e.g. to reduce environmental pollution) may adversely affect pig growth and bone characteristics.

Author contributions:

Conceptualization, M.S., G.S., E.R.G.; methodology, M.S., G.S. and M.Ś.; software, M.S., P.K.; validation, M.S. and M.Ś.; formal analysis, M.S., M.Ś. and P.K.; investigation, M.S., G.S. and P.K.; resources, M.S., G.S.; data curation, G.S. and M.S.; writing – original draft preparation, M.S., G.S. and P.K.; writing – review and editing, G.S., M.S., M.Ś. and E.R.G.; visualization, M.S., P.K.; supervision, G.S., E.R.G. and M.S.; project administration, G.S and M.S.; All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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