Evaluation of serum parameters to predict the dietary intake of calcium and available phosphorus in growing pigs

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Acknowledgments: This research received funding from the Tandem Ph.D. Program of the University of Veterinary Medicine, Vienna, Austria. The authors thank Sharma Suchitra, Simone Koger, Melanie Wild, Arife Sener, Annegret Lucke, Manfred Hollmann and Thomas Enzinger (Institute of Animal Nutrition and Functional Plant Compounds), Lukas Schwarz (University Clinic of Swine) as well as Sylvia Posseth and Tamara Strini (vetFarm) for excellent assistance with the animals, sampling and laboratory analysis.

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Abstract

Adequate provision of calcium (Ca) and phosphorus (P) is essential for bone formation and high growth performance in pigs. Nevertheless, reliable serum biomarkers for pig’s Ca and P intake are still missing. Here, we used phytase supplementation to alter the dietary available P (aP) level in order to investigate the effect of differences in dietary aP levels on serum parameters related to the Ca and P homeostasis in pigs. Moreover, we assessed whether serum parameters can be used to predict the Ca, total P (tP) and aP intake in barrows and gilts throughout the fattening period. In total, 216 pigs (115 gilts and 101 barrows) were randomly allotted to 1 of 2 diets in 3 replicate batches, each lasting 56 days (n = 108/diet). Pigs had free access to the diets without (Con) or with phytase (Phy; 650 phytase units/kg) via a transponder-based feeding system. Blood samples were collected on days 2, 23 and 52 and serum parameters were correlated to the daily Ca, tP, and aP intake. The intake of tP, aP, and Ca was overall 14.2, 13.8, and 14.2% higher in barrows compared to gilts, respectively (P < 0.001). Concurrently, phytase decreased the intake of tP and Ca by 8.4 and 6.7%, respectively, whereas it raised the intake of aP by 16.3% compared to Con diet (P < 0.001). Serum levels of fibroblast growth factor 23, alkaline phosphatase (ALP), vitamin D (VitD) and osteocalcin (OCN) decreased with age (P < 0.05). The higher aP intake of pigs fed Phy diet increased serum P on days 2 and 23 but decreased it on day 52 compared to Con diet (P = 0.004). Pigs fed Phy diet had higher serum ALP compared to pigs fed Con diet on days 23 and 52 (P < 0.05). Correlation analysis between serum parameters and Ca, tP and aP intake showed age- and sex-related associations. With 12 weeks of age serum P in both sexes, serum VitD in barrows and serum OCN and ALP in gilts correlated with aP intake (|r| > 0.38), whereas serum OCN correlated with Ca in both sexes’ intake (r > 0.50). At 20 weeks, serum Ca and ALP in gilts correlated with aP intake, whereas serum P, Ca and VitD correlated with Ca intake in both sexes (|r| > 0.39). In conclusion, present results showed that the daily Ca
and aP intake could be most reliably estimated from serum parameters for an approximate age of 12 and 20 weeks. Serum P and the Ca:P ratio at 12 weeks of age and serum VitD at 20 weeks of age may be used to predict pig’s daily aP intake in both sexes.

**Key words:** growing pig, mineral intake, phosphorus homeostasis, phytase, serum parameters, sex
List of abbreviations

ADFI, average daily feed intake; ADG, average daily gain; ALP, alkaline phosphatase; aP, available phosphorus; BW, body weight; Ca, calcium; Con, control; DM, dry matter; ELISA, enzyme-linked immunosorbent assay; FGF23, fibroblast growth factor 23; G:F ratio, gain to feed ratio; NEFA, non-esterified fatty acids; OCN, osteocalcin; P, phosphorus; Phy, phytase; tP, total phosphorus; VitD, vitamin D
Introduction

Leg weakness is a major economic problem in today’s pig production, affecting thriftiness of growing pigs and increasing the culling rate of breeding animals. Therefore, providing adequate amounts of minerals, especially calcium (Ca) and phosphorus (P), to pigs is critical to support adequate mineralization of the skeleton and hence optimal growth (Létourneau-Montminy et al., 2012, Li and Stahl, 2014). For the clinical diagnosis of leg weakness, physical examination of the living pig and certain serum parameters are assessed (Crenshaw and Rortvedt-Amundson, 2014). Histology and mechanical tests as well as bone ash measurements are more accurate than physical examination alone, but they require to kill the animal and hence cannot be used for herd screening in order to early detect nutritional inadequacies. Moreover, these analyses have partly long turn-around times and rapidity of diagnosis is an important issue in clinical diagnostics when dealing with acute problems in a pig herd (Crenshaw and Rortvedt-Amundson, 2014). In addition, it is not clear whether the measured serum parameters are reliably corresponding to abnormalities in pig’s Ca and P intake and are similar in barrows and gilts. Therefore, it is still difficult to assess whether pigs in a herd received adequate amounts of Ca and P via their diet by using little invasive methods. Currently, several serum parameters related to the Ca homeostasis, such as vitamin D (VitD), parathyroid hormone, and osteocalcin (OCN), are used as markers for bone health in practice (Sørensen et al., 2018; Lee et al., 2020). However, these regulatory factors do not sufficiently correspond to the dietary P intake (Oster et al., 2016; Amundson et al., 2017; Sørensen et al., 2018). As potential candidates, fibroblast growth factor 23 (FGF23), with its primary function to decrease systemic P and VitD levels (Erben, 2016), is used in the early detection of bone abnormalities in humans (Rupp et al., 2019), whereby evidence in pigs is still scarce. We could previously show that FGF23 has a certain role in the Ca homeostasis, but we could not find a clear association with the P metabolism (Vötterl et al., 2020). Further
regulators for bone mineralization that are linked to the P homeostasis are alkaline phosphatase (ALP), which enhances the local P concentration, and OCN which binds P in the bone (van Riet et al., 2013; Penido and Alon, 2012). In addition to FGF23, the hormone VitD increases Ca and P retention and absorption, in the case of Ca by aid of parathormone (Lederer 2014; Pu et al., 2016).

The requirement for total P (tP) to achieve optimal bone mineralization has been estimated to be 1 g/kg diet higher than to reach optimal body weight gain (O’Doherty et al., 2010; Varley et al., 2010). Due to the impact that the dietary ratio of Ca to tP has on Ca and P uptake and its systemic utilization (Veum, 2010; Vier et al., 2019), the optimal dietary Ca:tP ratio from 1:1 to 2.5:1 or Ca:aP ratio between 2:1 to 3:1 (NRC, 2012; Schneider et al., 2019) should guarantee optimal utilization of Ca and P for bone synthesis and mineralization. To render the otherwise non-digestible phytate-P more available to the pig, pig diets are typically supplemented with phytase (Dersjant-Li et al., 2015). Based on this, 500 phytase units (FTU) per kg diet should generate about 1.15 g aP/kg diet (Humer et al., 2015). Moreover, phytase supplementation may also improve dietary Ca availability (González-Vega and Stein, 2014; McGhee and Stein, 2019), which, in turn, may alter intestinal and systemic Ca availabilities.

Because imbalances in the dietary supply and availability impact absorption and body utilization of Ca and P, the identification of appropriate serum markers that correspond to both, the dietary Ca and aP intake, may help to detect imbalances in mineral intake on farms before skeletal problems become apparent. We therefore hypothesized that changes in the dietary aP intake are traceable in serum levels of regulatory factors related to both Ca and P homeostasis. In the present study, we used phytase supplementation to alter the dietary aP level in order to investigate the effect of differences in dietary aP levels on serum parameters related to the Ca and P homeostasis in pigs during the fattening period. Moreover, we
assessed whether serum parameters can be used to predict the Ca, tP and aP intake in barrows and gilts throughout the fattening period.

Materials and Methods

All procedures involving animal handling and treatment were approved by the institutional ethics committee of the University of Veterinary Medicine Vienna and the National authority according to the Law for Animal Experiments, Tierversuchsgesetz in Austria (BMWFV-68.205/01221-WF/V/3b/2017).

Animals and housing

A total of 216 crossbred growing pigs [Large White × Piétrain; 115 gilts and 101 barrows] from 19 litters at 11 weeks of age [average initial body weight (BW) 38.5 ± 7.1 kg] from the research and teaching farm of the University of Veterinary Medicine Vienna were used in three replicate batches (n = 72/replicate batch). Pigs were acclimatized to the experimental conditions (i.e. housing and feeding stations) for one week before the start of the experiment. Each replicate run lasted for 56 days (8 weeks). Pigs were housed in an outdoor climate fattening unit comprising 6 pens (19.18 m², 3.50 m × 5.48 m per pen) with slatted flooring. In each replicate batch, pigs were penned in groups of 12 littermates per pen. Pens were randomly allocated to diets; in each replicate batch 3 pens received one of the two diets, resulting in a total of 9 pens per diet across all 3 replicate batches (n=108 pigs/diet). Each pen was equipped with two nipple-drinkers. Water was freely available throughout the experimental period. The health status of the pigs was monitored daily by visual inspection and checking the feed intake records.
Diets and feeding

Two diets (Table 1) were formulated to meet or exceed the current recommendations for nutrient requirements (Flachowsky et al., 2006; NRC, 2012). The diets were either not supplemented (Con) or supplemented with a 6-phytase (Phy) derived from *Escherichia coli* (VM Phytase XP 897420, Garant-Tiernahrung GmbH, Pöchlarn, Austria), which was added at a concentration of 650 FTU/kg complete feed (Dersjant-Li et al., 2015). The dietary Ca:tP ratio of 1.3:1 for both diets, and the dietary Ca:aP ratio of 2.5:1 and 2.0:1 for the Con and Phy diets, respectively, were in the recommended range (NRC, 2012). Feed samples were collected 3-times on day 1, 22, and 49. The pigs had free access to pelleted feed via transponder-based feeders (Feed Intake Recording Equipment feeder, SCHAUER Agrotonic GmbH, Prambachkirchen, Austria). On the day of the transfer to the fattening unit, each pig received an individual radio-frequency identification ear tag (CI Tiris AF-Ohrchip 25.5 mm, SCHAUER Agrotonic GmbH, Prambachkirchen, Austria). One pig at a time got access to the feeder by means of the RFID ear tag. The amount of feed consumed by each pig was registered automatically by weighing the trough before and after the pig got access to the trough. Every visit of the pig at the feeder (amount of feed eaten and time) was registered. The data for each pig per day were summed up and expressed as consumed feed per day (g/d).

Body weight measurement and calculations

The BW of each pig was measured individually on experimental days 1, 8, 22, 35, and 49 using a transportable animal scale (Agreto Einzeltierwaage, Agreto electronics GmbH, Raabs, Austria). The average daily gain (ADG), average daily feed intake (ADFI), and gain:feed ratio (G:F ratio) as well as the Ca, tP, and aP intake were calculated for the whole experimental period and for the intervals day 1-8, day 9-22, day 23-35, and day 36-49.
Blood sampling

Blood samples were collected from the jugular vein of each pig on 1 of 2 consecutive sampling days by separating the pigs one by one and restraining them with a pig holder (36 pigs per day). Blood was collected on experimental days 2 and 3, 23 and 24, and 52 and 53 in serum tubes (S-Monovette 9 ml Z, Sarstedt AG & Co. KG, Nümbrecht, North Rhine-Westphalia, Germany). To simplify the presentation of the data, the sampling days for serum will be referred to as days 2, 23 and 52. During sampling, blood was kept on ice and centrifuged afterwards at 2,700 × g for 10 min at 4 °C (Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany). Serum aliquots were stored at -80 °C until analysis.

Chemical analysis

Dried diet samples were ground to pass a 1-mm sieve (Ultra-Zentrifugalmühle ZM 200, Retsch GmbH, Haan, North Rhine-Westphalia, Germany). Dry matter (DM), crude ash, crude protein, crude fat, tP, Ca, magnesium, potassium, sodium, neutral-detergent fiber, acid detergent fiber, total starch, resistant starch, and non-resistant starch were measured. To determine the DM (method 3.1) content, diet samples were oven-dried at 103 °C for 4 h, whereas crude ash (method 3.5) was determined by incinerating at 580 °C for 4 h (Naumann and Bassler, 2012). Crude fat (method 5.1.1/3.5.2) was analyzed by solvent extraction with petroleum ether (Naumann and Bassler, 2012). Calcium (method 10.3.1.), sodium (method 10.1.1), potassium (method 10.2.1), and magnesium (method 10.4.1) were measured with atomic absorption spectroscopy, whereas tP was measured photometrically (method 10.6.1; Naumann and Bassler, 2012). The content of crude protein was determined using the Kjeldahl method (method 4.1.1). The adapted methods of Van Soest et al. (1991) with FiberTherm FT 12 (C. Gerhardt GmbH & Co. KG, Königswinter, North Rhine-Westphalia,
Germany) were used for the analyses of neutral and acid detergent fiber (methods 6.5.1 and 6.5.2; Naumann and Bassler, 2012). The nitrogen-free extract fraction was calculated by subtracting crude ash, crude protein, crude fiber, and crude fat from the DM (Naumann and Bassler, 2012; Kamphues et al., 2014). Commercial enzymatic assay kits were used to analyze total starch, resistant starch, and non-resistant starch (K-RSTAR, Megazyme International Ireland Ltd., Bray, County Wicklow, Ireland). All analyses were done in duplicates and results are provided on DM basis. Analyses of phytase activity were conducted by LUFA Nord-West after the DIN EN ISO 30024 method (Institut für Futtermittel, LUFA Nord-West, Oldenburg, Lower Saxony, Germany). The amount of aP was calculated using data for aP contents in individual feed ingredients either without or with phytase addition to reduce inorganic P supply by one gram per diet (Schneider et al., 2019).

**Serum parameter analyses**

An auto-analyzer for clinical chemistry using enzymatic colorimetric assays (Cobas 6000/c501; Roche Diagnostics GmbH, Rotkirch, Canton of Zug, Switzerland) was used to determine serum content of P, Ca, ALP, cholesterol, non-esterified fatty acids (NEFA), triglycerides, and urea. The Ca:P ratio in serum was calculated to assess the systemic availability of Ca and P and their association with the regulatory hormones (Veum, 2010; Gerlinger et al., 2020; Vötterl et al., 2020). A porcine specific enzyme-linked immunosorbent assay (ELISA) was used to determine FGF23 (Porcine FGF23 ELISA kit, Wuhan Fine Biotech Co., Ltd., Wuhan, Hubei, China; CV < 10%). For calcitonin, a porcine-specific ELISA kit (Calcitonin ultrasensitive ELISA; DRG Instruments GmbH, Marburg, Hesse, Germany; CV < 8%) was used and the non-diluted samples were analyzed. Nevertheless, most samples contained calcitonin levels at the quantification limit of 0.7 pg/ml and were therefore not reported. Vitamin D (25-Hydroxy Vitamin Ds EIA, Immunodiagnostic Systems...
Holdings PLC, Tyne & Wear, NE35 9PD, United Kingdom; CV < 20%) and OCN (N-MID Osteocalcin ELISA, Immunodiagnostic Systems Holdings PLC, Tyne & Wear, NE35 9PD, United Kingdom; CV < 6%) were analyzed with commercial ELISA kits that were previously evaluated for pig serum (Kolp et al., 2017; Sørensen et al., 2018).

Statistical analyses

After testing the data for normal distribution and outlier using the Shapiro-Wilk test in SAS (version 9.4, SAS Inst. Inc., Cary, NC, USA), data were subjected to ANOVA using the MIXED procedure. Data were first analyzed as repeated measures over time (sampling days). Since effects for phytase and sex differed during the experimental period, data were analyzed with a second random model, separately per day. This model accounted for the fixed effects of phytase, sex, and the interaction phytase × sex. To account for a potential effect of the starting BW, it was implemented in the model as a covariate. Replicate batch was considered as the random effect and the individual pig nested within litter (pen) as the experimental unit assuming a compound symmetry variance-covariance structure (type = cs). Degrees of freedom were approximated by the Kenward-Roger method. Data were expressed as least square means ± standard error of the mean (SEM). The pairwise comparisons between least-square means were tested using the PDIF option in SAS. Differences at $P \leq 0.05$ and $0.05 < P \leq 0.10$ were defined as significance and trend, respectively. For the characterization of potential serum markers for Ca, tP, and aP intake, Pearson correlation coefficients ($r$) were calculated separately for gilts and barrows between daily Ca, tP, and aP intake and serum parameters separately for experimental days 1-8 (feed intake from day 1 to 7, blood sample on days 2 and 3), days 22-28 (feed intake from day 22 to 28, blood sample on days 23 or 24), and days 50-56 (feed intake from day 50 to 56, blood sample days 52 or 53) individually using PROC CORR of SAS. A significant correlation was defined as $P \leq 0.05$ and $|r| \geq 0.35$. 
Results

Animals and diets

In total, 16 pigs (i.e. 6 barrows and 10 gilts) were removed from the experiment mostly in the first 28 days of the experiment (replicate batch 1, \( n = 6 \); replicate batch 2, \( n = 3 \); and replicate batch 3, \( n = 7 \)) due to tail biting or low feed intake which was not related to dietary treatments. The Con and Phy diets provided 15.2 and 15.1 MJ metabolizable energy/kg diet, respectively, and had a crude protein content of 18.5 and 18.4% DM, respectively. The analyzed phytase activity was 257 and 596 FTU/kg complete feed for the Con and Phy diets, respectively, resulting in a 24.7% higher aP content in the Phy diet compared to the Con diet (Table 1).

Growth performance, feed intake, and daily consumption of Ca and P

The BW at the beginning of the experiment differed between sexes; therefore, the initial BW was considered as a covariate for the performance parameters (Table 2 and S1, Figure 1). Barrows had a 5.6% higher BW at the start of the experiment and weighed on average 4.1% more on days 9-22, days 23-35, and days 36-49 compared to the gilts (\( P < 0.05 \)). This resulted in an ADG which was 5.7% higher in barrows compared to gilts on days 1-49 (\( P = 0.014 \)). The greatest sex-related difference was on days 9-22 when the ADG of barrows was 18.1% higher than that of gilts (\( P < 0.001 \)). The ADG continued to increase in gilts from days 1-8 to days 36-49, whereas in barrows the highest growth rate was reached in the period of days 9-22 and declined afterwards (\( P < 0.001 \)). For the whole experimental period, the ADFI was 14.2% higher in barrows compared to gilts (\( P < 0.001 \)). As a result, the G:F ratio was 7.8% lower in barrows compared to gilts (\( P < 0.001 \)). Based on the ADFI, the intake of tP,
aP, and Ca over the whole experimental period was 14.2, 13.8, and 14.2% higher in barrows compared to gilts, respectively (Figure S2; $P < 0.001$).

When comparing the diet groups, pigs fed the Phy diet had a 6.1% higher BW compared to pigs fed the Con diet ($P = 0.005$). However, the phytase tended ($P = 0.098$) to lower pig’s BW by 1.8% on days 22-35 compared to the Con diet without phytase. Moreover, the phytase supplementation decreased the ADFI in the periods of days 9-22 and 23-35 by 6.1% ($P < 0.01$) and on days 36-49 by 10.1% ($P < 0.001$) compared to the Con diet. As result, phytase led to an overall improvement of the G:F ratio by 4.2% compared to the Con diet ($P = 0.026$). This was especially apparent on days 35-49, where the G:F ratio was 11.1% higher in pigs fed the Phy diet compared to those fed the Con diet ($P < 0.001$). For the whole experimental period, the intake of tP and Ca decreased in pigs fed the Phy diet on average by 8.4 and 6.7%, respectively ($P < 0.001$), whereas the intake of aP increased by 16.3% in the Phy group compared to the Con group ($P < 0.001$). When comparing the Ca and tP intake on the 14-day basis, phytase reduced the intake of tP by 8.2, 6.9, and 11.5% compared to the Con diet on days 9-22, 23-35, and 36-49, respectively ($P \leq 0.001$). By contrast, the increase in the daily intake of aP with the phytase supplementation was greatest with 21.0% from days 1-8 and lower for the following days with an increase of 17.0, 18.9, and 12.0% in pigs fed the Phy diet on days 9-22, 23-35 and 36-49, respectively, compared to the Con group ($P < 0.001$). Similarly, the intake of Ca was 6.4, 5.1, and 9.8% lower in the Phy group compared to the Con group from days 9-22, 23-35, and 36-49, respectively ($P < 0.05$).

**Serum parameters**

Except for FGF23, sex and phytase supplementation affected serum parameters. Additionally, age-related alterations occurred from day 1 to 49 (Table S2, Figure 2). Serum Ca was similar across sexes, whereas barrows had a 4.2 and 2.7%-higher serum P content than gilts on days
2 and 23, respectively \((P < 0.05)\), resulting in a 4.4\% \((P = 0.006)\) and 2.0\% \((P = 0.069)\) lower serum Ca:P ratio in barrows compared to gilts on days 2 and 23, respectively. Moreover, barrows had a 7.8\%--higher serum VitD content on day 23 and 9.9, 25.7, and 28.1\%--higher serum urea contents on days 2, 23 and 52 than gilts, respectively \((P < 0.05)\). Moreover, barrows contained 4.0\% more cholesterol in their serum than gilts on day 23 \((P = 0.016)\).

Over time, P, Ca, and OCN in serum increased from day 2 to day 23 but decreased towards day 52 compared to the previous measurement \((P < 0.001)\). In contrast, the Ca:P ratio, VitD, and triglycerides decreased in serum towards day 23 but increased again on day 52 compared to the previous sampling days \((P < 0.001)\). A continuous decrease over time was apparent for FGF23 and ALP in serum, while urea, cholesterol, and NEFA content in serum increased throughout the experiment \((P < 0.001)\).

Phytase supplementation increased serum P by 13.4 and 5.1\% on days 2 and 23, respectively \((P < 0.001)\), but reduced it by 3.4\% on day 52 \((P = 0.004)\) compared to the Con group. On day 2, the serum Ca content was 5.3\% lower in the Phy group compared to the Con group \((P < 0.001)\). The phytase × sex interaction on day 52 for serum Ca \((P = 0.029)\) indicated that the barrows of the Con group and gilts of the Phy group had higher serum Ca contents compared to the other two pig groups. The serum Ca:P ratio was 16.8 and 5.6\% lower on days 2 and 23, respectively, and 2.9\% higher on day 52 in the Phy group compared to the Con group \((P < 0.05)\). The phytase supplementation increased \((P = 0.028)\) the serum ALP by 7.3\% on day 23 and tended \((P = 0.063)\) to increase it by 6.4\% on day 52 compared to the Con group. Likewise, phytase tended \((P < 0.10)\) to increase serum OCN on days 23 and 52 compared to the Con group. Moreover, the serum urea content from pigs of the Phy group tended to by 6.9\% higher on day 2 \((P = 0.087)\) and was 8.2\% higher on day 52 \((P = 0.033)\) compared to pigs of the Con group. Serum cholesterol and triglycerides were 5.3 and 9.9\% lower on day 2, whereas on day 52 triglycerides were 10.3\%--higher in the Phy group compared to the Con.
group \((P < 0.05)\). Lastly, phytase supplementation increased serum NEFA by 71.6\% on day 52 compared to the Con group \((P < 0.001)\).

*Relationships between daily Ca and P intake on days 1-8, 22-35 and 36-49 with serum parameters on days 2 (week 1), 23 (week 4) and 52 (week 8)*

The sex-specific differences in serum parameters indicated that sex-related responses may interfere in the identification of potential serum markers for the dietary Ca and P intake. Therefore, Pearson correlations (Table 3) were calculated for each sex separately. In total, 47 correlations between serum parameters and Ca, tP, and aP intake were found in gilts but only 38 in barrows. Moreover, more correlations existed for days 1-7 (week 1) and days 50-56 (week 8) compared to days 22-28 (week 4). On days 1-7 (week 1), serum P content was positively correlated with aP intake, dietary Ca:tP ratio \((0.58 < r < 0.76)\) but negatively with the dietary Ca:aP ratio \((-0.76 < r < -0.61)\) in both sexes, whereas on days 22-28 (week 4) serum P only correlated with aP intake in barrows \((r = 0.42)\). On days 50-56 (week 8), serum P correlated with tP and Ca intake in both sexes \((0.39 < r < 0.41)\). Serum Ca levels in both sexes were negatively correlated with the dietary Ca:tP ratio \((-0.61 < r < -0.58)\) but positively with the dietary Ca:aP ratio \((0.58 < r < 0.61)\) on days 1-7 (week 1). Additionally, a positive correlation between serum Ca levels and tP and Ca intake existed in week 8 \((0.45 < r < 0.58)\) for both sexes, whereas in gilts serum Ca correlated also with the aP intake \((r = 0.50)\) on days 50-56 (week 8). Vitamin D positively correlated with aP intake on days 1-7 (week 1) in barrows \((r = 0.38)\) and negatively with intake of tP, aP, and Ca on days 50-56 (week 8) in both sexes \((-0.60 < r < -0.48)\). Only in gilts, ALP showed a negative correlation with tP, aP, and Ca intake on days 1-7 (week 1; \(r = -0.47)\), whereas a positive association between ALP and the aP intake existed on days 50-56 (week 8; \(r = 0.44\)). Serum OCN positively correlated with tP and Ca intake on days 1-7 (week 1) in both sexes \((0.50 < r < 0.60)\) and additionally in
gilts with the aP intake on days 1-7 (week 1; r = 0.44). Moreover, Pearson correlations were used to determine potential metabolic relationships between the dietary Ca and P intake and lipid and protein metabolism (Table S3).

Discussion
The importance of the dietary Ca and P supply and the impact on their systemic availability and skeletal mineralization in pigs are well-known (van Riet et al., 2013; Schlegel and Gutzwiller, 2017). From the available literature data, it was not clear how reliably serum parameters that are related to the Ca and P homeostasis can be used to predict the dietary supply with Ca and aP. Moreover, it was not clear whether different serum parameters were necessary to predict dietary Ca and aP intake in gilts and barrows due to sex-related differences in ADFI and growth rate. The latter was, for instance, suggested by the generally greater ADG and higher growth rate between days 9-22 of the barrows, whereas the gilts reached their highest ADG at a later stage in the present study. We could identify several relationships between the dietary Ca, tP, and aP intake and serum Ca, P, VitD, ALP, and OCN levels but apparently not with FGF23 levels, whereby all serum parameters remained in their physiological range. However, from the present correlations, it is obvious that the predictability of pig’s intake of Ca, tP, and aP by serum parameters depends on pig’s age and sex, not allowing the recommendation of one marker for the whole growing period for both sexes. The predictability of the intake of aP by serum parameters showed a greater sex-specificity, whereby these relationships again mostly existed on days 2 and 52. Serum P on day 2 and VitD on day 52 were the best predictors for the dietary aP intake in both sexes. In contrast, on day 23 the majority of correlations with the serum parameters were too weak to allow the prediction of the daily intake of Ca, tP, and aP. These findings are in line with the decreasing Ca and P requirements with increasing age and hence related to regulatory
mechanisms, reflecting the actual Ca and P requirements for skeletal growth and physiological functions (Horst et al., 1990; Pattanaungkul et al., 2000; Xu et al., 2002).

Barrows and gilts showed the expected sex-related ADFI and BW development, which were higher in barrows for the whole experimental period. Moreover, the present data supported the greater appetite of barrows throughout the fattening period despite similar growth, resulting in higher daily intakes of Ca, tP, and aP compared to the gilts. Accordingly, we would have expected that the higher supply of nutrients in barrows compared to gilts was mirrored in sex- and body weight gain-related serum profiles, especially as we only fed one fattening diet throughout the experimental period, oversupplying the pigs with nutrients towards the end of the experimental period. The dietary Ca and P content met the requirements (0.66% Ca, 0.56% tP for 25-50 kg pigs; NRC, 2012) at the start of the experiment but were above them (0.52% Ca, 0.47% P for 75-100 kg pigs; NRC, 2012) towards the end of the experiment. Nevertheless, serum Ca and P did not reflect consistently the elevated dietary intake of Ca and P over time in barrows compared to gilts. From the investigated parameters, only serum urea, a marker for protein turnover, corresponded to the increased protein intake in barrows compared to the gilts. The increased serum urea also reflected the lower amino acid requirements with increasing age. These discrepancies demonstrate the challenge to link the dietary intakes of Ca, tP and aP with serum profiles due to the regulatory action of the body to maintain serum levels in physiological ranges. Due to the many crucial functions in the body (e.g. enzyme activation and cell differentiation) serum Ca levels are tightly controlled by means of Ca-sensing receptors, which are evenly distributed in the body compared to serum P (Veum, 2010; Pu et al., 2016; Liu et al., 2018).

In contrast, P-sensing receptors seem to be confined to the intestine and parathyroid gland, thereby allowing a less strict regulation of serum P levels (Berndt and Kumar, 2009; Pu et al., 2016; Chande and Bergwitz, 2018). These differences in the regulation of serum Ca and P
were also suggested by the serum Ca:P ratio, which was below 1 throughout the experimental period across sexes and treatments despite dietary Ca:tP and Ca:aP ratios above 1. The present data support lower Ca and P requirements for bone formation when the animal reaches maturity (Clarke, 2008; Lederer, 2014). Against this background, serum FGF23, VitD, ALP, and OCN levels responded according to the maturational stage of the animal, reflecting skeletal growth and, except for FGF23, also reflected dietary Ca and aP provision on days 2, 23, and 52. Albeit ALP is rather a non-specific marker for tissue growth as all tissues and organs produce ALP during growth (Christenson, 1997; Penido and Alon, 2012; Gonzalo et al., 2018), it can be assumed that serum ALP and OCN, which is only synthesized by the bone, mirrored osteoblast activity and hence bone formation (Szulc et al., 2000; Allen, 2003; Owen and Reilly, 2018). Similarly, serum FGF23, a bone-derived hormone which suppresses renal phosphate reabsorption and VitD synthesis (Erben, 2018), was higher at the start of the experiment than at the end. However, the present data do not allow distinguishing whether the higher serum FGF23 was associated with more efficient P utilization for bone formation in the older pigs or with the adjustment of body VitD levels. Notably, serum ALP and FGF23 showed an age-specific decline which emphasizes the importance of animal’s age when evaluating serum markers. These findings further indicate that the level of FGF23 in the blood might be depending on skeletal growth as it is the case for ALP in growing animals (Allen, 2003).

In contrast to previous observations (Torres-Pitarch et al., 2012, Vigors et al., 2014), phytase supplementation decreased the ADFI while not compromising the ADG in the present study. This resulted in the trend for the improved G:F ratio, indicating increased digestibility and conversion of nutrients of phytase-supplemented diets. Phytase supplementation increases the intestinal aP availability and absorption (Vötterl et al., 2020), which likely induced the higher serum P of pigs receiving the Phy diet until experimental day 23. Afterwards, however,
serum P was lower in pigs fed the Phy compared to the Con diet, suggesting a reduced intestinal absorption but increased renal excretion as regulatory response in these pigs. The increased serum levels of ALP and OCN from day 23 in pigs receiving the Phy diet would support that more Ca and P was used for bone formation (Carter et al., 1996; Golub and Boesze-Battaglia, 2007; Clarke, 2008), which may be indicative of a more balanced systemic Ca and P availability with the Phy diet. This assumption may be further supported by the greater serum Ca:P ratio on day 52. Both, ALP and OCN, are used in human osteomalacia diagnosis but study results are inconsistent with respect to the interpretation of elevated values (Hlaing and Compston, 2014). Nevertheless, they may represent useful indicators for the dietary aP supply in growing pigs.

Pearson correlation analysis supported the importance to differentiate between sexes and stages of growth. The fact that none of the investigated serum parameters correlated to the daily intake of Ca, tP and aP across the 3 sampling time points emphasizes the need to define the age stages for which the respective serum marker can reliably predict the dietary intake of Ca, tP and aP. Moreover, the present correlations point out that sampling days 2 and 52 would be superior time points to indicate adequate supply with Ca and aP. These age stages may diverge when phase feeding is applied to fit the actual aP requirements over the fattening period, which need to be evaluated in further experiments. The pigs used in the present experiment came from one farrowing group with birth dates being 4 days apart at maximum. Accordingly, the days on which serum was collected corresponded to an age of 12, 16 and 20 weeks. Hence, the first and third time points represented the early growing and early finishing period; time points at which often a dietary change occurs and therefore would be useful for adjustments in the dietary Ca and aP amounts. Nevertheless, the validation of the identified serum markers should include the characterization of ‘time slots’ for their efficacy, using a tighter schedule of blood samplings. In fact, in practice, pigs of different ages are often mixed
due to non-uniform growth or body weight within age groups, and blood samplings may not be always possible at the exact age we sampled in this study. For experimental day 2 (12 weeks of age), the present correlations suggested a marker profile of serum P and the serum Ca:P ratio for the daily aP intake for both sexes. In gilts serum OCN and in barrows VitD could be used as additional marker in this profile to predict the aP intake. Serum OCN, and ALP only in gilts, may be further used to predict the Ca intake on experimental day 2. On experimental day 23 (16 weeks of age) only the serum Ca:P ratio for gilts and serum P for barrows may be used to predict aP intake. On experimental day 52 (20 weeks of age), the present correlations suggested a serum profile that comprises serum Ca and ALP in gilts and VitD in both sexes to predict pig’s daily aP intake. To predict pig’s daily intake of Ca, serum P, Ca and VitD could be used in both sexes on experimental day 52. Overall, the present correlations confirmed the applicability of certain serum parameters that are used in practice. However, the present findings also largely emphasize that their applicability differs with pig’s age and sex.

In conclusion, results demonstrated certain relationships between pig’s intake of Ca, tP, and aP and serum parameters. However, they also emphasized the dependencies of these relations on pig’s age and sex, not allowing the recommendation of one marker profile for the entire growing period and valid for both sexes. According to the present correlations, the daily Ca and aP intake could be most reliably estimated from serum parameters for an approximate age of 12 and 20 weeks. Serum P and the Ca:P ratio at 12 weeks of age and serum VitD at 20 weeks of age may be reliable markers to predict pig’s daily aP intake in both sexes. The present marker profiles should be validated in further studies, narrowing the age ranges for which these markers are applicable and evaluated for their applicability with different feeding regimes.
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Figure legend

Figure 1. Daily intake (g/d) of total phosphorus (tP; A), available phosphorus (aP; B) calcium (Ca; C), and the ratio of Ca:aP intake (D) in gilts (grey and cross striped columns) and barrows (black and cross striped columns) fed the control (Con) diet and gilts (grey and solid columns) and barrows (black and solid columns) fed the diet with phytase (Phy). Significant effects ($P \leq 0.05$) of sex are indicated by the symbol *; effects of phytase by the symbol †; and the interaction of sex and phytase by the symbol ‡.

Figure 2. Serum content of phosphorus (A), calcium (B), phosphorus:calcium ratio (Ca:P ratio; C), fibroblast growth factor 23 (FGF23; D), vitamin D (E), alkaline phosphatase (F) and osteocalcin (G), in gilts (dotted grey line with blank circle items) and barrows (dotted black with blank square items) fed the control (Con) diet and gilts (grey line with grey circle items) and barrows (black line with black square items) fed the diet with phytase (Phy). Significant effects ($P \leq 0.05$) of sex are indicated by the symbol *, effects of phytase by the symbol †, and the interaction of sex and phytase by the symbol ‡.
Table 1. Ingredients and analyzed nutrient composition of experimental diets

| Dietary treatment<sup>a</sup> | Con  | Phy  |
|------------------------------|------|------|
| Ingredient, %                |      |      |
| Barley                       | 45.06| 45.00|
| Wheat, 11% CP                | 35.51| 35.46|
| Soybean meal HP, 47% CP      | 8.11 | 8.10 |
| Soybean meal, 42% CP         | 7.01 | 7.00 |
| Calcium carbonate            | 1.28 | 1.28 |
| Rapeseed oil                 | 1.00 | 1.00 |
| Monocalcium phosphate        | 0.46 | 0.46 |
| Salt                         | 0.46 | 0.46 |
| Lysine-HCL 98                | 0.41 | 0.41 |
| Vitamin-mineral-premix<sup>b, c</sup> | 0.39 | 0.39 |
| L-Threonine                  | 0.13 | 0.13 |
| Magnesium oxide              | 0.10 | 0.10 |
| DL-Methionine                | 0.08 | 0.08 |
| Phytase, FTU/kg diet<sup>d</sup> | -   | 650  |

Analyzed nutrient content, g/kg DM

|                        | Con  | Phy  |
|------------------------|------|------|
| Dry matter, g/kg       | 893  | 893  |
| Crude ash              | 46.7 | 47.4 |
| Acid insoluble ash     | 3.63 | 3.61 |
| Crude protein          | 165  | 165  |
| Crude fiber            | 33.9 | 35.7 |
| Neutral-detergent fiber| 125  | 125  |
| Acid-detergent fiber   | 50.5 | 50.3 |
| Crude fat              | 33.0 | 33.1 |
| Nitrogen-free extract  | 618  | 617  |
| Total starch           | 450  | 457  |
| Resistant starch       | 3.07 | 3.07 |
| Non-resistant starch   | 437  | 437  |
| Phosphorus             | 5.68 | 5.59 |
| Available phosphorus   | 2.95 | 3.68 |
| Calcium                | 7.48 | 7.50 |
| Magnesium              | 2.70 | 2.70 |
| Sodium                 | 1.86 | 1.82 |
| Phytase, FTU/kg diet   | 257  | 596  |

Calculated energy content

|                        | Con  | Phy  |
|------------------------|------|------|
| Metabolizable Energy, MJ/kg | 15.2 | 15.1 |

<sup>a</sup>Con, control; Phy, phytase; FTU: phytase units.
The Vitamin-Mineral-Premix without phytase provided per kilogram of experimental diet (Garant-Tiernahrung GmbH, Pöchlarn, Austria): 6510 IU of vitamin A, 2003 IU of vitamin D3, 1561IU of vitamin E, 3.01mg of vitamin K3, 1.50mg of vitamin B1, 4.01mg of vitamin B2, 20.03mg of vitamin B3, 2.00mg of vitamin B6, 0.02mg vitamin of B12, 10.02mg pantothenic acid, 0.50mg of folic acid, 0.05mg of biotin, 1242.04mg of choline, 132.17mg of choline chloride, 160.46mg of Fe, 21.57mg of Cu, 122.06mg of Zn, 67.36mg of Mn, 0.86mg of Mo, 1.72mg of J, 0.10mg of Co, 0.57mg of Se.

The Vitamin-Mineral-Premix with phytase provided per kilogram of experimental diet (Garant-Tiernahrung GmbH, Pöchlarn, Austria): 6502IU of vitamin A, 2001IU of vitamin D3, 1561IU of vitamin E, 3.00mg of vitamin K3, 1.50mg of vitamin B1, 4.00mg of vitamin B2, 20.01mg of vitamin B3, 2.00mg of vitamin B6, 0.02mg of vitamin B12, 10.00mg of pantothenic acid, 0.50mg of folic acid, 0.05mg of biotin, 1240.43mg of choline, 132.00mg of choline chloride, 160.25mg of Fe, 21.54mg of Cu, 121.90mg of Zn, 67.27mg of Mn, 0.86mg of Mo, 1.72mg of J, 0.10mg of Co, 0.57mg of Se.

Phytase (VM Phytase XP 897420, Garant-Tiernahrung GmbH, Pöchlarn, Austria).
Table 2. Effect of dietary phytase and sex on body weight, body weight gain, and gain:feed ratio in growing pigs during the fattening period

| Dietary treatment<sup>a</sup> | Con | Phy | P – Value<sup>b</sup> |
|-------------------------------|-----|-----|-----------------------|
|                               | Gilts | Barrows | Gilts | Barrows | SEM | Phytase | Sex | Phytase × Sex |
| Body weight, kg               |     |     |     |     |    |         |     |            |
| Day 1                         | 36.2 | 38.7 | 38.9 | 40.6 | 0.80 | 0.005 | 0.009 | 0.549      |
| Day 8                         | 45.1 | 45.3 | 44.9 | 45.2 | 0.25 | 0.520 | 0.306 | 0.957      |
| Day 22                        | 57.5 | 59.8 | 56.8 | 59.4 | 0.56 | 0.314 | <0.001 | 0.816      |
| Day 35                        | 69.3 | 73.3 | 68.6 | 71.4 | 0.75 | 0.098 | <0.001 | 0.487      |
| Day 49                        | 83.7 | 86.7 | 82.9 | 84.8 | 0.98 | 0.193 | 0.016 | 0.624      |
| Average daily gain, kg/d      |     |     |     |     |    |         |     |            |
| Day 1-49                      | 0.94 | 1.00 | 0.92 | 0.96 | 0.021 | 0.185 | 0.014 | 0.602      |
| Day 1-8                       | 0.96 | 0.99 | 0.93 | 0.98 | 0.036 | 0.554 | 0.278 | 0.935      |
| Day 9-22                      | 0.89 | 1.04 | 0.86 | 1.03 | 0.031 | 0.384 | <0.001 | 0.748      |
| Day 23-35                     | 0.87 | 1.00 | 0.87 | 0.89 | 0.056 | 0.348 | 0.215 | 0.379      |
| Day 36-49                     | 1.00 | 0.92 | 0.99 | 0.93 | 0.059 | 0.969 | 0.262 | 0.942      |
| Average daily feed intake, kg/d|     |     |     |     |    |         |     |            |
| Day 1-49                      | 2.04 | 2.36 | 1.92 | 2.17 | 0.033 | <0.001 | <0.001 | 0.349      |
| Day 1-8                       | 1.75 | 1.87 | 1.67 | 1.84 | 0.051 | 0.276 | 0.009 | 0.651      |
| Day 9-22                      | 1.91 | 2.23 | 1.78 | 2.08 | 0.039 | 0.001 | <0.001 | 0.779      |
| Day 23-35                     | 2.04 | 2.41 | 1.98 | 2.22 | 0.045 | 0.010 | <0.001 | 0.209      |
| Day 36-49                     | 2.31 | 2.68 | 2.11 | 2.37 | 0.066 | <0.001 | <0.001 | 0.429      |
| Gain:feed ratio               |     |     |     |     |    |         |     |            |
| Day 1-49                      | 0.46 | 0.42 | 0.48 | 0.44 | 0.008 | 0.026 | <0.001 | 0.996      |
| Day 1-8                       | 0.55 | 0.54 | 0.55 | 0.53 | 0.015 | 0.738 | 0.475 | 0.833      |
| Day 9-22                      | 0.48 | 0.46 | 0.49 | 0.49 | 0.009 | 0.079 | 0.182 | 0.563      |
| Day 23-35                     | 0.44 | 0.43 | 0.45 | 0.44 | 0.010 | 0.499 | 0.221 | 0.724      |
| Day 36-49                     | 0.40 | 0.37 | 0.44 | 0.42 | 0.011 | <0.001 | 0.010 | 0.734      |

<sup>a</sup>Con, control diet; Phy, phytase diet; Values are presented as least square means with the standard error of the mean.

<sup>b</sup>Fixed effect of days was significant (P < 0.001) for all parameters.
Table 3. Pearson’s correlation between calcium (Ca), total phosphorus (tP) and available phosphorus (aP), ratio of calcium to total phosphorus (Daily Ca:tP ratio) and ratio of calcium to available phosphorus (Daily Ca:aP ratio) and serum phosphorus, serum calcium, serum calcium to phosphorus ratio, fibroblast growth factor 23, vitamin D, alkaline phosphatase, and osteocalcin in gilts and barrows.

| Item                  | Week | tP intake, g/d | aP intake, g/d | Ca intake, g/d | Daily Ca:tP ratio | Daily Ca:aP ratio | Barrows |
|-----------------------|------|----------------|----------------|----------------|-------------------|-------------------|---------|
| Serum P, mmol/L       | 1    | 0.24           | 0.58*          | 0.27           | 0.76*             | -0.76*            | 0.16    |
|                       | 4    | 0.12           | 0.32           | 0.15           | 0.33              | -0.33             | 0.31    |
|                       | 8    | 0.41*          | 0.2            | 0.39*          | -0.25             | 0.25              | 0.39*   |
| Serum Ca, mmol/L      | 1    | 0.09           | -0.22          | 0.06           | -0.58*            | 0.58*             | 0.31    |
|                       | 4    | 0.06           | -0.09          | 0.05           | -0.17             | 0.17              | 0.27    |
|                       | 8    | 0.57*          | 0.50*          | 0.58*          | 0.01              | -0.01             | 0.46*   |
| Serum Ca:P ratio      | 1    | -0.17          | -0.57*         | -0.21          | -0.86*            | 0.86*             | -0.01   |
|                       | 4    | -0.12          | -0.41*         | -0.16          | -0.46*            | 0.46*             | -0.19   |
|                       | 8    | -0.05          | 0.1            | -0.04          | 0.25              | -0.25             | -0.23   |
| Serum FGF23, pg/mL    | 1    | 0.11           | -0.03          | 0.1            | -0.22             | 0.22              | -0.27   |
|                       | 4    | -0.23          | -0.13          | -0.22          | 0.02              | -0.02             | 0.13    |
|                       | 8    | -0.14          | 0.01           | -0.13          | 0.2               | -0.2              | -0.07   |
| Serum VitD, ng/mL     | 1    | -0.12          | -0.08          | -0.12          | 0.07              | -0.07             | 0.12    |
|                       | 4    | 0.12           | 0.05           | 0.12           | -0.04             | 0.04              | 0.14    |
|                       | 8    | -0.48*         | -0.50*         | -0.49*         | -0.11             | 0.11              | -0.51*  |
| Serum ALP, U/L        | 1    | -0.47*         | -0.47*         | -0.47*         | -0.16             | 0.16              | -0.12   |
|                       | 4    | -0.06          | 0.06           | -0.05          | 0.16              | -0.16             | -0.02   |

Note: Asterisks indicate significant correlations.
| Serum OCN, ng/mL | 8 | 0.32 | 0.44* | 0.34 | 0.21 | -0.21 | 0.32 | 0.31 | 0.32 | 0 | 0 |
|------------------|---|------|-------|------|------|-------|------|------|------|---|---|
| 1                | 0.60* | 0.45* | 0.59* | -0.11 | 0.11 | 0.50* | 0.29 | 0.50* | -0.19 | 0.19 |
| 4                | 0.11 | 0.25 | 0.13 | 0.24 | -0.24 | 0.04 | 0.12 | 0.05 | 0.11 | -0.11 |
| 8                | 0.15 | 0.2 | 0.15 | 0.18 | -0.18 | 0.15 | 0.27 | 0.16 | 0.2 | -0.2 |

*Serum P, serum phosphorus; Serum Ca, serum calcium; FGF23, fibroblast growth factor 23; VitD, vitamin D; ALP, alkaline phosphatase; OCN, osteocalcin

Week 1: Correlation between calcium (Ca), total phosphorus (tP) and available phosphorus (aP) intake from days 1-7 and serum parameters from days 2+3 (in week 1); Week 4: Correlation between calcium (Ca), total phosphorus (tP) and available phosphorus (aP) intake from days 22-28 and serum parameters 23+24 (in week 4); Week 8: Correlation between calcium (Ca), total phosphorus (tP) and available phosphorus (aP) intake from days 50-56 and serum parameters days 52+53 (in week 8).

*| > 0.35 and P < 0.05.
Figure 1

A) IP intake, g/d

B) 4P intake, g/d

C) Ca intake, g/d

D) CuAP intake, g/d

Day 1-8, Day 9-22, Day 23-35, Day 36-49
