Dietary phosphorus level regulates appetite through modulation of gut and hypothalamic expression of anorexigenic genes in broiler chickens

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ABSTRACT Two experiments were designed to elucidate gut and hypothalamic molecular regulation of appetite by dietary phosphorus (**P**) concentration in broiler chickens. Birds (192 Cobb-500 broiler chickens) were randomly assigned to 3 experimental diets in experiment 1 (Exp. 1) and 24 broiler chickens were randomly assigned to 3 treatment groups in Exp. 2. Each diet comprised 8 replicate cages, with either 8 birds (Exp. 1) or 1 bird (Exp. 2) per replicate cage. In Exp. 1, diets contained 1.2 (P-deficient), 2.8 (P-marginal) or 4.4 (P-adequate) g/kg non-phytate P (**nPP**). In Exp. 2, birds fed the P-adequate diet were pair-fed (**PF**) to the feed consumption levels of birds fed the P-deficient diet. Feed intake and BW gain ($P < 0.001$) decreased in birds fed the P-deficient diet in Exp. 1. Birds fed the P-deficient diet had similar feed intake and BW gain with PF group fed the P-adequate diet (Exp. 2) but was significantly lower ($P < 0.001$) than birds fed the P-adequate diets. Sodium-phosphate cotransporter (NaPi-IIb) mRNA was upregulated ($P < 0.05$) in both experiments. Conversely, cholecystokinin (**CCK**) mRNA was downregulated ($P < 0.01$) in birds fed P-deficient diets. Anorexia-related hypothalamic cholecystokinin receptor (**CCKAR**) and melanocortin receptors (MC3R and MC4R) were upregulated ($P < 0.05$) in birds fed P-deficient diets, in both experiments. The current data show that dietary P deficiency decreases feed intake in broiler chickens by altering the expression of anorexigenic genes in the gut and hypothalamus of broiler chickens.

Key words: appetite, chicken, gut, hypothalamus, phosphorus

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INTRODUCTION

Voluntary feed intake in farm animals is very complex and is tightly regulated by the brain−gut axis acting through hypothalamic integration. The intrinsic cross-talk between the hypothalamus and the gastrointestinal tract ensures that energy balance is maintained. Generally, there are 2 primary populations of neurons integrating peripheral signals of nutritional status and influencing appetite through the release of signaling molecules, originating in the hypothalamus (Richards, 2003). These signals include the orexigenic neuropeptides; neuropeptide Y (**NPY**), agouti-related peptide (**AgRP**), peptide YY (**PYY**) (Takeuchi et al., 2000; Ando et al., 2001) and anorexigenic neuropeptides; pro-opiomelanocortin (**POMC**), cocaine- and amphetamine-regulated transcript (**CART**), corticotropin-releasing hormone (**CRH**), cholecystokinin (**CCK**), and ghrelin (Furuse et al., 1999; Richards, 2003). Ghrelin and CCK are highly expressed in the gastrointestinal tract and are essential to feed intake regulation in broilers and layers (Richards, 2003).

Previous reports show that feeding response in animals is affected by dietary phosphorus (**P**) concentrations. Maynard (1951) stated that a specific deficiency of P has a specific effect in causing loss of appetite. Similarly, Osborne and Mendel (1918) reported that a lack of sufficient dietary P causes cessation or growth retardation in rats. Recently in broiler chickens, several authors (Dilger et al., 2004; Adeola, 2010; Rousseau et al., 2016; Imari et al., 2020) have reported a drastic decrease in feed intake in birds fed diets deficient in dietary P. However, potential regulation of central and peripheral appetite regulators by dietary P remains unknown and warrants investigation.

Hence, in the current study, our hypothesis was that P deficiency directly affects feed intake response in broiler chickens through alterations in the expression of gut and brain appetite regulators. Two separate experiments were conducted to respectively investigate the hypothesis. Data obtained from the current study could aid in elucidating the delicate relationship that exists between dietary P

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level and appetite regulation and may open up new insights into other aspects of P metabolism in the chicken.

MATERIALS AND METHODS

Protocols of animal experiments were reviewed and approved by the Purdue University Animal Care and Use Committee.

Experimental Diets, Birds, Housing, and Design

Three corn-soybean meal-based diets (Table 1) were formulated to contain 1.2 (P-deficient), 2.8 (P-marginal), or 4.4 (P-adequate) g/kg non-phytate phosphorus (**nPP**). The Ca to nPP ratio was maintained across all three diets. Male day-old broiler chicks (Cobb 500, Siloam Springs, AR) were obtained from a commercial hatchery. The birds were individually tagged, weighed, and raised in heated battery brooders (model SB 4 T; Alternative Design Manufacturing, Siloam Springs, AR) with temperature and lighting maintained as previously described (Aderibigbe et al., 2020). The birds were fed a common starter diet, formulated to meet breeder nutrient specifications for 14 or 18 d (9.0 g/kg Ca; 8.4 g/kg total P and 4.4 g/kg nPP). Experiment 1 (**Exp. 1**) investigated the impact of dietary P concentration on feed intake response and appetite regulatory genes in broiler chickens. The second experiment (**Exp. 2),** a pair-feeding (**PF**) trial, was designed to investigate any direct relationship between dietary P-deficiency related feed intake reduction observed in Exp. 1 and appetite regulation in broiler chickens.

Table 1. Composition of experimental diets (g/kg, as-fed).

	Non-phytate P, g/kg		
Ingredients	1.2	2.8	4.4
Corn	597.7	574.5	560.7
Soybean meal (48% CP)	340.0	340.0	340.0
Soybean oil	20.0	30.0	30.0
Monocalcium phosphate[1]	0.0	7.5	15.3
Limestone[2]	4.3	10.0	16.0
Salt	4.0	4.0	4.0
Vitamin-mineral premix[3]	3.0	3.0	3.0
DL-Methionine	2.0	2.0	2.0
L-Lysine HCl	2.9	2.9	2.9
L-Threonine	1.1	1.1	1.1
Titanium dioxide premix[4]	25.0	25.0	25.0
Total	1,000.0	1,000.0	1,000.0
Calculated composition[5], g/kg			
CP	222.1	220.1	219.0
ME, kcal/kg	3,162.2	3,165.7	3,117.9
Ca	2.7 (2.8)	6.0 (6.6)	9.6 (9.8)
Total P	3.8 (3.8)	5.3 (5.5)	6.9 (7.0)
Non-phytate P	1.2	2.8	4.4
Ca: total P	0.7	1.1	1.4
Ca: non-phytate P	2.2	2.2	2.2
Total amino acids, g/kg			
Arg	14.2	14.1	14.0
His	5.8	5.7	5.7
Ile	9.0	8.9	8.9
Leu	18.9	18.7	18.5
Lys	13.9	13.9	13.8
Met	5.4	5.3	5.3
Cys	3.6	3.5	3.5
Phe	10.3	10.2	10.2
Tyr	8.5	8.4	8.4
Thr	9.2	9.2	9.1
Trp	2.9	2.9	2.9
Val	10.0	9.9	9.9
Met + Cys	8.9	8.9	8.8
Phe + Tyr	18.8	18.6	18.5

[1]16% Ca, 21% P.
[2]38% Ca.
[3]Supplied the following per kg diet: vitamin A, 5,484 IU; vitamin D3, 2,643 ICU; vitamin E, 11 IU; menadione sodium bisulfite,4.38 mg; riboflavin, 5.49 mg; pantothenic acid, 11 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B12, 13.2 ug; biotin, 55.2 ug; thiamine mononitrate, 2.2 mg; folic acid, 990 ug; pyridoxine hydrochloride, 3.3 mg; I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 ug.
[4]1 g of titanium dioxide added to 4 g corn.
[5]Analyzed values are in brackets.

Experiment 1 One-hundred and ninety-two birds were allotted to the 3 diets (Table 1) in a randomized complete block design consisting of 8 replicate cages and 8 birds per cage on d 14 post hatching. Experimental diets, fed as mash, and water were provided ad libitum to all birds. Feed intake records were taken every 12 h and the experiment was terminated on d 18 when there was a significant decrease (approximately 30% reduction) in the average feed intake of birds fed the P-deficient diet (1.2 g/kg nPP) compared to other groups.

Experiment 2 A successive PF trial was conducted to delineate any direct impact of dietary P deficiency on the expression of brain-gut appetite regulators in broiler chickens without the confounding effect of differences in amount of feed consumed. On d 18 post hatching, 24 birds previously fed the P-adequate diet (4.4 g/kg nPP) were weighed and individually allotted to three treatment groups in a randomized complete block design consisting of 8 replicate cages and 1 bird per cage. Birds in treatment group 1 were fed ad libitum a P-deficient diet (Table 1) formulated to contain 1.2 g/kg nPP. Birds in treatment group 2 were fed a P-adequate diet (Table 1) formulated to contain 4.4 g/kg nPP but pair-fed to the amount of feed consumed by the birds in treatment group 1 to equalize the feed intake of the 2 groups. The average daily feed intake of birds fed the P-deficient diet was offered to the PF group in subsequent days. Birds in the third treatment group were fed ad libitum a P-adequate diet formulated to contain 4.4 g/kg nPP.

Sampling Procedures

The studies were terminated on d 18 or 21 post hatching in Exp. 1 or 2, respectively. In both experiments, birds were individually weighed and 1 bird (with median BW in Exp. 1) per replicate was selected for sample collection. First, the birds were anesthetized with sodium pentobarbital via cardiopuncture and then decapitated. The hypothalamus was dissected from the ventral surface of the brain. Two transverse cuts were made at the apex of the optic chiasm and the rostral margin of the mammillary bodies. Next, 2-mm bilateral cuts were made on either side of the midline and the whole

Table 2. Gene-specific primers used in real −time quantitative PCR.

Gene	Accession No.	Forward	Reverse	Size (bp)
CCK	NM_001001741.1	GGAAGGAAGGAGGAAGCGAT	GAGGGAGCTCACCGACAG	75
NPY	NM_205473.1	TCAAGCCCAGAGACACTGAT	GGGTCTTCAAACCGGGATCT	80
AGRP	NM_001031457.1	CGGTTGTGCACGTTGCC	CTGAGGTCCGAGGTGAGGAT	96
PYY	NM_001361182.1	GGTATGGGAAGCGCAGCA	CGTCGATGTCGGACCACAG	87
POMC	NM_001031098.1	TCCATGGAGCATTTCCGCTG	GGTAACTCTCAGCCGACTCC	100
Ghrelin	NM_001001131.1	CCTCTCTGCTAACCTGTCTGG	AGGCAGTGCTTCAAATGTGT	72
NaPi-IIb	NM_204474.2	CACCTCTTGTTGGCATTGGTG	GCTGTTGTGGTTGTGCCAAT	85
CCKAR	NM_001081501.1	ACCAGCATCGCCAAATACGA	GTTGCAACGGCACTTTCCTT	79
MC4R	NM_001031514.1	CAAGCGTGTAGGGGTCATCA	CAGATGATGACAACGCTGCTG	99
MC3R	XM_004947236.3	CCGTTCCACCGTTCACCTAA	GGGACCTTGGTGTGGGATTT	75

Aabbreviations: AGRP, agouti-related peptide; CCKAR, cholecystokinin receptor; CCK, cholecystokinin; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor; NaPi-IIb, sodium-phosphate cotransporter; NPY, neuropeptide Y; PYY, peptide YY; POMC, proopiomelanocortin.

hypothalamus removed according to the chicken brain atlas (Yuan et al., 2009). The hypothalamus was immediately snap frozen in liquid nitrogen and stored at −80°C for RNA extraction. For the gut markers of appetite regulation, whole of the jejunal segments was removed, flushed gently with ice-cold 10% phosphate-buffered saline (VWR International, Radnor, PA). The segments were cut longitudinally in half exposing the lumen, and mucosal contents were scraped with a metal spatula. Mucosal contents were immediately placed in 2 mL of Trizol reagent (Invitrogen, Grand Island, NY) and stored at −80°C prior to RNA isolation.

Total RNA Extraction, Reverse Transcription, and Real-Time PCR Analysis

Expression of genes in the hypothalamus and jejunal mucosa was quantified using quantitative real-time polymerase chain reaction (**qPCR**). Trizol reagent (Invitrogen, San Diego, CA) was used to extract the total RNA following the manufacturer's protocol. RNA concentrations were determined by Nano-Drop 1000 (Thermo Scientific, Wilmington, DE) by taking the optical density at 260 nm and 280 nm. RNA with A260/A280 ratio above 1.8 was retained, and RNA integrity was verified by 1% agarose gel electrophoresis. Afterward, 2 mg of total RNA from each sample were reverse transcribed into cDNA product with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The cDNA was then diluted 1:10 with nuclease-free water (Ambion) and stored at −20°C until use. Real-time qPCR was performed with Bio-Rad CFX machine (Biorad, Tamecula, CA) with the SYBR green-based mix (Biotool, Houston, TX) in a total reaction volume of 20 μL and programmed at the following cycling parameters: 10 min at 95°C; 40 cycles of 95°C for 30 s, primer-specific annealing temperature for 30 s, and 72°C for 30 s. A melt curve analysis was performed for each gene at the end of the PCR run. Nucleotide sequences for genes of interest used in the current study are listed in Table 2. Primers were designed with the Primer Blast software (NCBI-NIH, Bethesda, MD). Samples were analyzed in duplicate, and a difference lesser than or equal to 5% was considered acceptable. Relative gene expression was subsequently calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with normalization against glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) as the housekeeping gene.

Statistical Analyses

Using values from previous publications on feed intake response of broiler chickens to dietary P deficiency, we determined (Dell et al., 2002) that 8 replicates per treatment would be sufficient for the impact of dietary P level on feed intake to achieve 80% power with a significance level of 0.05. Data were analyzed using the Proc GLM procedures of SAS (SAS Inst. Inc., Cary, NC) for randomized complete block design with dietary treatment as the main effect. Initial body weight was used as the blocking factor. Cage was used as the experimental unit for all analyses. For gene expression, fold change of dietary treatments was expressed relative the P-adequate diet, which was set at 1. Statistical significance was declared at $P \leq 0.05$, with $0.05 < P \leq 0.10$ considered as a tendency. When the main effect of the dietary treatment was significant, means were separated using Tukey's HSD. In experiment 1, linear and quadratic effects of graded dietary nPP concentration were determined by orthogonal polynomial contrast. Graphs were generated using GraphPad Prism 5.0 software.

RESULTS

Growth Performance, Feed, and P Intake

There were significant decreases ($P < 0.001$) in feed intake, final BW and BW gain in birds fed P-deficient diet (1.2 g/kg nPP) compared to other treatments (Exp. 1; Table 3). However, there was no difference between birds fed diets containing 2.8 and 4.4 g/kg nPP. There were linear increases ($P < 0.01$) in feed intake, final BW, BW gain, and total P intake with increasing dietary nPP level. In Exp. 2 (Table 4), response of birds fed P-deficient diets (1.2 g/kg nPP) was not different from the PF group, but similar to Exp. 1, had lower final BW ($P < 0.01$), BW gain ($P < 0.001$) and feed intake ($P < 0.001$) compared to birds fed P-adequate diets (4.4 g/kg nPP). Total P intake was similar between PF

Table 3. Growth performance of broiler chickens in Exp. 1, from d 14 to 18 post hatching[1].

	Non-phytate P, g/kg				*P*-value		
Item	1.2	2.8	4.4	SEM	Diet	Linear	Quadratic
d 14 BW	412.8	412.4	412.3	0.46	0.421	0.480	0.271
d 18 BW	577.0[b]	651.9[a]	675.9[a]	10.11	< 0.001	< 0.001	0.060
BW gain, g/bird	164.2[b]	239.5[a]	262.7[a]	10.21	< 0.001	< 0.001	0.056
Feed intake, g/bird	222.7[b]	299.8[a]	321.7[a]	16.12	< 0.001	0.001	0.182
Gain: Feed, g/kg	772.7	814.9	826.3	50.42	0.735	0.465	0.807
Total P intake[2], g/bird	0.85[c]	1.59[b]	2.22[a]	0.073	< 0.001	< 0.001	0.541

[1]Data represent least square means of 8 replicate cages.
[2]Arithmetic product of feed intake (g/bird) and dietary P (g/kg) concentration.
[a,b]Means within the same row with different superscripts are different at $P < 0.05$.

Table 4. Growth performance of broiler chickens in Exp. 2 (Pair-feeding trial), from d 18 to d 21 post hatching[1].

	Non-phytate P, g/kg				*P*-value	
Item	1.2	PF[2]	4.4	SEM	Diet	PF vs. 4.4
d 18 BW	707.5	708.4	707.8	5.29	0.993	0.948
d 22 BW	884.6[b]	878.8[b]	973.6[a]	19.58	0.006	0.004
BW gain, g/bird	177.1[b]	170.4[b]	265.8[a]	18.97	0.005	0.003
Feed intake, g/bird	290.3[b]	302.9[b]	404.7[a]	14.1	< 0.001	< 0.001
Gain: Feed, g/kg	615.7	562.8	656.6	48.05	0.535	0.273
Total P intake[3], g/bird	1.10[b]	2.09[a]	2.14[a]	0.058	< 0.001	0.513

[1]Data represent least square means of 8 replicate cages.
[2]PF, pair-fed group in which birds on the 4.4 g non-phytate/kg diet were pair-fed to the feed intake of the birds on the 1.2 g non-phytate/kg diet.
[3]Arithmetic product of feed intake (g/bird) and total dietary P (g/kg).
[a,b]Means within the same row with different superscripts are different at $P < 0.05$.

group and birds fed P-adequate diets (4.4 g/kg nPP) but were higher ($P < 0.001$) than in birds fed P-deficient diets (1.2 g/kg nPP).

Relative Gene Expression of Appetite Regulators

Figures 1–4 depict the relative mRNA expression of intestinal P transporter and appetite regulatory genes in Exp. 1. The expression of NaPi-IIb mRNA was upregulated ($P < 0.05$) in birds fed P-deficient diets (1.2 g/kg nPP) compared to other treatments. Conversely, expression of intestinal CCK mRNA was downregulated ($P < 0.001$) in birds fed P-deficient diets (1.2 g/kg nPP). However, mRNA expression of intestinal Ghrelin and PYY, or hypothalamic POMC was unaffected by dietary P concentration. There was a tendency for an effect of dietary P concentration on hypothalamic mRNA expressions of NPY ($P = 0.094$) and AGRP ($P = 0.069$). Hypothalamic expressions of CCK receptor (**CCKAR**), and melanocortin receptors (**MC3R** and **MC4R**) were upregulated ($P < 0.05$) in birds fed P-deficient diets but was similar between the birds fed marginal (2.8 g/kg nPP) and P-adequate (4.4 g/kg nPP) diets.

Figures 5–8 show the relative mRNA expression of intestinal P transporter and appetite regulatory genes in Exp. 2. Like Exp. 1, mRNA expression of NaPi-IIb was upregulated ($P < 0.001$) and CCK was downregulated ($P < 0.01$), respectively, in birds fed P-deficient diets (1.2 g/kg nPP) compared to other treatments. The expression of intestinal Ghrelin and PYY, or

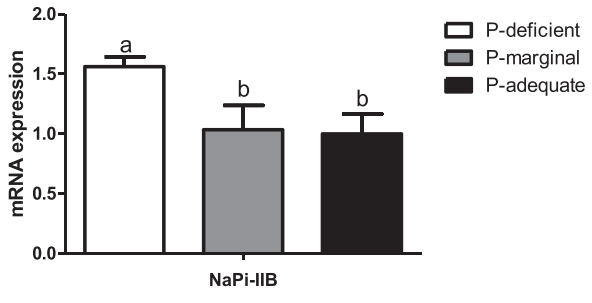

Figure 1. Effect of dietary phosphorus (P) level on mRNA expression of sodium-phosphate cotransporter (NaPi-IIb) in jejunal mucosa in experiment 1. Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). [a, b] indicates significant difference ($P < 0.05$) between the given pair of treatment. There was linear ($P < 0.05$) effect of increasing dietary P level on NaPi-IIb expression.

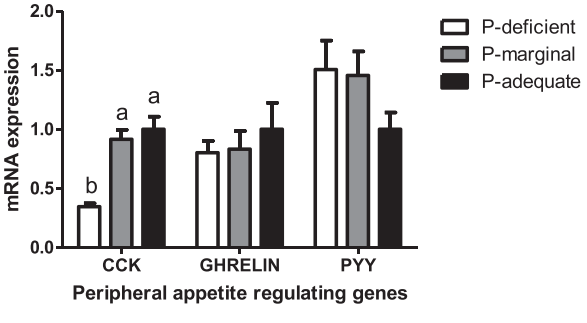

Figure 2. Effect of dietary phosphorus (P) level on peripheral appetite regulating genes in jejunal mucosa in experiment 1. Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). [a,b] indicates significant difference ($P < 0.05$) between the given pair of treatment. There were linear and quadratic ($P < 0.05$) effects of increasing dietary P level on CCK expression. Abbreviations: CCK, Cholecystokinin; PYY, peptide YY.

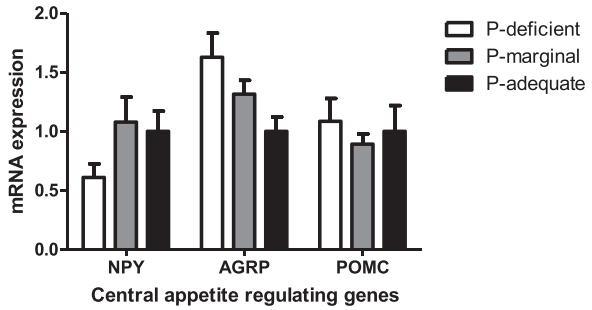

Figure 3. Effect of dietary phosphorus (P) level on central appetite regulating genes in the hypothalamus in experiment 1. Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). There was linear ($P < 0.05$) effect of increasing dietary P level were observed on AGRP expression. Abbreviations: AGRP, Agouti-related peptide; NPY, neuro-peptide Y; POMC, proopiomelanocortin.

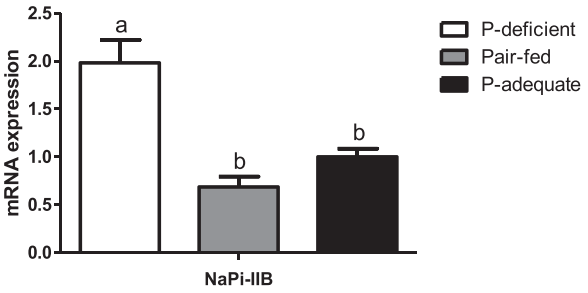

Figure 5. Effect of dietary phosphorus (P) level on mRNA expression of sodium-phosphate cotransporter (NaPi-IIb) in jejunal mucosa in experiment 2 (Pair-feeding study). Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). [a,b] indicates significant difference ($P < 0.05$) between the given pair of treatment.

hypothalamic NPY, AGRP, and POMC mRNA were unaffected by dietary P concentration. However, like in Exp. 1, hypothalamic CCKAR, MC3R, and MC4R were upregulated ($P < 0.01$) in birds fed P-deficient diets compared to other dietary treatments.

DISCUSSION

Our current data suggest a strong relationship between appetite regulation in broiler chickens and dietary P deficiency. This was observed as differential expression of anorexigenic genes in the gut and hypothalamus of chickens fed P-deficient diets, compared to other treatment groups, which supports our stated hypothesis. However, that we detected mRNA alterations does not necessarily imply that expression of the protein parallels the transcript. Importantly, there may be other factors associated with appetite that contribute to differences in feeding behavior that were not evaluated in the current study.

Appetite regulation is essential in animals because of its importance in determining the levels and consumption pattern of nutrients that are important for sustenance of life. Appetite is controlled by complex mechanisms that involve the nutritional status of the body, neurotransmitters, and a change in feeding behavior (Richards and Proszkowiec-Weglarz, 2007). Like mammals (Leibowitz and Wortley, 2004; Bellinger and Langley-Evans, 2005), a variety of orexigenic and anorexigenic neuropeptides have been identified in birds (Kuenzel, 1994; Richards, 2003). However, response to the neuropeptides has been observed to differ between chicken breeds. For instance, although the orexigenic AGRP stimulates feeding behavior in layer chicks, it has no such effect in broiler chicks (Tachibana et al., 2001) whereas the anorexigenic effects of CRH are stronger in layer chicks than in broiler chicks (Tachibana et al., 2007). The mechanisms underlying such differences between species remain unclear. In addition to its major function as a digestive and absorptive organ, the gut also plays an important role in short-term regulation of feed intake via hormones such as CCK, and ghrelin, which transmit meal-related signals to the hypothalamus (Jensen, 2001). Previously, reports have shown that dietary macronutrients such as amino acids, fat, carbohydrates, and calcium can affect appetite in animals (Anderson, 1979; White et al., 2003; McConn et al., 2018). However, few reports exist in literature on P-

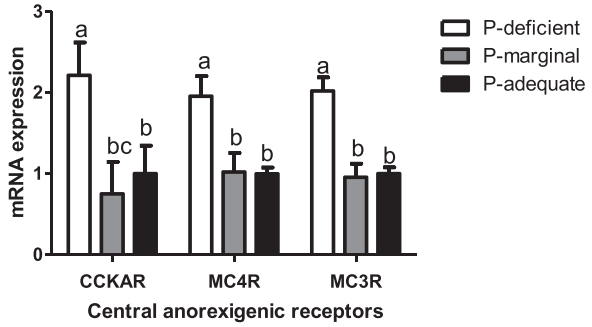

Figure 4. Effect of dietary phosphorus (P) level on mRNA expression of receptors of anorexigenic pathway in the hypothalamus in experiment 1. Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). [a,b] indicates significant difference ($P < 0.05$) between the given pair of treatment. There were linear and quadratic ($P < 0.05$) effects of increasing dietary P level was observed on CCKAR and MC3R expression. There was linear effect ($P < 0.05$) of increasing dietary P level on MC4R expression. Abbreviations: CCKAR, cholecystokinin receptor; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor.

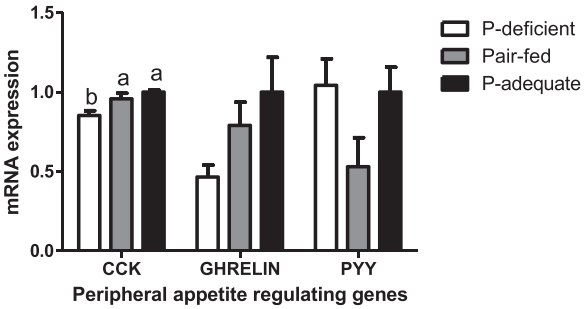

Figure 6. Effect of dietary phosphorus (P) level on peripheral appetite regulating genes in jejunal mucosa in experiment 2 (Pair-feeding study). Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). [a,b] indicates significant difference ($P < 0.05$) between the given pair of treatment. Abbreviations: CCK, Cholecystokinin; PYY, peptide YY.

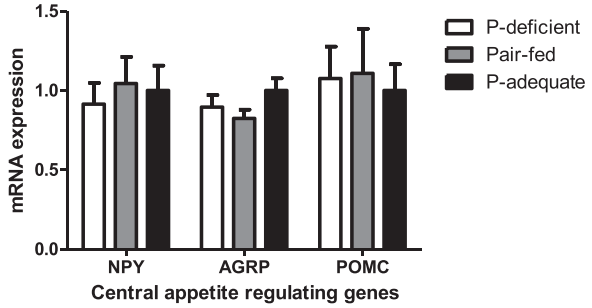

Figure 7. Effect of dietary phosphorus (P) level on central appetite regulating genes in the hypothalamus in experiment 2 (Pair-feeding study). Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). Abbreviations: AGRP, Agouti-related peptide; NPY, neuro-peptide Y; POMC, proopiomelanocortin.

related appetite in animals, especially in broiler chickens which show a drastic decrease in feed intake when fed a P-deficient diet. To our knowledge, the current study has not been reported before regarding the possible role of gut-brain axis for appetite regulation in response to dietary P deficiency-induced feed intake reduction in broiler chickens.

Consistently, in both experiments, and like previous reports (Dilger et al., 2004; Adeola, 2010; Rousseau et al., 2016; Imari et al., 2020), birds fed P-deficient diets had a drastic decrease in feed intake. Although the reason for this observation is unclear, one possible explanation is that as blood phosphate concentration becomes low over time, due to mild chronic hypophosphatemia, bones become weak because of increased resorption, which may eventually lead to loss of appetite in the birds. However, this seems counterintuitive, as birds will typically increase feed intake to satisfy requirement for a nutrient that is deficient in the diet. This is especially true for Ca (Wilkinson et al., 2013), and in fact, birds would more likely adjust their feed intake to satisfy a Ca appetite as dietary P fluctuates and scarcely the other way around (Wilkinson et al., 2014; Rousseau et al., 2016). For instance, in the study by Wilkinson et al. (2014), birds fed diets with 5.5 g/kg of nPP consumed 37% more of a

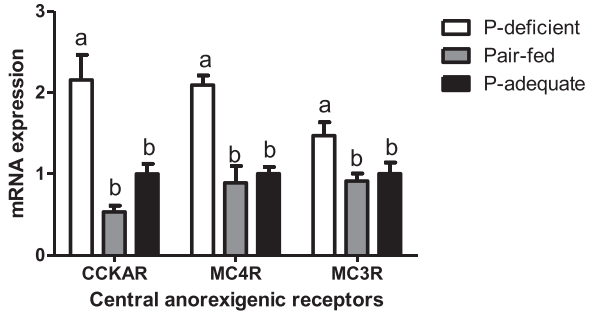

Figure 8. Effect of dietary phosphorus (P) level on mRNA expression of receptors of anorexigenic pathway in the hypothalamus in experiment 2 (Pair-feeding study). Gene expression represents fold change relative to the adequate P treatment. Error bars represents means ± SEMs (n = 8). [a,b] indicates significant difference ($P < 0.05$) between the given pair of treatment. Abbreviations: CCKAR, cholecystokinin receptor; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor.

Ca source than birds fed 2.5 g/kg of nPP, indicating that birds were not only responding to dietary Ca concentration but also to the amount of nPP in the mixed ration. It is likely that birds increased their consumption of the Ca source as dietary nPP concentration increased to avoid narrow Ca:nPP ratios.

Although there were marked reduction in feed and P intake in birds fed P-deficient diets, there was an upregulation of intestinal NaPi-IIb. On a functional level, upregulation of intestinal P absorption due to a deficient-P diet has been described in many species (Caverzasio et al., 1987; Cross et al., 1990; Danisi and Murer, 1991) and are associated with the abundance of NaPi-IIb in the apical membrane. A low P diet would lead to a rapid decrease of the plasma phosphate concentration and activation of the renal 1,25-hydroxylase, resulting in an increased level of vitamin D3 (Matsumoto et al., 1980; Portale et al., 1989). Therefore, adaptation of small intestinal phosphate transport to low P diet has been explained to occur via vitamin D3 (Cross et al., 1990). In the current study, intestinal transcripts of satiety signals such as ghrelin and PYY were unaltered in response to dietary P concentration, which suggests that these hormones may not be directly involved in P appetite. In vertebrates, peripheral PYY is purported to act as a satiety factor released from the gastrointestinal tract after feeding to curb appetite via afferent vagal Y-receptors or directly within the arcuate nucleus of the hypothalamus (Batterham et al., 2002; Batterham and Bloom, 2003; Simpson et al., 2012). However, unlike in mammals where ghrelin increases food intake through the melanocortin system, ghrelin in birds induces anorexia through the release of corticotropin-releasing factor (Kaiya et al., 2002; Saito et al., 2002). Interestingly, intestinal CCK expression was downregulated in birds fed P-deficient diet. This observation is contrary to the known anorexigenic function of CCK (Denbow, 1994; Kuenzel, 1994; Jensen, 2001) and therefore does not explain the decreased feed intake of birds fed the P-deficient diet. This observation warrants further investigation.

Because satiety signals in the gut are relatively short-lived when compared to central appetite regulators, we also investigated appetite regulatory genes in the brain. In the current study, hypothalamic gene expression of orexigenic (AgRP and NPY) and anorexigenic (POMC) appetite hormones were not influenced by dietary treatments. Although, there was a tendency for an alteration in NPY and AGRP expressions, this was not consistently observed in the PF trial and therefore any potential effect of dietary P level was discarded. The reason for the lack of effect in these genes remains unclear and warrants further investigations. NPY is a very potent orexigenic peptide in mammals in birds (Kuenzel et al., 1987) and AgRP plays a similar role (Tachibana et al., 2001). However, AGRP does not influence feed intake in broiler chicks compared to layer-type chicks (Tachibana et al., 2001; Honda et al., 2007). Although expression of the anorexigenic POMC gene was not influenced by dietary P level, and therefore could not

explain the reduction in feed intake response of the birds fed the P-deficient diets, a processed peptide of POMC, the α-melanocyte-stimulating hormone (**α-MSH**) is strongly anorexigenic in broiler chickens (Honda et al., 2007; Tachibana et al., 2007; Honda et al., 2012) and may play a role in the P appetite responses in the birds.

In the current study, there was upregulation of hypothalamic CCKAR, MC3R and MC4R in birds fed P-deficient diets. Upregulation of CCKAR directly contradicts the peripheral CCK expression, which was downregulated in birds fed P-deficient diets. While this observation is unclear, this could be due to a negative feedback of the hypothalamic receptor to CCK production in the gut. Alternatively, neuronal synthesis of CCK in the brain has been reported (Innis et al., 1979; Jonson et al., 2000) and may stimulate the CCKAR independently of the CCK originating in the gut. Perhaps, decreased appetite in birds fed P-deficient diet is due more to changes in hypothalamic, and less to peripheral, anorexigenic CCK concentrations. It is also important to state that high-growth haplotypes, like broiler chickens, have been associated with a decreased level of CCK satiety signaling compared with the other genotypes (Dunn et al., 2013). The melanocortin system plays a critical role in whole body energy balance, in addition to appetite-regulatory effects and is mediated via the MCRs (Seeley et al., 2004). In mammals, the anorexigenic effect of α-MSH is mediated by hypothalamic melanocortin receptor; MC4R (Adan et al., 2006; Lee et al., 2006). In addition to MC4R, both MC3R and MC5R are expressed in the chicken brain (Takeuchi and Takahashi, 1998; Ka et al., 2009), although α-MSH has more affinity to MC4R in the chicken brain than MC3R or MC5R (Ling et al., 2004). The melanocortin receptors are strongly anorexigenic in chickens (Tachibana et al., 2001), and could likely explain the decreased feed intake response in birds fed the P-deficient diet. This may suggest a central role of melanocortin receptors in P-induced appetite regulation. However, as previously noted, POMC expression was unaltered by dietary P intake, and therefore presumably the α-MSH concentrations. Because α-MSH concentrations was not measured in the current study, it is not totally clear how the melanocortin receptors are activated to cause the observed changes in feed intake response in the birds. This potentially suggests possible involvement of other genes in dietary P-induced appetite regulation.

One strong candidate is lipocalin-2 (**LNC-2**), a bone-specific osteoblast-derived hormone, with strong anorexigenic functions (Yoshikawa et al., 2011; Mosialou et al., 2017). Previously thought to be an adipokine, expression profiling showed that LNC-2 is expressed at least 10-fold higher in bone than in fat or any other tissue (Yan et al., 2007). Given the central role of osteoblasts in plasma P regulation, and our current results, the physiological role of LNC-2 in P-induced appetite regulation cannot be overlooked. Additionally, it has been reported that increased circulating levels of LNC-2 can cross the blood-brain barrier and act directly on anorexigenic melanocortin receptors with similar affinity to α-MSH (Mera et al., 2018). Although this would require a separate investigation, our current results suggest a potential role for LNC-2 in dietary P induced appetite: First, elevated plasma 1,25 $(OH)_2$ D upregulates NaPi-IIb in response to dietary P deficiency and this increases the intestinal utilization of available P. Additionally, 1,25 $(OH)_2$ D directly stimulates osteoblasts proliferation in the bone (Stern, 1990; Van Leeuwen et al., 2001). 1,25 $(OH)_2$ D also promotes bone resorption by increasing the number and activity of osteoclasts (Suda et al., 1992) in the presence of receptor activator of nuclear factor-κB ligand (**RANKL**) (Kogawa et al., 2010). Osteoclasts perform bone resorption by dissolution and degradation of hydroxyapatite and other organic material releasing Ca and P into the blood to maintain homeostatic balance. Increased osteoblast proliferation increases circulating LNC-2 levels, which crosses the blood-brain barrier. This acts on the melanocortin receptors to decrease feed intake in the birds. Taken together, there appears to be strong evidence for a potential role for LNC-2 in the response to P deficiency in this study.

In summary, these results confirm the impact of dietary P deficiency to induce feed intake suppression in broiler chickens. Notably, dietary P deficiency downregulated CCK gene expression in the gut but upregulated the anorexigenic CCKAR, MC3R, and MC4R genes in the hypothalamus. The current study provides valuable information toward the understanding of appetite regulation in broiler chickens. However, altered gene expression may not always translate to changes in protein synthesis, therefore these data warrants careful interpretation. Given its direct action on melanocortin anorexigenic pathway, a possible role of osteoblast-derived LNC-2 in phosphorus-induced appetite regulation in broiler chickens requires further investigation.

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DISCLOSURES

The authors have no conflicts of interest to report.

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