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Genetic background influences metabolic response to dietary phosphorus restriction☆

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Abstract

Dietary phosphorus (P) is essential to bone growth and turnover; however, little research has focused on the genetic mechanisms controlling P utilization. Understanding the interactions between genetics and dietary P that optimize bone integrity could provide novel interventions for osteoporosis. Thirty-six pigs from two sire lines known to differ in bone structure [heavier boned (HB) and lighter boned (LB)] were assigned to one of the three diets (P adequate, P repletion or P deficient). After 14 days, bone marrow and intact radial bones were collected. Differences between these lines in growth rate, bone integrity and gene expression within bone marrow were observed. In HB, but not LB, pigs, the P-deficient diet decreased weight gain ($P<.01$). For both lines, P deficiency caused a reduction in radial bone strength ($P<.01$), but HB P-deficient animals had greater ($P<.10$) bone integrity than P-deficient LB pigs. In HB, but not LB, pigs, dietary treatment affected the expression of *CALCR* (calcitonin receptor) ($P<.05$), *VDR* (vitamin D receptor) ($P<.04$) and *IGFBP3* (insulin-like growth factor binding protein 3) ($P<.06$). There was also a trend of increased *IL6* (interleukin-6), *TFIIB* (transcription initiation factor IIB) and *SOX9* (sex determining region Y-box 9) expression with P deficiency in HB, but not LB, pigs. Both genetic backgrounds responded similarly to P deficiency with an increase in the expression of *OXTR* (oxytocin receptor) and *IGF1* (insulin-like growth factor 1). Differences in growth rate, bone integrity and gene expression within the bone marrow suggest a difference in the homeorhetic control of P utilization between these genetic lines. Understanding these differences could lead to novel treatments for osteoporosis and aid in the development of tests for identifying those at risk for this disease.

Keywords: Bone; Gene expression; Phosphorous; Pigs; Osteoporosis

1. Introduction

Bone mineral turnover occurs throughout life, with rates of deposition exceeding rates of resorption during growth and adolescence. Peak bone mineral density (BMD) in humans occurs relatively early in life with nearly 90% of maximum skeletal mass accumulated by the age of 18 years [1]. Because BMD is used to diagnose osteoporosis, the lifetime risk of developing osteoporosis may be established during the first few decades of life. Acquisition of peak bone mass during growth depends on a variety of factors, including nutrition and genetics [2,3].

Although most nutritional studies on bone development have focused on calcium (Ca), phosphorus (P) is also essential in supporting bone growth and turnover. Phosphorus deficiency can be caused by major alterations in energy metabolism, particularly related to weight reduction programs [4] or the consumption of vegetarian diets in which a majority of the P is present as phytic acid (which is of limited biological value) [5]. A genetic contribution to the acquisition of peak bone mass and the etiology of osteoporosis has also been recognized [2]. Genetic linkage studies [6,7] and candidate gene association studies [8,9] have implicated several loci and candidate genes in the regulation of bone mass and the pathogenesis of osteoporotic fractures.

Understanding the interactions between genetics and diet that optimize bone integrity could provide novel interventions for the prevention of osteoporosis. The objective of this study was to determine the effect of the interactions between dietary P and genetic background on growth performance,

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bone strength and gene expression in bone marrow. In this study, we used rapidly growing young pigs from two different genetic backgrounds known to differ in gross bone conformation. Similar differences in bone conformation have been seen in humans between racial groups. The phenotypic differences seen between the two lines of pigs utilized allowed us to examine P deficiency×Genetic interactions between genetically divergent populations.

2. Materials and methods

2.1. Animals

All animal protocols were approved by Iowa State University's Committee on Animal Care. Thirty-six female piglets of two genetic lines were obtained by crossing two sire lines with a single female line. These two sire lines are known to differ in bone structure, with one considered heavier boned (HB) and the other considered lighter boned (LB). The HB line is of Duroc origin and the LB line is a true hybrid line with at least four different breeds contributing to its formation. All breeding females and semen from the two sire lines were purchased from Pig Improvement Company (Franklin, KY). At 21 days of age, the piglets were fed a basal diet that met or exceeded their nutritional requirements [10] for 7 days prior to study initiation. At the initiation of the study, animals were assigned to one of the three treatments based on genetic background and initial body weight. These treatments consisted of being fed either a P-adequate diet, a P-deficient diet or a P-deficient diet for 7 days followed by the P-adequate diet for 7 days (P-repletion diet). All diet compositions and analyzed Ca and P levels are listed in Table 1. Animals were housed individually in 0.43×1.22-m pens in environmentally controlled rooms. Body weights were determined initially and weekly throughout the trial, and average daily gain (ADG) was calculated for each week individually and for the 2-week trial period. Individual feed consumption was recorded weekly and used to calculate feed/gain ratio for each week and across the trial period.

Table 1
Composition of diets, as-fed basis

Ingredient	Diet: Adjustment (%)	P adequate (%)	P deficient (%)
Corn	66	66	66.65
Soybean meal, 48% CP	23	23	23
Spray-dried plasma protein	5	5	5
Dicalcium phosphate	1.5	1.5	0
Limestone	1.2	1.2	2.05
L-Lysine–HCl	0.15	0.15	0.15
DL-Methionine	0.05	0.05	0.05
Corn Oil	2	2	2
Vitamin/mineral premix[a]	0.35	0.35	0.35
Salt	0.5	0.5	0.5
Nutritive values[b]			
Crude protein	20.3	20.3	20.4
Lysine, available	1.17	1.17	1.17
Methionine, available	0.31	0.31	0.31
Ca_{total}	0.88	0.88	0.87
P_{total}	0.71	0.71	0.43
$P_{available}$	0.41	0.41	0.14
Ca_{total}, analyzed[c]	0.95	0.91	1.0
P_{total}, analyzed[c]	0.65	0.64	0.40

[a] Provided vitamins and minerals to meet or exceed all requirements for pigs of this size [10].
[b] Calculated based on NRC (1998), Nutrient Requirements of Swine.
[c] Total mineral content analysis performed by Eurofins Scientific, Des Moines, IA.

At days 0, 7 and 14 of the study, venous blood samples were collected after an overnight fast into Vacutainer Plus tubes containing sodium heparin (BD Vacutainer, Franklin Lakes, NJ) by venipuncture. Plasma was obtained by centrifugation at 2000×*g* and 4°C in a Kendro SuperT21 Centrifuge (Kendro, Newton, CT) and stored at −20°C until used for analysis. On day 14 of the study, after collecting blood samples and recording body weight, all animals were euthanized by captive bolt. Intact radial and metacarpal bones were collected, placed in individual plastic bags and stored at 4°C. Bone marrow samples were collected from the proximal end of the right humerus and snap frozen in liquid nitrogen. These samples were stored at −80°C until RNA extraction.

2.2. Biochemical analysis

Calcium concentration, alkaline phosphatase (AKP) activity and inorganic P concentration was determined for all plasma samples. Plasma Ca levels were determined using a Cole-Palmer Digital Flame Analyzer, model 2655-00 (Cole-Palmer Instrument, Chicago, IL). Alkaline phosphatase activity was assayed by the method of Bowers and McComb [11] in which the rate of formation of yellow-colored structures by the hydrolysis of *p*-nitrophenol phosphate to *p*-nitrophenol is proportional to the level of AKP activity in the plasma. The rate of appearance of this yellow color was determined at 700 nm in a PowerWave HT microplate scanning spectrophotometer (Bio-Tek, Winooski, VT). Inorganic P concentrations were determined by the method of Gomori [12] modified for use with a microplate spectrophotometer. Briefly, plasma was deproteinated with 12.5% trichloroacetic acid and assayed using Elon solution (*p*-methylaminophenol sulfate).

2.3. Bone measurements

The radii were manually cleaned of all soft tissue and tested for tensile strength using a 5-kN Flexure Fixture, configured for three-point bend tests, attached to an Instron Universal Testing Machine Model 4502 equipped with a 10-kN load cell (Instron, Canton, MA). Load applied at bone breaking was determined using Series IX, v 8.08.00 software (Instron). Metacarpals were stored at −20°C until analysis for bone ash. Ash determination was made following an ether extraction by drying the metacarpals at 70°C for 48 h and then ashing the bones in a muffle furnace at 700°C for 48 h.

Table 2
Primers used for quantification of gene expression by real-time PCR

Gene name	Primer sequences for real-time PCR[a]	GenBank accession number
60S ribosomal protein (*RPL35*)	I-AACCAGACCCAGAAAGAGAAC II-TTCCGCTGCTGCTTCTTG	AY550044
Calcitonin receptor (*CALCR*)	I-TGCTCATGCCATTACTAGGGCAGT II-ATGACAGGGCCGTGGATGATGTAA	NM_214354
Vitamin D receptor (*VDR*)	I-TTGCCAAACACCTCAAGCACAAGG II-TGCTCTACGCCAAGATGATCCAGA	AJ606306
Insulin-like growth factor binding protein 3 (*IGFBP3*)	I-ACTCCACTCTATCCACACCAAGATG II-GTCCGTGCTCTGAGACTCGTAG	AF085482
Interleukin 6 (*IL6*)	I-ACAGCAAGGAGGTACTGGCAGAAA II-AAGCAGGTCTCCTGATTGAACCCA	AF518322
Transcription initiation factor IIB (*TFIIB*)	I-TGAATGTGGCCTGGTTGTAGGTGA II-TCCGGTACCCTTGCCAATCATAGT	NM_001514
SRY (sex determining region Y)-box 9 (*Sox-9*)	I-AGAAGGAGAGCGAAGAGGACAAGT II-CTTGACGTGCGGCTTATTCTTGCT	AF029696
Oxytocin receptor (*OXTR*)	I-CATGAACTTGTGCAGCGCTTCCTT II-AGACAAAGGTGGACGAGTTGCTCT	X71796
Insulin-like growth factor 1 (*IGF1*)	I-TTCGCATCTCTTCTACTTGGCCCT II-CGTACCCTGTGGGCTTGTTGAAAT	NM_214256
Receptor activator of nuclear factor-kappaB ligand (*RANKL*)	I-TGGATCACAGCACATCAGAGCAGA II-TGGTACCAAGAGGACAGACTCACT	NM_003701
Osteoprotegerin (*OPG*)	I-AACGGCAACACAGCTCACAAGAAC II-TGCTCGAAGGTGAGGTTAGCATGT	BC030155
Osteocalcin (*BGLAP*)	I-CTACCCAGATCCTCTGGAGCCC II-TATGCCATAGAAGCGCCGATAG	AY150038
A-Raf-1 (*ARAF1*)	I-ACGAGATGCAAGTGCTCAGGAAGA II-GCACCACTGTGTGATGATGGCAAA	NM_214329
Insulin-like growth factor binding protein 5 (*IGFBP5*)	I-AGCAAGCCAAGATCGAGAGAGACT II-TCAGCTTCTTTCTGCGGTCCTTCT	NM_214099

[a] All primers listed in the 5′ to 3′ direction. I is the forward primer, II is the reverse primer.

2.4. RNA isolation and cDNA synthesis

RNA isolation from 30 to 70 mg of bone marrow was performed using the Ambion RNAqueous kit (Ambion, Austin, TX). Extracted total RNA was treated with deoxyribonuclease I to eliminate genomic DNA (Ambion DNA-free kit) and then reverse transcribed with Superscript II (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacture's instructions. The cDNA was then incubated for 20 min at 37°C with *Escherichia coli* RNase H and stored at −80°C until analysis by real-time PCR.

2.5. Real-time PCR

In order to measure specific transcript levels, semiquantitative real-time PCR was performed using the MyiQ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primer oligonucleotides (Table 2) were designed using "PrimerQuest" software available from Integrated DNA Technologies (Coralville, IA). Reactions were performed using 12.5 μl of 2× SYBR Green Supermix (Bio-Rad), 50–900 nM of each primer and 100 ng of the above-described cDNA reaction in a final volume of 25 μl.

Table 3
Effect of dietary P and genetic background on growth performance of young pigs

	Body weight initial (kg)	S.E.	Average daily weight gain (kg)	S.E.	Average daily feed intake (kg)	S.E.	Feed/gain	S.E.
LB line								
P+	6.24	0.30	$0.28^{y,*}$	0.035	0.40	0.034	1.46^{y}	0.059
RP	6.23	0.21	0.34^{x}	0.035	0.47	0.034	1.41^{y}	0.059
P−	6.41	0.22	0.28^{y}	0.034	0.45	0.034	$1.62^{x,*}$	0.059
HB line								
P+	6.92	0.44	$0.36^{a,*}$	0.033	0.51	0.032	1.45^{y}	0.057
RP	7.12	0.39	0.35^{a}	0.034	0.51	0.032	1.49^{y}	0.057
P−	6.85	0.20	0.25^{b}	0.032	0.45	0.032	$1.84^{x,*}$	0.057

Values are means and S.E.'s of six individually housed pigs. Average daily weight gain and average daily feed intake were analyzed using initial BW as a covariate.

[a,b] Means without a common superscript are different ($P<.01$).

[x,y] Means without a common superscript are different ($P<.1$).

* Means between sire lines are different ($P<.1$).

Table 4
Effect of genetic background and diet on plasma indicators of P status

	Initial			Week 1			Week 2		
	PIP (mg/L)	Ca (mM)	AKP (U/L)	PIP (mg/L)	Ca (mM)	AKP (U/L)	PIP (mg/L)	Ca (mM)	AKP (U/L)
LB line									
P+	76 (4.3)	3.37 (0.05)	117 (10.3)	71^{a} (5.9)	3.17 (0.04)	67^{b} (5.8)	83^{a} (4.6)	3.63 (0.29)	77^{b} (8.2)
RP	80 (4.0)	3.43 (0.12)	116 (10.3)	51^{b} (3.5)	3.20 (0.06)	84^{a} (5.8)	76^{a} (3.2)	3.37 (0.08)	76^{b} (8.2)
P−	78 (2.5)	3.18 (0.10)	116 (10.3)	44^{b} (5.0)	3.23 (0.01)	84^{a} (5.8)	53^{b} (6.3)	3.41 (0.12)	114^{a} (8.2)
HB line									
P+	74 (5.3)	3.39 (0.06)	100 (10.2)	65^{a} (3.4)	3.23 (0.05)	64^{b} (5.6)	83^{a} (3.1)	3.61 (0.15)	80^{b} (8.0)
RP	74 (3.4)	3.27 (0.06)	99 (10.2)	44^{b} (2.7)	3.28 (0.03)	75^{a} (5.6)	87^{a} (2.3)	3.43 (0.09)	72^{b} (8.0)
P−	70 (4.9)	3.35 (0.04)	91 (10.2)	39^{b} (2.8)	3.02 (0.16)	83^{a} (5.6)	51^{b} (4.5)	3.40 (0.05)	111^{a} (8.0)

Values are means and S.E.'s of six individually housed pigs.
[a,b] Means without a common superscript are different ($P<.01$).

Prior to quantification by real-time PCR, optimal primer concentrations for each primer set were determined, and we verified that the linearity of amplification for each gene of interest was similar to that of the control gene, 60S ribosomal RNA (RPL35), which we verified to not be affected by treatment or genetic background. All amplified products were sequenced to confirm their identities. The thermal cycling conditions allowed for 45 cycles of 30 s of melting at 95°C followed by 30 s of annealing and extension at 60°C. After the 45 amplification cycles, all samples were subjected to a melt curve analysis in which they were heated at 1°C/30-s increments from 60°C to 94°C to validate the absence of nonspecific products. Normalized gene expression is presented using the $2^{-\Delta Ct}$ method [13]. The cDNA samples from two pigs, one for the HB P-adequate group and the other from the HB P-repletion group, were excluded in this analysis due to inadequate RNA quality.

2.6. Statistics

Data were analyzed using the PROC MIXED procedures of SAS (SAS Institute, Cary, NC) [14] with genetic background (sire line), treatment and interaction between genetic background and treatment considered as fixed effects. The effect of sow nested within sire line was treated as a random effect. For ADG, average daily feed intake and feed/gain values, initial body weight was considered as a covariate in the model. For bone strength and bone ash values, body weight at week 2 was considered as a covariate in the model. Means and S.E.'s are presented. Significance was set at an α value of .1, whereas differences described as trends were identified at an α value of .2.

3. Results

3.1. Growth performance

Among the HB pigs, the P-deficient group had significantly ($P<.01$) lower ADG than the other two treatment groups (Table 3). However, the LB P-deficient pigs did not exhibit any growth depression when compared to P-adequate pigs, and the P-repletion group tended to have higher ($P<.1$) ADG than either of the other two treatment groups. In addition, the HB P-adequate pigs exhibited significantly ($P<.1$) higher ADG than the LB P-adequate animals. There were no significant differences in feed intake between the sire lines or treatment groups; however, P-deficient animals had higher ($P<.1$) feed/gain than animals in the other dietary treatments. The HB P-deficient pigs also had higher ($P<.05$) feed/gain than the LB P-deficient animals.

3.2. Plasma assays

Plasma Ca concentrations were not affected by dietary treatment, genetic background or their interaction (Table 4).

Table 5
Genetic background and dietary P influence bone strength and ash percentage

	Body weight final (kg)	S.E.	Radial Strength (kN)	S.E.	Metacarpal ash %	S.E.
LB line						
P+	10.13	0.53	0.477^{a}	0.0258	37.1^{a}	0.78
RP	10.93	0.24	0.429^{b}	0.0250	34.8^{b}	0.76
P−	10.34	0.39	$0.253^{c,*}$	0.0254	27.8^{c}	0.77
HB line						
P+	11.97	0.78	0.576^{a}	0.0281	36.9^{a}	0.80
RP	12.02	0.62	0.498^{b}	0.0269	33.4^{b}	0.80
P−	10.36	0.52	$0.319^{c,*}$	0.0254	26.4^{c}	0.77

Values are means and S.E.'s of six individually housed pigs.
[a,b,c] Means without a common superscript are different ($P<.01$).
* Means between sire lines are different ($P<.1$).

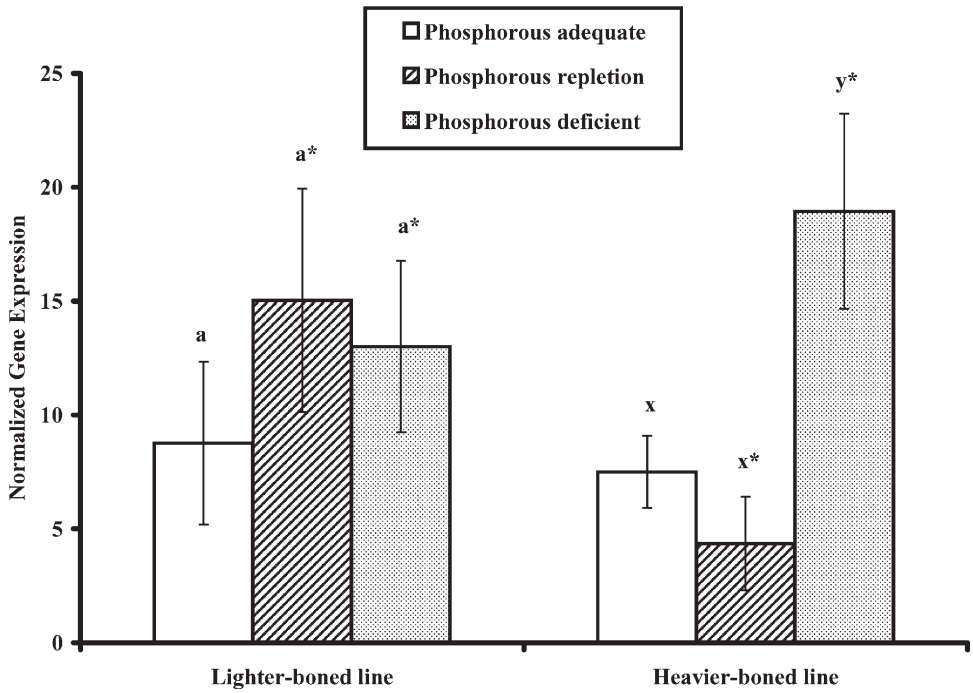

Fig. 1. Effect of dietary P and genetic background interactions on calcitonin receptor gene expression in bone marrow. $n=6$, except for HB P adequate and P repletion where $n=5$. Within sire line, columns not sharing a common letter are significantly different ($P<.05$). Significant differences ($P<.08$) within treatment between sire lines are indicated by an asterisk (*).

Initially, there were no differences in the plasma inorganic phosphorous (PIP) level or AKP activity between any of the treatment groups. After 7 days on treatment diets, pigs receiving the P-adequate diet had higher ($P<.01$) PIP and lower ($P<.01$) AKP than those receiving the P-deficient diets. At the completion of the study, the P-deficient groups had lower ($P<.01$) PIP and higher ($P<.01$) AKP than the other treatments groups, and the P-repletion groups were able to achieve PIP and AKP similar to those of the P-adequate groups. There were no genetic background effects or Genetic background×Treatment interactions for any of these plasma indicators of P status.

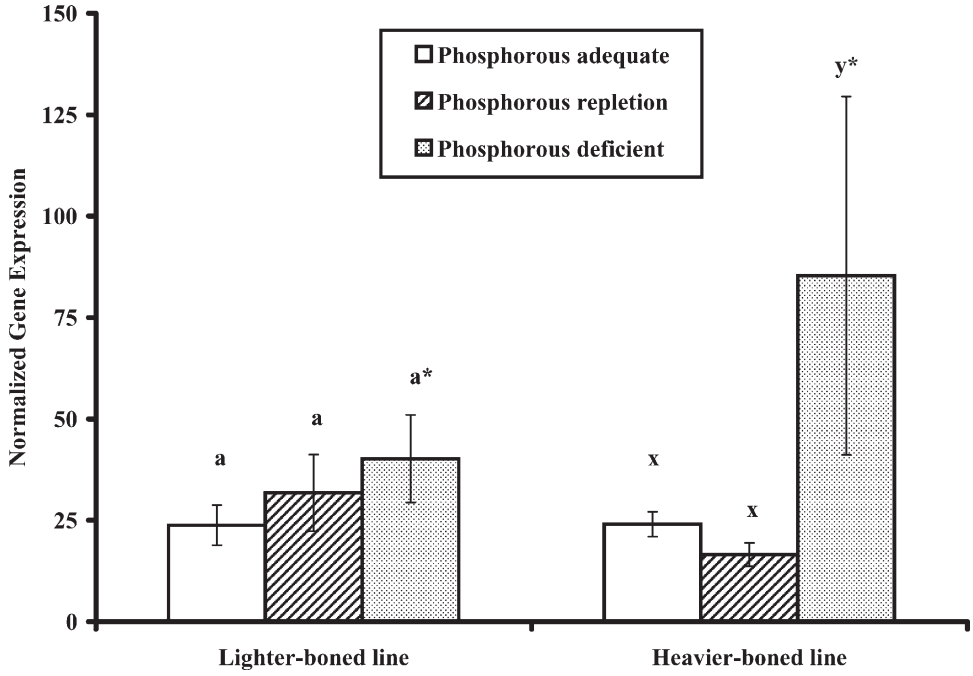

Fig. 2. Vitamin D receptor gene expression. $n=6$, except for HB P adequate and P repletion where $n=5$. Within sire line, columns not sharing a common letter are significantly different ($P<.06$). Significant differences ($P<.05$) within treatment between sire lines are indicated by an asterisk (*).

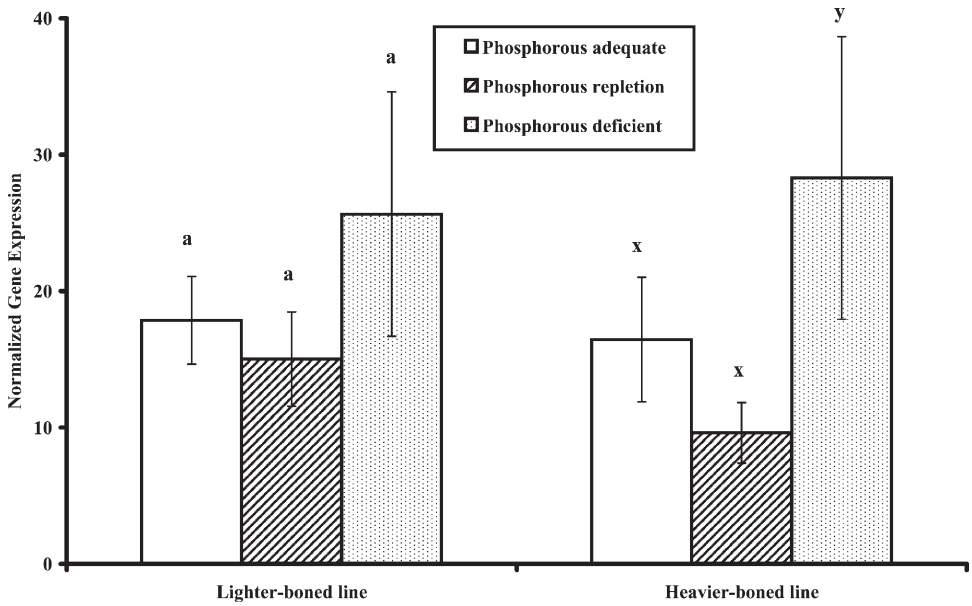

Fig. 3. Insulin-like growth factor 1 gene expression. $n=6$ except for HB P adequate and P repletion where $n=5$. Within sire line, columns not sharing a common letter are significantly different ($P<.11$). In the LB pigs, there was a trend of increased transcript concentration in the P-deficient pigs compared to the other treatment groups.

3.3. Bone measurements

Bone strength and ash percentage were affected by dietary treatment in pigs from both genetic backgrounds (Table 5). The radial strength and metacarpal ash percentage of the P-deficient pigs were significantly lower ($P<.01$) relative to the other two treatment groups. In addition, there was a significant ($P<.1$) Genetic background×Treatment interaction in bone strength among the P-deficient pigs. The P-deficient HB pigs exhibited stronger radial bones than the LB P-deficient pigs. We did not observe any Genetic background×Treatment interactions for metacarpal ash percentage.

3.4. Real-time PCR

3.4.1. Calcitonin receptor

Levels of *CALCR* (calcitonin receptor) mRNA were significantly affected by dietary treatments among the HB, but not LB, pigs (Fig. 1). The P-deficient HB pigs had increased levels of *CALCR* message when compared to both the P-adequate and P-repletion groups ($P<.05$ and .02, respectively). The P-deficient HB pigs also had significantly higher ($P<.08$) concentrations of *CALCR* transcript than the P-deficient LB pigs. Additionally, the HB P-repletion pigs had significantly lower ($P<.05$) *CALCR* mRNA levels than

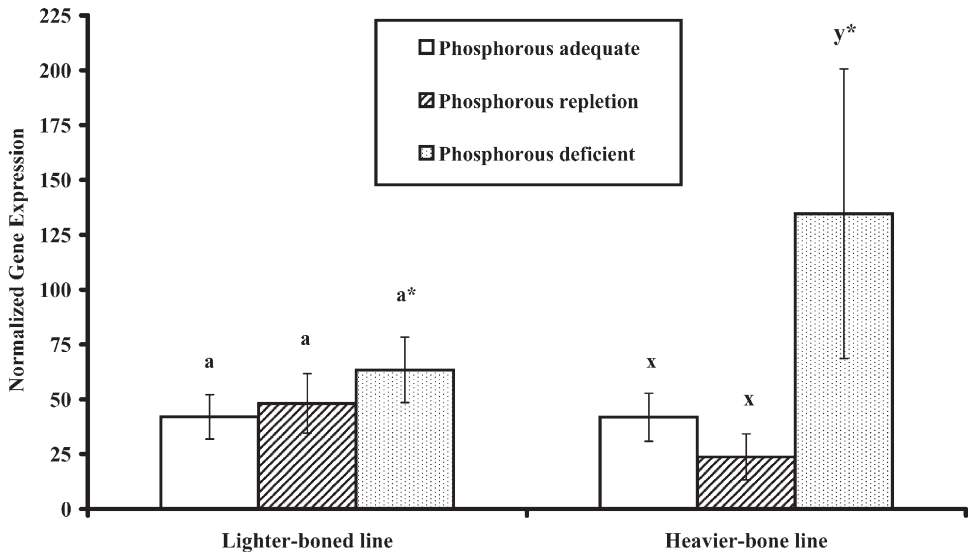

Fig. 4. Insulin-like growth factor binding protein 3 gene expression. $n=6$ except for HB P-adequate and P repletion where $n=5$. Within sire line, columns not sharing a common letter are significantly different ($P<.06$). Significant differences ($P<.07$) within treatment between sire lines are indicated by an asterisk (*).

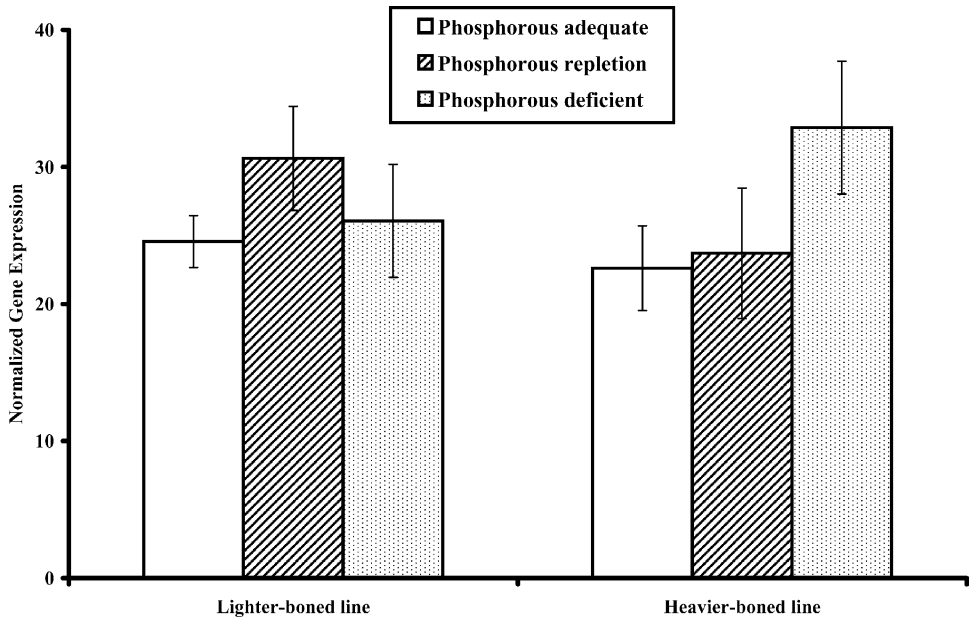

Fig. 5. Transcription initiation factor IIB gene expression. $n=6$ except for HB P adequate and P repletion where $n=5$. No significant differences. However, in the HB line, there was a trend of increased *TFIIB* expression in the P− pigs when compared to the other two treatment groups.

the LB P-repletion group. Although the level of *CALCR* mRNA was affected by the interactions between genetic background and dietary treatment in both the P-deficient and repletion groups, it was not affected by these interactions in the P-adequate group.

3.4.2. Vitamin D receptor

Levels of *VDR* (vitamin D receptor) mRNA were not affected by dietary treatments in LB animals; however, in the HB pigs, P deficiency caused a significant increase in *VDR* mRNA compared to the P-adequate and the P-repletion groups ($P<.06$ and .04, respectively) (Fig. 2). The P-deficient HB pigs also had higher ($P<.05$) concentrations of *VDR* transcript than the P-deficient LB pigs. This interaction between genetic background and dietary treatment affecting the level of *VDR* message in the P-deficient animals was not seen in the other dietary treatments.

3.4.3. Insulin-like growth factor 1 and insulin-like growth factor binding protein 3

In the LB line, the amount of *IGF1* (insulin-like growth factor 1) and *IGFBP3* (insulin-like growth factor binding

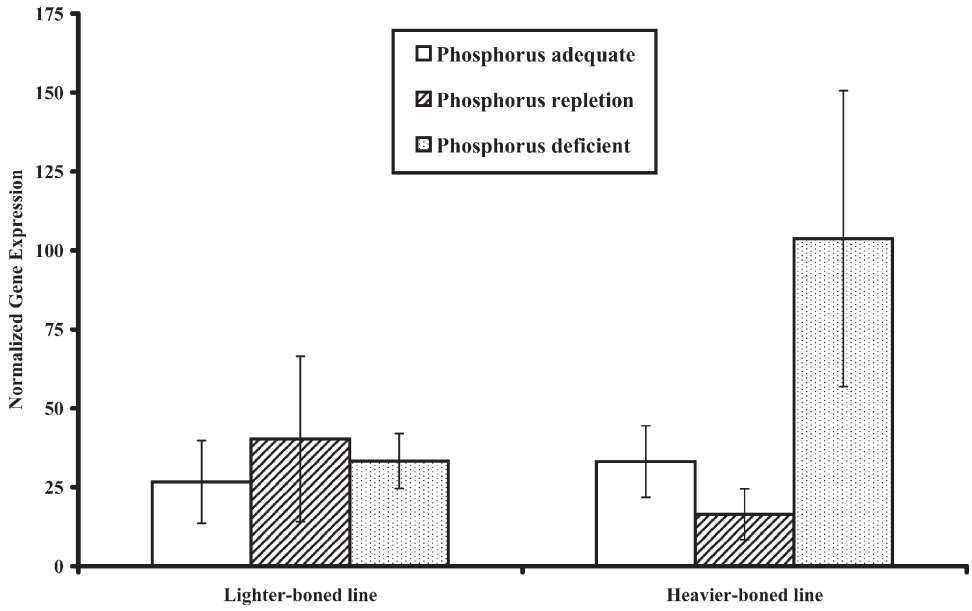

Fig. 6. SRY (sex determining region Y)-box 9 gene expression. $n=6$ except for HB P adequate and P repletion where $n=5$. No significant differences. However, in the HB line, there was a trend of increased *Sox-9* expression in the P− pigs when compared to the other two treatment groups.

protein 3) messages in bone marrow was not affected by dietary treatment (Figs. 3 and 4). However, the P-deficient HB pigs had increased levels of *IGF1* message when compared to both the P-adequate and P-repletion groups ($P<.11$ and .02, respectively). The P-deficient HB pigs also had increased levels of *IGFBP3* transcript compared to the P-adequate and P-repletion groups ($P<.06$ and .03, respectively). These animals also had higher ($P<.07$) levels of *IGFBP3* transcript than the LB P-deficient animals.

3.4.4. Oxytocin receptor

Levels of *OXTR* (oxytocin receptor) mRNA in the bone marrow were not significantly affected by any dietary treatments or genetic background. However, there was a trend ($P<.16$) of increased transcript concentration in the P-deficient pigs compared to the other treatment groups.

3.4.5. Interleukin-6, transcription initiation factor IIB and sex determining region Y-box 9

The amount of *IL6* (interleukin-6), *TFIIB* (transcription initiation factor IIB) (Fig. 5) and *SOX9* (sex determining region Y-box 9) (Fig. 6) messages was not affected by dietary treatment or genetic background. Although not significantly different, there was a trend ($P<.20$) for increased expression of these genes in the P-deficient HB pigs when compared to the other HB animals.

Neither dietary treatments nor genetic backgrounds had a significant impact on the levels of *RANKL* (receptor activator of nuclear factor-kappaB ligand), *OPG* (osteoprotegerin), *BGLAP* (osteocalcin), *ARAF1* (A-Raf-1) or *IGFBP5* (insulin-like growth factor binding protein 5) mRNA.

4. Discussion

We have demonstrated that genetic background influences the metabolic response to dietary P deficiency in young pigs. In this study, traditional indicators of P status, PIP and AKP validated that our treatment diets were effective in causing P deficiency. There was no effect of genetic background on either of these indicators of mineral status; however, there were significant differences in growth performance based on the interaction between sire line and dietary treatment. In HB animals, P deficiency caused a significant reduction in ADG; however, the LB animals' growth rate was not affected by P deficiency. Because there was no reduction in ADFI, this reduction in ADG was not due to a depressed feed intake sometimes seen with P-deficient diets, suggesting altered homeorhetic control of P utilization between these genetic backgrounds. Previous research has demonstrated both a prompt growth-retarding effect of P deficiency [15], as well as a lack of this effect [16]. Although Stahl et al. [16] suggested that the lack of a growth retarding effect of P deficiency to young pigs may be due to genetic background, to the best of our knowledge, this is the first report demonstrating that genetic background influences the growth response to dietary P deficiency.

As expected, P-deficient pigs had significantly weaker bones than the P-adequate or the P-repletion pigs, regardless of their genetic background. However, the P-deficient pigs of the HB line had significantly ($P<.10$) stronger bones than the LB P-deficient pigs. From these data, it appears that the HB animals sacrifice their growth rate in order to spare P and maintain bone integrity, whereas the LB animals sacrifice bone strength to provide P for soft-tissue growth. Whereas altered utilization/partitioning of P in the body is likely, differences in the efficiency of absorption of dietary P in the gastrointestinal tract and its resorption in the kidney could also differ between these animals, offering another explanation to the differences in growth response to P deficiency.

The changes in gene expression seen in the bone marrow of these animals may help explain the altered homeorhetic control of P metabolism seen between these genetic backgrounds. The levels of *CALCR*, *VDR* and *IGFBP3* transcripts were increased in the bone marrow of P-deficient HB pigs but were not altered in the LB animals, further suggesting a difference in the regulation of P metabolism between these genetic backgrounds, as well as providing a possible explanation for the better bone integrity of HB pigs during P deficiency. Calcitonin acts directly on bone by inhibiting the activity of osteoclasts via calcitonin receptors (CALCR) located on their cell surface [17,18]. The response of osteoclasts to calcitonin is related to the amount of calcitonin receptors available for binding with calcitonin on the osteoclast surface [19]. The increase in *CALCR* message seen in the P-deficient HB pigs may indicate an increased inhibition of the activity of osteoclasts, thereby reducing bone resorption and helping to preserve bone mineral stores. In the P-deficient LB animals, *CALCR* expression was unresponsive to P deficiency, potentially allowing for normal osteoclast function and bone resorption in spite of dietary P restriction, thereby providing P for soft-tissue growth.

An increase in *VDR* message, similar to that of *CALCR*, was also seen in HB, but not LB, P-deficient animals. The hormonal form of vitamin D regulates many aspects of Ca, and therefore P, homeostasis such as absorption from the gastrointestinal tract, resorption from the kidneys and deposition in bone. Vitamin D receptor mediates most of the actions of vitamin D through transcriptional control of target genes, although the importance of VDR to mediate several nongenomic responses to the hormonal form of vitamin D has also been demonstrated [20,21]. An increase in the expression of VDR during P deficiency has been shown in other animals [22]. One transcriptional effect of VDR is the regulation of *IGFBP3* expression [23]. The HB P-deficient animals also had increased levels of *IGFBP3* transcript. Insulin-like growth factor binding proteins (IGFBPs) bind to insulin-like growth factor 1 and regulate the access of IGF1 to its receptor, extend the half-life of IGF1 and transport IGF1 to various tissues. Insulin-like growth factor 1 is considered to be a potent stimulator of bone formation. Although we did not expect to see an

increase in *IGF1* message with P deficiency, the P-deficient HB pigs had increased levels of *IGF1* message when compared to the other two treatment groups. If the increase in *IGFBP3* message also seen in this treatment group corresponded to an increase in active IGFBP3, the increase in *IGF1* transcripts may be due to cells sensing a functional deficiency of IGF1 due to its complexing with IGFBP3.

In addition to these genes that are known to have a direct effect on bone turnover, we also examined the expression of genes involved in transcriptional regulation, such as the general transcription factor IIB (*TFIIB*), sex determining region Y-box 9 (*SOX9*) and A-Raf-1 (*ARAF1*). Whereas the expression of *ARAF1* was not affected by P deficiency or by the genetic differences between our pigs, there was a trend ($P<.20$) for increased *SOX9* and *TFIIB* expression in the HB P-deficient pigs when compared to the other HB animals. Transcription initiation factor IIB is one of the ubiquitous factors required for transcription initiation by RNA polymerase II. In addition, TFIIB has been shown to interact with the VDR [24]. Sex determining region Y-box 9 (SOX9) is a transcription factor that has an essential role in chondrocyte differentiation and is required for formation of normal mesenchymal condensations, for conversion of mesenchymal cells to chondrocytes, for proliferation of chondrocytes and for suppression of premature conversion of these chondrocytes to hypertrophic chondrocytes [25]. In addition, SOX9 regulates the transcription of type II collagen, a chondrocyte specific gene [26,27]. A role for SOX9 in endochondral bone development has also been suggested [28,29]. The differential expression of these transcription factors in response to nutrient deficiency and genetic background deserves further examination, as it may help to explain the differences in the regulation of mineral metabolism that can be attributed to genetic background.

Although receptor activator of nuclear factor-kappaB ligand (RANKL), osteoprotegerin (OPG), osteocalcin (BGLAP) and insulin-like growth factor-binding protein 5 (IGFBP5) are all known to be important proteins involved in bone metabolism [30–41], in this model, we found that the expression of these genes were not affected by P deficiency or by the genetic differences between our pigs. This suggests that these genes are not responsible for differences in the homeorhetic control of P metabolism in young growing pigs. Further research is needed to examine if these genes may be involved in the regulation of mineral metabolism at different stages of bone growth and development.

In addition to the differences caused by the interaction of genetic background and P deficiency, another interesting Genetic background×Treatment interaction was observed in the P-repletion groups. The HB P-repletion pigs maintained ADGs that were not significantly different from that of P-adequate pigs; however, the LB P-repletion animals had a significantly higher growth rate than even their siblings in the P-adequate group. This could indicate a possible up-regulation of a P transport system by dietary P restriction in these animals. Improved absorption of P from their diet and/or increased resorption of P in the kidney could help explain the increased growth in these pigs. Up-regulation of P transporter genes in response to dietary P has been described in both rats and trout [22,42].

There is a great need to develop an animal model for human osteoporosis. Pigs are an excellent model for investigating the nutritional factors that may affect bone. The bone remodeling cycle in pigs is histologically similar to that of humans, but is more rapid, allowing relatively short experimental times. Pigs possess a definable peak bone mass and closely resemble humans in their size, diet, gastrointestinal function and estrous cycle [43–46]. Additionally, pigs are one of the few animal species in which spontaneous fractures have been reported [47].

Whereas most nutritional studies on bone growth and development have focused on Ca, P is also essential in supporting bone growth and integrity. Whereas the average adult consumption of P in the United States is generally considered adequate, between 10% and 15% of older women have intakes of less than 70% of the recommended daily allowance [48], and a 1986 survey estimated that only 10% of adults in the United States take P-containing supplements [49]. Currently, osteoporosis patients are given high-dose Ca supplements that consist of carbonate or citrate salts. Although P makes up more than half of the mass of bone mineral, very few dietary supplements contain P [50,51]. In patients given high-dose Ca supplements but not meeting their RDA of P, it is possible that dietary P bioavailability will be dramatically reduced due to the formation of insoluble tri-Ca phosphate or phytic acid salts in the small intestine [50]. Therefore, the amount of absorbed P may be too low to meet the needs for soft-tissue growth and new bone mineralization [50]. Induced P insufficiency under these circumstances would not only limit bone mineral deposition, but also enhance osteoclastic bone resorption [52]. Attention to the nutritional adequacy of the diets of such patients is necessary if they are to realize the full potential of osteoporosis therapies. Studies have found that rats fed P-containing salts promoted significantly greater improvement in bone development than rats fed supplements with only Ca [51].

These differences in growth and gene expression between the genetic backgrounds while under P deficiency suggest a difference in the mechanisms of homeorhetic control of P utilization between these genetic lines. The three most significant changes in gene expression that were based on dietary P deficiency and its interaction with genetic background were seen in *CALCR*, *VDR* and *IGFBP3*. Polymorphisms in the human *CALCR* gene and *VDR* gene have been shown to be associated with BMD [53–58]. Based on the expression data, *CALCR*, *VDR* and *IGFBP3* are good candidates for single nucleotide polymorphism studies.

Understanding the interaction between nutrition and genetic background that lead to maximal bone integrity is critical. With a better understanding of its effect on bone, dietary P requirements could be more precisely defined, and

we may be able to identify human populations that have different dietary requirements in order to optimize their BMD and prevent osteoporosis. This work also has great implications in animal agriculture. The sire lines utilized in this study likely produce over 25% of the pigs in the United States annually. With increasing governmental and public concerns over the environmental impact of P in the excreta from animal agriculture, more accurately defining P requirements to reduce excess, P excretion is of great importance [59]. Identifying genotype-specific dietary P requirements could lead to strategies to increase the efficiency of growth in domestic animals while doing so in a more environmentally friendly manner.

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