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Ileal phosphorus digestibility of soybean meal for broiler chickens remains consistent across institutions in a collaborative study regardless of non-phytate phosphorus concentration in the pre-experimental starter diet

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ABSTRACT The same experimental protocol was used in 4 institutions to evaluate the impact of non-phytate phosphorus (**nPP**) concentration in the starter diet on regression method-derived ileal P digestibility of soybean meal (**SBM**) during the subsequent grower phase. A total of 1,536 Ross 308 male broiler chickens on d 0 post hatching were allotted to 2 pre-experimental starter diets that contained 3.5 or 4.5 g nPP/kg (96 replicate cages per diet, 8 birds per cage) for 18 d. Subsequently, 576 birds from each starter diet were selected and allocated to 3 experimental semi-purified grower diets containing 400, 510, or 620 g SBM/kg (32 replicate cages per diet, 6 birds per cage) for 3 d until collection of ileal digesta. Statistical analysis was conducted as a randomized complete block design with the starter period as whole plot and the grower period as split-plot. The only significant 2-way interaction was between grower diet and experimental institution ($P < 0.05$) on BW gain and gain to feed ratio. The main effect of institution and grower diet impacted ($P < 0.05$) feed intake, the digestibility of DM, P, and calcium, and disappearance of inositol hexakisphosphate (**InsP$_6$**) in the grower diets. Birds fed the 3.5 g nPP/kg starter diet had lower ($P < 0.05$) BW gain and feed intake during the grower period, but presented higher ($P < 0.05$) digestibility of P and disappearance of InsP$_6$ compared with the birds that were fed the 4.5 g nPP/kg starter diet. Regression method-derived ileal P digestibility of SBM was determined to be 46 or 42% for the respective 3.5 or 4.5 g nPP/kg pre-experimental starter diet and was not affected by the nPP concentration or by the institution. In conclusion, the experimental protocol used in the current study resulted in similar estimates across multiple institutions and is thus endorsed for future application in studies that aim to expand the database of digestible P content in plant source feed ingredients.

Key words: broiler, digestibility, phosphorus, regression, soybean meal

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INTRODUCTION

Phosphorus (**P**), the second most abundant body mineral after calcium, functions in a variety of important physiological and metabolic processes including skeletal structural support; part of cellular energy currency; integral involvement in carbohydrates, proteins, and lipid metabolism; components of cell membranes, DNA, RNA, and many enzyme systems; and enzyme regulation in broiler chickens, among others. Phosphate, the nutritional currency of P (Zhai et al., 2022) is supplied in diets as inorganic mineral phosphate and/or phytin, the collective term for mixed salts of Mg, Ca, and K of *myo*-inositol with 6 phosphate groups, which is the main storage form of plant P with low utilization by birds (Eeckhout and Paepe, 1994; Maenz and Classen, 1998). The respective nomenclatures phytic acid, phytate, and phytin have been used to refer to free acid, salt, and the mixed salts of Mg, Ca, and K (Adeola and Sands, 2003) for the main storage form in plants. Utilization of these forms of dietary P by birds, of necessity, relies on absorption from the gastrointestinal tract.

Fundamentally, an accurate estimation of the amount of dietary P that is utilized by birds is crucial to prevent deficiency or reduce waste from oversupply. Like many minerals, P has traditionally presented a variety of

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challenges as quantitative determination of utilization can be arduous (Ammerman, 1995). This is particularly true in poultry due to the simultaneous excretion of both feces and urine in the excreta. As a result of the challenges, non-quantitative methods such as expressing P utilization relative to a standard (i.e., slope-ratio approach) have been used to estimate P availability in feed ingredients (Ammerman et al., 1995).

A protocol proposed by the Working Group No 2: Nutrition of the European Federation of Branches of the World's Poultry Science Association for the determination of P availability (WPSA, 2013) involves quantifying the digestibility of P in feed ingredients by collecting digesta from the terminal portion of the small intestine (ileum) of poultry. This technique eliminates the potentially confounding factor of P originating from urine, which is encountered when P digestibility is measured from excreta in poultry. In a ring test in which all participating laboratories supposedly followed the standardized assay protocol, diets mixed in one location were sent to 17 laboratories, chemical analysis, and data analyses for the estimate of ileal P digestibility was at a central location (Rodehutscord et al., 2017). The result of the ring test revealed wide variability in values for ileal P digestibility of soybean meal (**SBM**) among the different research laboratories. The methods proposed in WPSA (2013) may be inadequate due to highly variable estimates of P digestibility of the same feed ingredient among different laboratories (Rodehutscord et al., 2017), indicating that P digestibility values for major feed ingredients cannot be independently verified or agreed upon while employing the standardized assay protocol. Although the exact causes of this large variability are unknown, the wide variability may be principally due to differences in the pre-experimental nutrition and bird husbandry conditions in these participating laboratories. Pre-experimental dietary factors that differed among the laboratories, which could have potentially caused the differences observed in the values for ileal P digestibility of SBM among the different research laboratories were listed in Rodehutscord et al. (2017). One such dietary factor is the concentration of non-phytate P (**nPP**) in the starter diet fed during the pre-experimental period.

Broiler chickens have shown some ability to adapt to diets low in P and Ca by increasing the efficiency of digestion and/or absorption of these nutrients (Yan et al., 2005). Two physiological mechanisms could allow the bird to meet its P requirement when its diet is low in P. First, given the presence of brush border phosphatases in the intestinal tract of the bird (Maenz and Classen, 1998; Bobeck et al., 2016) and the rich supply of phytate P in most poultry diets, perhaps a physiological need for P leads to greater expression of these phosphatases with subsequent greater digestion of P from phytate (Onyango et al., 2006; Rodehutscord et al., 2022). Secondly, there is evidence that the sodium-dependent active P transporters (e.g. Sodium-phosphate cotransporter IIb (**NaPi-IIb**)) are modulated by dietary nPP content, with the efficiency of P absorption increased at low dietary nPP concentrations (Yan et al., 2007; Huber et al., 2015). Therefore, some of the variability between laboratories conducting ileal digestible P assays could potentially be due to differences in the efficiency of P absorption from the variation in nPP concentrations of the starter diet fed during the pre-experimental period, that carry over to the experimental period when birds consume the test diets for the P digestibility experiment.

From the foregoing, the objective of the current study was to determine the impact of 2 dietary concentrations of nPP during the starter pre-experimental period (d 0 −18 post hatching) on regression method-derived ileal digestibility of P in SBM fed to broiler chickens during the subsequent experimental period (d 18−21), and on the consistency of a standardized protocol for the determination of P digestibility conducted at 4 institutions. The null hypothesis tested in the experiment is that feeding broiler chickens a starter diet that is below the required nPP concentration at 3.5 g nPP/kg during the pre-experimental period will result in a subsequent regression method-derived ileal digestibility of P in SBM during the grower period that is not different from feeding a starter diet that is adequate in the required nPP concentration at 4.5 g nPP/kg across the 4 participating institutions.

MATERIALS AND METHODS

Experimental Design

This research was conducted at 4 institutions (Auburn University, Auburn, AL; Purdue University, West Lafayette, IN; University of Illinois at Urbana-Champaign, Urbana, IL, and University of Kentucky, Lexington, KY). All protocols used in the study were approved by the Animal Care and Use Committee of each institution.

The same batch of all the diets (2 pre-experimental starters and 3 experimental growers) were mixed at Purdue University and shipped to all participating institutions. The 4 institutions procured male Ross 308 broiler chicks from the same commercial hatchery, although they originated from different hatches and were received at different times. Birds were individually tagged with identification numbers, weighed, and allotted on d 0 post hatching to 2 pre-experimental starter diets (Table 1) formulated to contain 3.5 g nPP/kg (low nPP) or 4.5 g nPP/kg (adequate). Each pre-experimental starter diet was fed to 96 (24 per institution) replicate cages with 8 birds per cage in a randomized complete block design with initial BW as the blocking factor. Birds were raised in battery cages, located in a thermostatically controlled room, and had unlimited access to feed and water.

On d 18 post hatching, all 1,536 birds were weighed and the ratio of the average BW of birds on the 3.5 g nPP/kg diet (768 birds) to those on the 4.5 g nPP/kg diet (768 birds) was calculated. Subsequently within each pre-experimental starter diet group, 576 birds were

Table 1. Ingredients and nutrient composition of pre-experimental starter and experimental grower diets fed to broiler chickens on an as-fed basis.

	Starter diet		Experimental grower diet		
	Non-phytate P, g/kg		Soybean meal level, g/kg		
Ingredient, g/kg	3.5	4.5	400	510	620
Ground corn	564.1	555.9	-	-	-
Soybean meal, 48% CP[1]	360.0	360.0	400.0	510.0	620.0
Cornstarch	-	-	431.1	319.2	207.3
Dextrose	-	-	80.0	80.0	80.0
Soybean oil	45.0	45.0	30.0	30.0	30.0
Dried egg albumen	-	-	18.0	18.0	18.0
Ground limestone	9.4	13.0	6.9	8.8	10.7
Monocalcium phosphate	10.5	15.1	-	-	-
Salt	4.0	4.0	3.0	3.0	3.0
L-Lysine HCl	1.3	1.3	-	-	-
DL-Methionine	2.5	2.5	2.7	2.7	2.7
L-Threonine	0.2	0.2	0.3	0.3	0.3
Vitamin-mineral premix[2]	3.0	3.0	3.0	3.0	3.0
Chromic oxide premix[3]	-	-	25.0	25.0	25.0
Total	1,000.0	1,000.0	1,000.0	1,000.0	1,000.0
Analyzed nutrients					
Dry matter, g/kg	888	885	890	890	890
P, g/kg	5.8	6.7	2.5	3.2	3.6
Ca, g/kg	8.0	8.4	4.6	5.6	7.2
Ins(1,2,3,4,5)P_5-P, μmol/g	-	-	0.2	0.3	0.3
Ins(1,2,4,5,6)P_5-P, μmol/g	-	-	0.4	0.5	0.6
InsP_6-P, μmol/g	-	-	7.2	8.6	10
Calculated nutrients					
ME_n, kcal/kg	3,107	3,079	3,289	3,092	2,896
Crude protein, g/kg	221	220	209	261	313
Total P, g/kg	5.9	6.9	2.4	3.1	3.7
Non-phytate P, g/kg	3.5	4.5	0.9	1.2	1.5
Ca, g/kg	7.0	9.0	4.8	6.1	7.4

[1]Analyzed to contain 5.98 g total P/kg; 5.8 g Ca/kg.
[2]Provided the following quantities per kg of complete diet: vitamin A, 5,145 IU; vitamin D_3, 2,580 IU; vitamin E, 17.15 IU; menadione, 4.38 mg; riboflavin, 5.49 mg; D-pantothenic acid, 11.0 mg; niacin, 44.1 mg; choline chloride, 771 mg; vitamin B_{12}, 0.01 mg; biotin, 0.06 mg; thiamine mononitrate, 2.20 mg; folic acid, 0.99 mg; pyridoxine hydrochloride, 3.30 mg; I, 1.11 mg; Mn, 107 mg; Cu, 4.44 mg; Fe, 73.5 mg; Zn, 179 mg; Se, 0.43 mg.
[3]Prepared as 1 g chromic oxide added to 4 g cornstarch for a 20% premix.

selected such that the calculated ratio of the average BW of birds between the 2 groups during the pre-experimental starter period was maintained (Table 2). Selected birds within each pre-experimental starter diet group were then allotted to 3 experimental grower diets containing graded concentrations of SBM at 400, 510, or 620 g/kg, with 32 (8 per institution) replicate cages per treatment and 6 birds per cage. It was a 2 × 3 factorial arrangement of treatments with 2 pre-experimental starter diets and 3 experimental SBM grower diets in a split-plot arrangement such that the 2 pre-experimental starter diets were the main plots, and the 3 experimental SBM grower diets were the subplots. The corn and soybean meal used in the pre-experimental starter, and SBM used in the experimental grower diets were medium ground by a hammermill grinder. The mash diets were mixed in a horizontal ribbon mixer according to the manufacturer's recommendations at the Purdue University Feed Mill for uniformity and shipped to all the participating institutions. Birds were fed semi-purified experimental grower diets containing SBM as the test ingredient from d 18 to 21 post hatching.

Birds were weighed individually and euthanized by CO_2 asphyxiation on d 21 and dissected to excise the ileum, which is the portion of the distal small intestine from the Meckel's diverticulum to the ileocecal junction. Ileal digesta samples were flushed out of the distal two-thirds of the ileum up to 2 cm proximal to the ileocecal junction with distilled water. Collected ileal digesta samples were pooled within cage and immediately stored at −20°C.

Laboratory Analysis

Ileal digesta samples were freeze-dried and ground to pass through a 0.5-mm screen (Retsch ZM 100, GmbH & Co. K.C., Haan, Germany). Diets, SBM, and ileal digesta samples from the 4 institutions were analyzed at Purdue University, to minimize inter-lab variation, for DM, chromium, P, and Ca. Dry matter content was determined by drying the samples at 105°C overnight in a forced-air drying oven (Precision Scientific Co., Chicago, IL; method 934.01; AOAC, 2006). Grower diets and ileal digesta were analyzed for chromium concentration using a spectrophotometer (Spark 10 M; Tecan Group Ltd., Männedorf, Switzerland) after wet-ash digestion as described by Fenton and Fenton (1979). Subsequently, the concentration of P in samples was also determined from digested samples by spectrophotometry with absorbance read at 630 nm. Calcium concentration in samples was determined by flame atomic absorption spectrometry using Varian Spectr. AA 220FS (Varian Australia Pty Ltd., Victoria, Australia).

Table 2. Summary of experiment layout, replications, and BW of birds for the 2 pre-experimental starter and 3 experimental grower diets across the 4 experimental institutions.[1]

			Starter period			
Institution	Starter diet, g of nPP/kg	Grower diet SBM level, g/kg	Replicate cages	Birds per cage	D 0 BW, g/bird	D 18 BW, g/bird
1	3.5		24	8	37	553
	4.5		24	8	37	563
2	3.5		24	8	42	559
	4.5		24	8	42	569
3	3.5		24	8	41	670
	4.5		24	8	41	687
4	3.5		24	8	42	618
	4.5		24	8	42	643
			Grower period			
			Replicate cages	Birds per cage	D 18 BW, g/bird	D 21 BW, g/bird
1	3.5	400	8	6	610	765
1	3.5	510	8	6	610	803
1	3.5	620	8	6	610	824
1	4.5	400	8	6	620	784
1	4.5	510	8	6	620	830
1	4.5	620	8	6	620	847
2	3.5	400	8	6	577	744
2	3.5	510	8	6	576	768
2	3.5	620	8	6	577	768
2	4.5	400	8	6	593	756
2	4.5	510	8	6	589	788
2	4.5	620	8	6	590	796
3	3.5	400	8	6	723	837
3	3.5	510	8	6	725	885
3	3.5	620	8	6	724	914
3	4.5	400	8	6	739	858
3	4.5	510	8	6	739	914
3	4.5	620	8	6	739	908
4	3.5	400	8	6	651	787
4	3.5	510	8	6	651	797
4	3.5	620	8	6	650	820
4	4.5	400	8	6	672	811
4	4.5	510	8	6	671	828
4	4.5	620	8	6	672	864

[1]nPP: nonphytate phosphorus; SBM: soybean meal.

The ileal digesta samples from 2 of the 4 participating institutions as well as diet samples were sent to University of Hohenheim that contributed via analyses of samples for inositol phosphate (**InsP**) concentration. Concentrations of $InsP_6$ and $InsP_5$ in the grower diets and ileal digesta samples were analyzed following EDTA extraction at pH 10 using high-performance ion chromatography as described by Zeller et al. (2015) with slight modifications described by Sommerfeld et al. (2018).

Calculations and Statistical Analysis

The apparent ileal digestibility (**AID**) of P, Ca, and DM, and the disappearance of $InsP_6$ in the diets were calculated using the index method (Kong and Adeola, 2014).

$$AID\ or\ Disappearance\ (\%) = 100 - \left[\left(\frac{Cr_i \times P_o}{Cr_o \times P_i}\right) \times 100\right]$$

where Cr_i is the chromium concentration in the diets and Cr_o is the concentration of chromium in ileal digesta; P_i is the P, Ca, DM, or $InsP_6$ concentration in the diets, and P_o is P, Ca, DM, or $InsP_6$ concentration in ileal digesta. Apparent ileal digestible P (**AIDP**) in the diets were calculated by the multiplication of the dietary concentration of P ($\mathbf{P_{diet}}$) in g P/kg diet and respective AID of P divided by 100.

Data were analyzed as a randomized complete block design. Growth performance data during the starter pre-experimental period (d 0−18 post hatching) were analyzed using the following model: $Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{jk} + \varepsilon_{ijk}$, where μ is the overall mean; α_i is the effect of the i^{th} block (23 df); β_j is the effect of the j^{th} institution (3 df); γ_k is the effect of k^{th} pre-experimental starter diet (1 df); $(\beta\gamma)_{jk}$ is the interaction effects of institution and pre-experimental starter diet (3 df); and ε_{ijkl} is the error term. The interaction effect, $(\beta\gamma)_{jk}$, was pooled into the error term in the absence of an interaction.

Data from the grower period (d 18−21 post hatching), during the feeding of the 3 experimental SBM grower diets, were analyzed as a split-plot experiment using the following model: $Y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \delta_l + (\beta\delta)_{jl} + (\gamma\delta)_{kl} + (\beta\gamma\delta)_{jkl} + \varepsilon_{ijklm}$, where μ is the overall mean; α_i is the effect of the i^{th} block (7 df); β_j is the effect of the j^{th} institution (3 df); γ_k is the effect of k^{th} pre-experimental starter diet (1 df); $(\alpha\beta)_{ij}$ is the interaction effects of block and institution (21 df), which is the error term for testing

the effects of institution; $(\alpha\gamma)_{ik}$ is the interaction effects of block and pre-experimental starter diet (7 df), which is the error term for testing the effects of pre-experimental starter diet; $(\beta\gamma)_{jk}$ is the interaction effects of institution and pre-experimental starter diet (3 df); $(\alpha\beta\gamma)_{ijk}$ is the interaction effects of block, institution, and pre-experimental starter diet (21 df), which is the whole-plot error term for testing the interaction effects of institution and pre-experimental starter diet; δ_l is the effect of the l^{th} experimental grower SBM diet (2 df); $(\beta\delta)_{jl}$ is the interaction effects of institution and experimental grower SBM diet (6 df); $(\gamma\delta)_{kl}$ is the interaction effects of pre-experimental starter diet and experimental grower SBM diet (2 df); $(\beta\gamma\delta)_{jkl}$ is the interaction effects of institution, pre-experimental starter diet, and experimental grower SBM diet (6 df); and ε_{ijklm} is the split-plot error term. The interaction effects were pooled into the error term when there was a lack of interaction. Effects of the increasing concentrations of SBM on P digestibility were determined using linear contrast.

Coefficient of ileal digestibility of P in SBM was determined by regressing AIDP in the diets on the concentration of P in the diet (P_{diet}) to generate a slope, that represents the regression method-derived ileal P digestibility of SBM, and the intercept as described by Dilger and Adeola (2006) and Bolarinwa and Adeola (2012). The following SAS statements were used for the combined regression analyses of all the data from the 4 institutions and 2 pre-experimental starter diets:

Proc GLM; Class INS ST; Model AIDP = INS*ST* Pdiet /solution ss3;

where AIDP is the apparent ileal digestible P (the dependent variable, g P/kg diet), P_{diet} is the concentration of P in the diet (the regressor independent variable, g P/kg diet), the solution option was used to generate the 8 slopes for the interaction of 4 INS (institutions) and 2 ST (pre-experimental starter diets) and the intercept.

Proc GLM; Class INS; Model AIDP = INS* Pdiet /solution ss3;

where AIDP and P_{diet} are as defined above, the solution option was used to generate the 4 slopes for 4 INS (institutions) and the intercept.

Proc GLM; Class ST; Model AIDP = ST* Pdiet /solution ss3;

where AIDP and P_{diet} are as defined above, the solution option was used to generate the 2 slopes for 2 ST (pre-experimental starter diets) and the intercept.

The slope was multiplied by 100 to convert coefficient to percent ileal digestibility of P in SBM and statistical significance of difference was set at $P < 0.05$ for all analyses.

RESULTS

The d 0 and 18 BW of birds are presented in Table 2. All 4 institutions that conducted the animal experiments utilized the same strain of broiler chicken, male Ross 308, in order to minimize any variation due to bird strain that may exist. Across institutions, d 0 BW ranged from 37 to 42 g and expectedly, BW range on d 18 was much wider from 553 to 687 g (Table 2). In anticipation of these differences across participating institutions and a pre-experimental starter diet-induced changes in BW gain, it was planned that each institution maintains the ratio of the average BW of all the birds on the 3.5 g nPP/kg diet to those on the 4.5 g nPP/kg diet during the subsequent d 18 allotment to the experimental grower diets within each pre-experimental starter diet. These ratios were 0.98 for each of institution numbers 1, 2, and 3 and 0.97 for institution number 4 (Table 2).

There were main effects ($P < 0.05$) of institution, pre-experimental starter diet, and experimental grower diet on BW gain, but no 3-way interactions were observed in any response criteria during the grower period from d 18 to 21 post hatching (Table 3). The birds that received 4.5 g nPP/kg during the pre-experimental starter period had greater ($P < 0.05$) BW gain and feed intake in the grower period than those that received pre-experimental 3.5 g nPP/kg. Further, there were linear increases ($P < 0.001$) in BW gain, feed intake, and gain to feed ratio as dietary SBM in the experimental grower diet increased from 400 to 620 g/kg during the grower period from d 18 to 21 post hatching (Table 3).

Institution and experimental grower diet affected ($P < 0.05$) the digestibility of DM, Ca, and P in the grower diet (Table 4). The pre-experimental starter diet only affected ileal P digestibility of grower diet, with a higher ($P < 0.001$) digestibility in birds that received the 3.5 g nPP/kg pre-experimental starter diet than those that received the 4.5 g nPP/kg diet during the pre-experimental starter period. Increasing dietary SBM concentration in the experimental grower diets linearly decreased ($P < 0.01$) the digestibility of DM, Ca, and P regardless of the pre-experimental starter diet that the birds received (Table 4).

Inositol phosphate analysis conducted on diets and ileal digesta samples from institution numbers 3 and 4 revealed greater ($P < 0.01$) disappearance of $InsP_6$ in birds that received the pre-experimental starter containing 3.5 g nPP/kg diet than those that received the 4.5 g nPP/kg diet during the pre-experimental starter period (Table 5). In addition, institution and starter diet affected ($P < 0.01$) the concentrations of Ins(1,2,3,4,5)P_5 and Ins(1,2,4,5,6)P_5 in ileal digesta. Increasing dietary SBM concentration in the experimental grower diets linearly decreased ($P < 0.05$) the disappearance of $InsP_6$ (Table 5). The concentration of Ins(1,2,3,4)P_4 was below the limit of quantification (0.2 μmol/g) in majority of the ileal digesta samples, and no other inositol phosphates were detected.

The regression method-derived ileal P digestibility (%) of SBM for broiler chickens that received 2 pre-experimental starter diets containing 3.5 or 4.5 g nPP/kg and experimental grower diets with increasing concentrations of SBM at 4 institutions is outlined in Table 6. There was no effect of pre-experimental starter dietary nPP concentration on ileal P digestibility of SBM in any of the 4 institutions. Ileal P digestibility of SBM ranged from 42.8 to 46.2% across the 4 institutions

Table 3. Growth performance of broiler chickens that received a pre-experimental starter diet containing 3.5 g/kg non-phytate P (**nPP**) or 4.5 g/kg nPP, and experimental grower diets containing increasing concentrations of soybean meal (**SBM**) from d 18 to 21 at 4 institutions.

			Grower period				
Institution	Starter diet, g of nPP/kg	Grower diet SBM level, g/kg	Replicate cages	Birds per cage	BW gain, g/bird	Feed intake, g/bird	Gain/feed, g/kg
1	3.5	400	8	6	156	255	612
1	3.5	510	8	6	193	263	736
1	3.5	620	8	6	214	271	789
1	4.5	400	8	6	164	258	636
1	4.5	510	8	6	209	271	775
1	4.5	620	8	6	227	283	802
2	3.5	400	8	6	168	238	703
2	3.5	510	8	6	192	241	796
2	3.5	620	8	6	191	243	790
2	4.5	400	8	6	163	235	696
2	4.5	510	8	6	199	241	821
2	4.5	620	8	6	206	245	837
3	3.5	400	8	6	113	229	492
3	3.5	510	8	6	160	253	630
3	3.5	620	8	6	190	270	705
3	4.5	400	8	6	119	245	485
3	4.5	510	8	6	175	270	649
3	4.5	620	8	6	169	257	651
4	3.5	400	8	6	136	204	666
4	3.5	510	8	6	146	217	675
4	3.5	620	8	6	170	224	758
4	4.5	400	8	6	139	218	637
4	4.5	510	8	6	157	222	708
4	4.5	620	8	6	191	240	799
SEM[1]					7.3	5.9	23.6
P-value							
Institution					<0.001	<0.001	<0.001
Starter diet					0.014	0.010	0.276
Grower diet					<0.001	<0.001	< 0.001
Interactions							
Institution*Starter diet					0.425	0.378	0.474
Institution*Grower diet					0.003	0.162	0.006
Starter diet*Grower diet					0.450	0.841	0.302
Institution*Starter diet*Grower diet					0.191	0.165	0.548
Contrasts							
Linear for 3.5 g nPP/kg					< 0.001	0.002	< 0.001
Linear for 4.5 g nPP/kg					< 0.001	0.009	< 0.001
1			48	6	194	267	725
2			48	6	186	241	774
3			48	6	154	254	602
4			48	6	157	221	711
	3.5		96	6	169	242	698
	4.5		96	6	177	249	708
		400	64	6	145	235	618
		510	64	6	179	247	723
		620	64	6	195	254	767
RMSE[2]					23.3	19.5	71.4

[1]Standard error of mean.
[2]Root means square of error; each least squares mean represents 48 replicate cages per institution, or 96 replicate cages per pre-experimental starter diet, or 64 replicate cages per grower diet with 6 birds per cage.

with no statistical difference. Furthermore, pre-experimental starter dietary nPP concentration had no statistical effect on regression method-derived ileal P digestibility of SBM at 46 or 42% for the pre-experimental starter diet containing 3.5 or 4.5 g nPP/kg, respectively (Table 6).

DISCUSSION

A starter diet that is below the required nPP concentration at 3.5 g nPP/kg during the pre-experimental period will result in a subsequent regression method-derived ileal digestibility of P in SBM during the grower period that is not different from feeding an nPP-adequate starter diet (4.5 g nPP/kg) to broiler chickens across the 4 participating institutions is the null hypothesis that was tested in the current study. There was a lack of sufficient statistical evidence to reject the null hypothesis that ileal P digestibility of SBM is not affected by pre-experimental starter nPP concentration at 3.5 g nPP/kg diet compared with 4.5 g nPP/kg diet. The hypothesis was borne out of the publication by Rodehutscord et al. (2017) that reported the results of a 17-institution collaborative project in which the ileal digestibility of P in the same SBM ranged from 19 to

Table 4. Apparent ileal digestibility (%) of dry matter, calcium, and phosphorus in experimental grower diets containing increasing concentrations of soybean meal (**SBM**) for broiler chickens that received 2 pre-experimental starter diets containing 3.5 g/kg non-phytate P (**nPP**) or 4.5 g/kg nPP at 4 institutions.

Institution	Starter diet, g of nPP/kg	Grower diet SBM level, g/kg	Dry matter	Calcium	Phosphorus
1			75.2	58.2	59.2
2			78.3	61.2	56.3
3			76.7	55.6	56.0
4			79.6	61.2	57.4
	3.5		77.4	60.1	59.2
	4.5		77.5	58.0	55.2
		400	82.1	66.4	59.4
		510	76.9	55.7	58.2
		620	73.3	55.0	54.1
RMSE[1]			2.26	9.41	5.93
P-value					
Institution			<0.001	0.009	0.036
Starter diet			0.712	0.127	<0.001
Grower diet			<0.001	<0.001	<0.001
Contrasts					
Linear for 3.5 g nPP/kg			<0.001	<0.001	0.002
Linear for 4.5 g nPP/kg			<0.001	<0.001	0.001

[1]Root means square of error; each least squares mean represents 48 replicate cages per institution, or 96 replicate cages per pre-experimental starter diet, or 64 replicate cages per grower diet with 6 birds per cage.

51% among institutions. Given that the same recommended assay protocol was followed by all institutions, the experimental SBM diets were mixed at a central location, shipped to all 17 institutions and therefore same for all the participants, and together with the chemical analysis and data processing conducted at one central location, Rodehutscord et al. (2017) reasoned that the encountered variability must result from differences in pre-experimental factors such as concentration of nPP in the starter diets for the birds at the participating institutions.

Optimizing dietary nutrient utilization is essential to improve the efficiency of poultry production and reduce feed costs and waste. Advancing the application of digestible mineral matrix in broiler chicken nutrition hinges on ensuring the accuracy and repeatability of digestibility values for feedstuffs determined in animal trials. In this context, several factors have been shown to impact the digestion and absorption of P, such as dietary concentration of P (Yan et al., 2005; Li et al., 2016; Rousseau et al., 2016), Ca:P ratio (Liu et al., 2013; Perryman et al., 2017), age of birds and length of experiments (Li et al., 2018; Babatunde et al., 2019), assay methodology (Mutucumarana et al., 2015; An et al., 2020). Additionally, elements pertaining to the composition of the starter diet can potentially impact the determination of P digestibility in feed ingredients.

To minimize bird strain variation, all 4 participating institutions used Ross 308 from the same hatchery, albeit within 7 weeks of one another. The BW on d 0 that ranged from 37 to 42 g perhaps resulted from the travel distances and conditions during shipping. It was anticipated that feeding pre-experimental starter diet containing 3.5 vs. 4.5 g nPP/kg for 18 d would affect

Table 5. Inositol phosphate (**InsP**) concentration in ileal digesta, and inositol hexakisphosphate (**InsP$_6$**) disappearance (%) in experimental grower diets containing increasing concentrations of soybean meal (**SBM**) for broiler chickens that received 2 pre-experimental starter diets containing 3.5 g/kg non-phytate P (**nPP**) or 4.5 g/kg nPP at 2 institutions.

			Concentration in ileal digesta			Disappearance
Institution	Starter diet, g of nPP/kg	Grower diet SBM level, g/kg	Ins(1,2,3,4,5)P$_5$, μmol/g DM	Ins(1,2,4,5,6)P$_5$, μmol/g DM	InsP$_6$, μmol/g DM	InsP$_6$, %
3			0.75	0.36	17.73	51.9
4			1.04	0.43	25.00	40.1
	3.5		0.84	0.36	20.04	49.0
	4.5		0.95	0.44	22.69	43.0
		400	0.90	0.38	20.48	50.4
		510	0.84	0.35	21.09	45.1
		620	0.94	0.46	22.52	42.5
RMSE[1]			0.188	0.107	3.104	6.90
P-value						
Institution			< 0.001	0.005	< 0.001	< 0.001
Starter diet			0.004	< 0.001	< 0.001	< 0.001
Grower diet			0.092	0.001	0.032	< 0.001
Contrasts						
Linear for 3.5 g nPP/kg			0.656	0.052	0.018	0.022
Linear for 4.5 g nPP/kg			0.455	0.022	0.199	0.025

[1]Root means square of error; each least squares mean represents 48 replicate cages per institution, or 48 replicate cages per pre-experimental starter diet, or 32 replicate cages per grower diet with 6 birds per cage.

Table 6. Regression method-derived ileal phosphorus digestibility (%) of soybean meal for broiler chickens that received 2 pre-experimental starter diets containing 3.5 g/kg non-phytate P (**nPP**) or 4.5 g/kg nPP and experimental grower diets containing increasing concentrations of soybean meal at 4 institutions.

Institution	Starter diet, g of nPP/kg	Ileal digestibility of P, %
1	3.5	48.9
	4.5	43.5
2	3.5	43.7
	4.5	41.9
3	3.5	45.6
	4.5	40.0
4	3.5	46.0
	4.5	42.4
SEM[1]		3.07
Intercept, g P/kg diet		0.45
SEM of intercept[2]		0.100
R-square		0.60
Pooled by institution		
1		46.2
2		42.8
3		42.8
4		44.2
SEM[1]		3.11
Intercept, g P/kg diet		0.45
SEM of intercept[2]		0.106
R-square		0.55
Pooled by starter diet		
	3.5	46.0
	4.5	42.0
SEM[1]		2.94
Intercept, g P/kg diet		0.45
SEM of intercept[2]		0.102
R-square		0.59

[1]Standard error of mean of ileal P digestibility (%); the calculated standard error of the difference of 2 means ($\sqrt{2}$ x SEM) is indicative of a lack of difference in the ileal P digestibility of soybean meal among institutions or pre-experimental starter diets.

[2]Standard error of mean of the intercept of the regression of the apparent ileal digestible P (the dependent variable, g P/kg diet) on the dietary concentration of P (the regressor independent variable, g P/kg diet).

growth performance prior to introducing the experimental diets. This expectation informed the decision during the planning stages for each institution to maintain the ratio of the average BW of all the birds on the 3.5 g nPP/kg diet to those on the 4.5 g nPP/kg diet during the subsequent d 18 allotment to the experimental grower diets within each pre-experimental starter diet. Indeed, birds on the 3.5 g nPP/kg diet were lighter in BW than those on the 4.5 g nPP/kg pre-experimental diet.

Of relevance is the lack of a 3-way interaction among institutions, pre-experimental starter diet, and experimental grower diet on the 3-d growth performance. Expectedly, there were main effects of institution, pre-experimental starter diet, and experimental grower diet on BW gain presumably due to the differences in BW on d 18. All experimental diets were prepared at the Purdue University feed mill and shipped to the other institutions to ensure homogeneity, additionally, all laboratory analyses of ingredients, diets, and ileal digesta samples were conducted at Purdue University to mitigate variability. Nevertheless, there was an effect of institution on the growth performance and digestibility criteria. These may be attributed to uncontrollable differences among the experimental stations, such as the starting BW of the birds, along with the management of birds, which include the lighting of the room, animal handling, room temperature, and bird space. These, together with differences in the battery cages between institutions may have affected the amount of stress posed on the birds, which can impact the growth performance and digestion of nutrients in broiler chickens (Puvadolpirod and Thaxton, 2000; Virden and Kidd, 2009).

The ileal P digestibility of experimental grower diets ranged between 54% and 59%, and that of Ca ranged between 55% and 66% in the current study. Using diets like those used in the current study, Rodehutscord et al. (2017) reported 48 to 82% for P and 39 to 71% for Ca ileal digestibility, whereas ileal P digestibility range of 33 to 62% was recently reported by Olukosi et al. (2022). There was a higher P digestibility of experimental grower diets and, thus a higher intestinal uptake of P in the group that received the 3.5 g nPP/kg than the 4.5 g nPP/kg pre-experimental starter diet, which is in agreement with previous studies (Yan et al., 2005; Rao et al., 2006; Rousseau et al., 2016; Sommerfeld et al., 2019,2020; Omotoso et al., 2023; Novotny et al., 2023b). Feeding low dietary P to young broilers has been shown to increase P absorption by augmenting the expression of genes encoding the transcellular transport of P in the intestine (Onyango et al. 2006; Yan et al., 2007; Rousseau et al., 2016; Hu et al., 2018; Sommerfeld et al., 2020). This is part of the physiological mechanism for maintaining Ca and P homeostasis, whereby the secretion of parathyroid hormone in response to low serum inorganic P increases P reabsorption in the kidneys, and stimulates the synthesis of calcitriol ($1,25(OH)_2D_3$), that in turn enhances intestinal P absorption and promotes bone resorption (Proszkowiec-Weglarz and Angel, 2013). This was demonstrated by Omotoso et al. (2023), who found higher serum levels of calcitriol in birds that received a starter diet with 50% of nPP requirements for 17 d compared to birds fed adequate or higher dietary levels of nPP.

Statistical differences among experimental diets do not always automatically translate into differences in regression-derived estimates of digestibility as observed in the current study. In regression-derived estimates of ileal P digestibility of SBM, the slope of the regression analysis designates the change in digestible P for every unit increase in dietary P, which in this case is attributed to a higher intake of SBM thus explaining differences in dietary P. However, the main effect of starter diet significantly affected the digestibility of P in the grower diet because it only considers the digestibility variable and does not focus on the relationship between digestible and dietary P.

Analyses of inositol phosphate in diets and ileal digesta samples from 2 institutions revealed higher disappearance of $InsP_6$ in the birds that received the 3.5 g nPP/kg pre-experimental diet, which matched higher P digestibility of experimental diets. This observation agrees with Rodehutscord et al. (2017). Similarly, Yan et al. (2005) reported increases in the disappearance of phytate P measured on d 18, 21, and 23 in birds that were fed a low P starter diet. The lower nPP supply in

the starter diet might have conditioned the birds towards an increased endogenous intestinal mucosal phytase activity that persisted and instigated during the grower phase a higher InsP6 disappearance in birds that received the 3.5 g nPP/kg pre-experimental diet. Previous studies have reported a wide range of phytate disappearance in broiler chickens fed diets not supplemented with exogenous phytase (16−76%) that is attributable to the diversity of intestinal microflora, age of birds, concentration of P and Ca in the diet, and composition of the diet (Rutherfurd et al., 2002; Shastak et al., 2014; Zeller et al., 2015; Sommerfeld et al., 2018). For instance, Rutherfurd et al. (2002) reported phytate disappearance of 19, 42, 24, and 44% in diets based on corn, wheat, rice bran, and SBM, respectively. Without the supplementation of exogenous phytase, $InsP_6$ is hydrolyzed by endogenous and bacterial phytases in the gut and when present, plant phytases. However, plant phytases are largely considered to be inactivated during feed processing or in the acidic pH of the proventriculus (Zeller et al., 2015). Approximately 76% of $InsP_6$ was hydrolyzed before the lower ileum of broilers, with most of this hydrolysis occurring in the duodenum and jejunum (Zeller et al., 2015). Further, Morgan et al. (2015) reported age related gradual increases in intestinal phytase activity and phytate degradation in the gizzard, duodenum, and ileum of birds until d 12, which was accompanied by increases in P and Ca utilization.

In the current study, $InsP_6$ disappearance was 49% in the birds that received the 3.5 g nPP/kg pre-experimental diet compared with 43% in the 4.5 g nPP/kg group, which linearly decreased with higher dietary SBM. This was accompanied by a reduction in P digestibility, which may be a consequence of the increase in dietary P from SBM, especially phytate P that increased from 7.2 to 10.0 μmol/g DM, coupled with an increase in Ca from limestone. Earlier reports indicated that even small increases in dietary Ca concentration can decrease phytate P disappearance. Tamim et al. (2004) observed reductions in phytate P digestion from 69.2 to 25.4% with the addition of 5 g/kg of limestone. Similarly, previous studies showed that lower dietary Ca and P improve phytate P digestion in broilers (Applegate et al., 2003; Shastak et al., 2014; Sommerfeld et al., 2018, 2019; Novotny et al., 2023a). Applegate et al. (2003) found that intestinal phytase activity was 9% greater in birds fed diets with 4 g/kg Ca compared to 9 g/kg, and phytate hydrolysis was 12% greater in birds fed the low Ca diet. Also, Sommerfeld et al. (2018) noted significant decreases, up to 16%, in the disappearance of $InsP_6$ when monosodium phosphate was added to diets and an even greater reduction of 35% with the combined addition of monosodium phosphate and limestone.

Evaluating capacity for phytate hydrolysis exclusively from intestinal enzymes, Sommerfeld et al. (2019) used gnotobiotic broilers fed gamma-irradiated feed and found that $InsP_6$ disappearance was 42% in birds fed a low P and Ca diet, whereas, in birds fed diets supplemented with P and Ca, it was only 17%. This effect is due to the chelation of phytate with Ca^{2+} ions, forming insoluble complexes that impair the activity of phytases, and the negative feedback of higher concentrations of inorganic phosphate on phytase activity (Wodzinski and Ullah, 1996; Bedford and Rousseau, 2017; Novotny et al., 2023a). It is therefore possible that the Ca and P concentration in the starter diet affected the P digestion capacity of broilers in the subsequent period, contributing to variability of P digestibility values as reported by Rodehutscord et al. (2017). Additionally, fiber from SBM may have affected P and InsP digestion via soluble fraction related increased digesta viscosity, mineral binding to insoluble fiber components, or physical entrapment of substrates, that reduces the access of endogenous enzymes to substrates (Baye et al., 2015; Bournazel et al. 2018). However, because fibrous plant feedstuffs generally contain significant amounts of phytate, it is difficult to separate the effect of fiber from that of phytate as fiber at moderate concentrations improves gizzard functional development and may beneficially alter the microbiota and intestinal fermentation (Bournazel et al., 2018).

Standardized methods for determining P utilization in feed ingredients are of relevance in formulating P-adequate broiler chicken diets that avoid deficiency or environmental-issue-related oversupply (Adeola, 1999; WPSA, 2013; Rodehutscord et al., 2017). Observations from a 17-institution collaborative ring test reported by Rodehutscord et al. (2017) revealed a large variability in the estimates of regression method-derived P digestibility of the same feed ingredient among different laboratories and several factors were listed as possible culprits for the observed differences in P digestibility estimates. These factors, according to Rodehutscord et al. (2017), include: 1) Strain of broiler chickens; 2) sex of broiler chickens; 3) age of broiler chickens at sampling; 4) method of euthanasia; 5) pre-experimental housing condition regarding floor pens with access to litter versus cages without access to litter; 6) pre-experimental dietary Ca concentration; 7) pre-experimental dietary coccidiostat; 8) pre-experimental dietary phytase; and 9) pre-experimental dietary P concentration.

Schedle et al. (2016) indicated that P digestibility responses are not different between male and female broilers fed low P diets (item #2 above). Further, Künzel et al. (2019) reported that adding coccidiostats to pre-experimental diet had no effects on regression method-derived P digestibility of SBM or the activity of endogenous or exogenous phytase despite influencing the microbiota composition of the gastrointestinal tract in broiler chickens (item # 7 above). More recently, Olukosi et al. (2022) showed that phytase in the pre-experimental diet did not affect assayed regression method-derived P digestibility of SBM (item # 8 above). In the current study, pre-experimental dietary P concentration had no effects on regression method-derived P digestibility of SBM (item # 9 above). It is noteworthy that the current study as well as those reported in Rodehutscord et al. (2017) and Olukosi et al. (2022) used experimental diets that were identical in ingredient composition and similar in dietary SBM concentrations.

Of relevance is the consistency among institutions in the regression method-derived P digestibility of SBM for each of the 3.5 and 4.5 g nPP/kg pre-experimental diet and the absence of institution by pre-experimental diet interaction, which reinforces the lack of an influence of pre-experimental dietary P concentration on regression method-derived P digestibility of SBM. The current range from 43 to 46% P digestibility of soybean across institutions is considerably less variable than the 19 to 51% in the ring test (Rodehutscord, 2017), which is attributable to more stringent standardization of protocols and fewer participants. Furthermore, these estimates are similar to some reported values in the literature of 45% (Rostagno et al., 2017; Haetinger and Adeola, 2023), but lower than other reports that range from 55 to 65% (Rutherfurd et al., 2002; Liu et al., 2013; Babatunde et al., 2020). Perhaps these discrepancies result from differences in bird management, diets, SBM P concentration, euthanasia procedure, or analytical methods. Following the standardized protocol in 4 institutions in the current study mitigated procedure- and soybean-related variability in determining the P digestibility of SBM. It can be concluded that this was successful, as the experimental institution did not exert a significant effect on the regression method-derived P digestibility.

Severe P deficiency during the starter phase leads to impaired bone growth and mineralization, lower bone-breaking strength, reduced weight gain, and increased mortality (Yan et al., 2005; Sung et al., 2024). In addition, low dietary P has been shown to lead to decreased levels of triiodothyronine in the serum of broilers that is indicative of hypothyroidism associated with P deficiency which, in turn, increases bone fracture risk and reduces weight gain (Omotoso et al., 2023). However, mild reductions in dietary nPP such as the level of 3.5 g/kg used in the current study had only slight effects on growth performance. Further, this effect was more notable during the grower phase, when the diets were severely deficient in P, with superior weight gain and feed intake in the group that received the 4.5 g nPP/kg during the starter phase.

In summary, the regression method-derived P digestibility of SBM was not affected by the concentration of nPP in the pre-experimental starter diet given that the regression method-derived ileal digestibility of P in SBM during the grower period was not different between broiler chickens that received 3.5 g nPP/kg pre-experimental diet at 46% and those that received pre-experimental starter diet containing 4.5 g nPP/kg at 42%. Furthermore, the regression method-derived ileal digestibility of P in SBM during the grower period was not different across the 4 participating institutions regardless of the concentration of non-phytate phosphorus in the pre-experimental starter diet. The experimental protocol used in the current study, which is based on Rodehutscord et al. (2017), resulted in similar estimates across multiple institutions and is thus endorsed for future application in studies that aim to expand the database of digestible P content in plant source feed ingredients.

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DISCLOSURES

The authors declare no conflicts of interest.

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