Evaluation of a new generation phytase on phytate phosphorus release for egg production and tibia strength in hens fed a corn-soybean meal diet

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ABSTRACT To test the effect of several inclusion levels of *Citrobacter braakii* phytase (CBP), on phytate P release, 420 50-wk-old-Bovans White hens were randomly allocated to 7 treatments with 5 replicates of 12 hens each. The experimental period lasted 12 weeks, first 8 for adaptation and last 4 for data collection. Feed and water were provided ad libitum. Treatments were: (1) a 0.12% basal corn-soybean meal diet deficient only in non-phytate P. Treatments 2 and 3 were added with constant increases of 0.11% inorganic P, to get a linear hen response to P addition. Treatments 4 to 7 were the addition of 300; 600; 1,200; and 1,800 phytase units (FYT)/kg to the basal diet. Variables analyzed were hen productive performance (HPP) and tibia resistance to fracture (TRF), and mineral content. Data were analyzed as a Complete Randomized Design (CRD). The results from treatments 1 to 3 were analyzed by a regression model to test for a significant linear response ($P < 0.05$). Then for every level of CBP added (treatments 4 to 7), the linear regression equation was solved to find out the equivalent value of released P. Based on hen health and welfare, the response variables that yielded realistic P equivalence values for the CBP levels used in the present trial were the tibia data. Following the significant ($P < 0.001$) linear response, the equations; TRF, kg (Y = 28.16X + 17.42 R^2 = 0.84); Tibia Ca, % (Y = 11.6X + 14.2 R^2 = 0.80); Tibia P, % (Y = 11.6X + 6.1 R^2 = 0.81); and T ash, % (Y = 33.3X + 38.1 R^2 = 0.80). Under the experimental conditions of this trial, the HPP variables were not a sensitive parameter to measure P release; whereas, tibia parameters showed the following average P release values per level of CBP inclusion in the corn- soybean meal diet; 300 FYT/kg = 0.099%, 600 FYT/kg = 0.141%, 1,200 FYT/kg = 0.182%, and 1,800 FYT/kg = 0.198%.

Key words: *Citrobacter Braakii*, phytase hens, phosphorus

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INTRODUCTION

Addition of phytase enzyme to feedstuffs has become a common practice in animal production, being the first phytase activities from fungal origin, designed to release a fixed amount of 1 kg of phosphorus per metric ton of feed. Later, phytase activities from bacterial source, such as *E. coli*, proved to be more efficient to degrade the phytate molecule, allowing the release of higher amounts pf phosphorus in the feed (Augspurger et al., 2003). Regarding the response of the laying hen to the P level in their feed, either alone or in combination with phytase enzyme, there are several issues that have to be taken into account, for example, the hen response to a very low P level in the feed. Feeding Hy Line W 36 hens with 78 mg/h/d non-phytate P (**NPP**), with no phytase addition, took 12 wk to observe a significant decrease on egg production compared to hens fed 420 mg/h/d NPP; this trial lasted 16 weeks (Gordon and Roland, 1997). Whereas, feeding Dekalb Delta White hens with 105 mg/h/d NPP, with no phytase addition, took 8 wk to observe a significant decrease on egg production, compared to hens fed 486 to 500 mg/h/d (Boling et al., 2000a,b; Snow et al., 2004). In these trials, the hens fed low NPP levels showed a complete stop on egg production after 17 (Snow et al., 2004) or 22 wk (Boling et al., 2000a,b) on trial. Another important parameter to consider is the effect of feeding different P levels on bone composition, because the hen has been subject of genetic selection; to become a very efficient egg producer, its bone metabolism has become very active and efficient.

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Each eggshell contains about 2.3 g of Ca (Miller, 1992). A modern laying hen may produce 426 eggs in a 90 wk production cycle (Bovans White, 2017), which means the deposit of 980 g of Ca. Considered that the hen skeleton represents around 11% of a hen´s empty body weight (Gregory and Robins, 1998). The hen deposits were almost 5.2 times the weight of its skeleton as Ca in the eggshell. This extraordinary utilization of Ca is possible due to the development of the medullary bone; this bone type is unique to female birds in reproductive status and it has also been found in fossils of non-avian dinosaurs, such as the theropods *Tyrannosaurus rex* and *Allosaurus fragilis*, but not in any other animal or fossil (Schweitzer et al., 2016). At the onset of sexual maturity, the function of osteoblasts changes from forming lamellar cortical bone to produce the woven medullary bone (Whitehead, 2004). A unique feature of the medullary bone is its capacity for being quickly resorbed by the osteoclasts and deposited by the osteoblasts (Whitehead and Fleming, 2000). It is important to mention that for the medullary bone formation, it is necessary the presence of Ca and P in the blood stream, because hydroxyapatite crystals of calcium phosphate form bone. When the hen is fed low levels of P for long periods, she cannot build up back her medullary bone reserves. So, in time, the hen starts taking the Ca she needs for eggshell formation from its structural bone, both cortical and trabecular; and because these 2 forms of bone cannot be resorbed and deposited as easily as the medullary bone, the hen starts showing weak bones (Whitehead and Fleming, 2000). Therefore, when doing the evaluation of the effect of phytase enzyme on the laying hen P utilization, it will be necessary to consider on one hand the time it takes for the hen to show the effect of low P intake on egg production, as well as its effect on bone strength and composition. So, based on the former information, the objective of the present work was to evaluate the effect of feeding a 6-phytase from *Citrobacter braakii* (RONOZYME® HiPhos) on laying hen performance, bone mineralization, as well as to obtain relative P release equivalence values when compared to inorganic P added from Monodicalcium phosphate (MDCP).

MATERIALS AND METHODS

All Animal procedures were approved by the Universidad Nacional Autónoma de México (UNAM) committee on Laboratory Animal Care.

Birds and Diets

Four hundred twenty number of 50-wk-old-Bovans White hens were used to perform the present trial. The hens were kept in cages (3 birds per cage) measuring 1,800 cm^2, which provided 600 cm^2 cage space per bird. The cages were built in groups of 4 cages, which was deemed the experimental unit (12 hens/replicate). During the experimental period, 16 h of light and 8 h of dark were provided. The trial was conducted at UNAM experimental poultry farm, located in Mexico City (2,200 m above sea level) with temperate weather (18 to 22°C). The experimental facility was an open hen house with curtains. All hens were fed a corn-soybean meal (**SBM**) diet, formulated to fulfill all laying hen nutritional requirements (NRC, 1994), but P. A basal diet with 0.12% NPP was formulated, containing 1 kg per metric ton of MDCP (Table 1). The corn and the SBM were analyzed for AA, protein, and phytic P with NIR technology (AminoDAT Platimun Versión 5.0, Evonik industries). Corn and SBM total P, and MDCP total Ca and P content were analyzed with the official AOAC methodology (AOAC, 2007). Calculation of NPP content in the diet was done by subtracting phytic acid content from total P value. The experimental diets were formulated with the analytical values found in the mentioned ingredients. Limestone in the feed was provided as 50% particulate; 3 to 5 mm particle size and 50% ground < 3 mm particle size. The first 3 treatments had increasing levels of inorganic P going from 0.12% NPP to 0.34% NPP, whereas treatments 4 to 7 had the same level of NPP; 0.12% NPP, but with the addition of increasing levels of phytase activity from *Citrobacter braakii*, going from 300 to 1,800 phytase units (**FYT**)/kg of feed (1 FYT is defined as the activity that releases 1 μmol inorganic phosphate from 5.0 mM sodium phytate/m at pH 5.5 and 37°C (Engelen et al., 1994). To keep all feeds with the same composition but MDCP, an inert filer, silica was used to substitute the MDCP as needed. The hens were fed the experimental feeds in a period of 12 wk; based on the information regarding the time it takes to observe the hen response to the level of P in the feed (Gordon and Roland 1997; Boling et al., 2000a,b; Snow et al., 2004), it was decided to use the first 8 wk as an adaptation period, performing the hen productive performance data analysis on the last 4 wk of the trial. At the beginning and at the end of the experimental period, all eggs per replicate were collected for 4 d, to assure a sample of a least 30 eggs per replicate to measure egg shell strength. At the end of the experiment, 3 hens per replicate were killed by cervical dislocation, then both tibias were dissected out for subsequent analysis. The parameters analyzed were as follows: egg production, egg mass, feed intake, NPP intake, feed conversion, mortality, egg shell resistance to fracture (RF), tibia RF, Ca, P, and tibia ash content (AOAC, 2007).

Tibia and egg shell RF were analyzed by a QC-SPA shell strength and packaging analyzer (Technical services and supplies Ltd. Chessingham Park Dunnigtong York, England).

Statistical Analysis

All data were analyzed by analysis of variance procedures for a complete randomized design (Steele and Torrie, 1980). Statistical significance was determined at $P < 0.05$. When a statistical significance for the model

Table 1. Ingredient and nutrient compositions of the experimental diets.

	Diet						
Ingredients	1	2	3	4	5	6	7
	%						
Yellow corn[1]	66.43	66.43	66.43	66.43	66.43	66.43	66.43
SBM[1]	20.30	20.30	20.30	20.30	20.30	20.30	20.30
$CaCO_3$[2]	10.60	10.39	10.20	10.60	10.60	10.60	10.60
Soy oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00
NaCl	0.28	0.28	0.28	0.28	0.28	0.28	0.28
Vit-Min[3]	0.30	0.30	0.30	0.30	0.30	0.30	0.30
NaHCO3	0.15	0.15	0.15	0.15	0.15	0.15	0.15
DL Met	0.14	0.14	0.14	0.14	0.14	0.14	0.14
BioLys 65%	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Silica[4]	0.65	0.33	–	0.65	0.64	0.64	0.63
Monodicalcium phosphate (MDCP)[5]	0.10	0.63	1.15	0.10	0.10	0.10	0.10
Phytase[6]	–	–	–	0.00	0.01	0.01	0.02
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Nutrients[7]							
ME, kcal/kg	2800.00	2800.00	2800.00	2800.00	2800.00	2800.00	2800.00
CP, %	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Dig Lys, %	0.68	0.68	0.68	0.68	0.68	0.68	0.68
Dig Met, %	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Dig SAA, %	0.55	0.55	0.55	0.55	0.55	0.55	0.55
Dig Thr, %	0.48	0.48	0.48	0.48	0.48	0.48	0.48
Ca, %	4.20	4.20	4.20	4.20	4.20	4.20	4.20
P, %	0.35	0.46	0.57	0.35	0.35	0.35	0.35
NPP, %	0.120	0.230	0.340	0.120	0.120	0.120	0.120
Phytase, FYT/kg	–	–	–	300.00	600.00	1200.00	1800.00
Phytase found, FYT/kg	BDL	BDL	BDL	345.30	580.60	1350.00	1900.40

[1]Total P analyzed with the official AOAC (2007) methodology.
[2]50% particle size between 3 to 5 mm (particulate) and 50% particle size < 3 mm (ground).
[3]Vitamin-mineral premix contained the following by kg of feed: vitamin A (12,000 IU), vitamin D_3 (3,500 UI), vitamin E (30 mg), vitamin K_3 (3.5 mg), thiamine (2.5 mg), riboflavin (7.0 mg), pyridoxine (3.5 mg), vitamin B_{12} (0.02 mg), niacin (30 mg), pantothenate (10 mg), folate (1.0 mg), biotin (0.1 mg), choline (300 mg), copper (15 mg), iron (60 mg), manganese (110 mg), iodine (1.0 mg), zinc (80 mg), and selenium (0.3 mg).
[4]Inert filler; SiO_2.
[5]Total Ca and P analyzed with the official AOAC (2007) methodology; 21% P, 17% Ca.
[6]*Citrobacter Braakii* phytase; Ronozyme® HiPhos 10,000 FYT/g. DSM Nutritional Products.
[7]Formulated from analyzed corn and SBM values for AA, protein, and phytic P with NIR technology (AminoDAT Platimun Versión 5.0, Evonik industries).

was present, Fisher's Least Significant Difference means separation test was used to determine significant differences among individual treatment means (Steele and Torrie, 1980).

Phosphorus Equivalence Calculation

The responses recorded from the first 3 treatments, were used to create a linear regression equation (Steele and Torrie, 1980) between each variable measured as function of the NPP level in the feed, as follows:

$$Y = a + bX.$$

Where
Y = dependent variable (any response variable),
a = intercept (the value of y when x = 0),
b = slope of the line,
X = explanatory variable (NPP % in the feed).
Then for the remaining 4 treatments (basal feed supplemented with the corresponding phytase level), each response point was solved with the respective regression equation X = (Y−a)/b to find the respective NPP % equivalence value.

RESULTS AND DISCUSSION

As expected, due to the P feed formulation strategy designed for this research, the NPP intake was statistically different ($P < 0.0001$) for each one of the first 3 dietary treatments, going from 112 to 345 mg/h/d (Table 2). Due to the 8-weeks adaptation period to the feed, it was possible to show the P intake effect on the parameters analyzed. For egg production, feed efficiency (Table 2), egg mass (Table 3), and tibia RF (Table 4), only the treatment with the lowest level of NPP intake (112 mg/h/d) was different ($P < 0.05$) from the other 2 NPP intakes (225 and 345 mg/h/d). Whereas, for feed intake (Table 2), egg weight (Table 3), tibia Ca and tibia P content (Table 4), the treatment with highest NPP intake (345 mg/h/d) was different ($P < 0.05$) to the lowest NPP intake level (112 mg/h/d), finding for tibia ash content a statistical difference ($P < 0.001$) for any of the 3 NPP intake levels (Table 4). Regarding the phytase treatments (300; 600; 1,200; and 1,800 FYT/kg), the NPP intake was an average of 121.8 mg/h/d with no statistical difference ($P > 0.05$) to NPP intake in treatment 1 (112 mg/h/d). The addition of the phytase enzyme to these 4 treatments

Table 2. Effect of feeding different amounts of NPP and phytase on laying hen performance[1].

Treatment	Dietary NPP %	Inorganic P addition %	Phytase addition FYT/kg	NPP intake mg/h/d	Egg production %	Feed intake g/h/d	Gain:feed g/kg
1	0.12	0.00	0.0	112[c]	66.5[b]	93.2[b]	429[b]
2	0.23	0.11	0.0	225[b]	77.4[a]	97.6[a,b]	484[a]
3	0.34	0.22	0.0	345[c]	81.7[a]	101.4[a]	504[a]
4	0.12	0.00	300	122[c]	80.3[a]	101.9[a]	487[a]
5	0.12	0.00	600	122[c]	82.4[a]	102.0[a]	498[a]
6	0.12	0.00	1200	120[c]	83.1[a]	99.8[a]	511[a]
7	0.12	0.00	1800	123[c]	83.0[a]	102.2[a]	504[a]
			SEM	3.0	2.46	1.45	13.3
			P <	0.00001	0.0005	0.0010	0.0028

[1]Numbers with distinct letter per column were statistically different (P < 0.05).

Table 3. Effect of feeding different amounts of NPP and phytase on laying hen performance[1].

Treatment	Dietary NPP %	Inorganic P addition %	Phytase addition FYT/kg	Egg		Hen weight	
				weight g	mass g/h/d	week 1 g/h	week 4 g/h
1	0.12	0.00	0.0	60.3[c]	40.1[b]	1,602	1,621
2	0.23	0.11	0.0	61.1[b,c]	47.2[a]	1,601	1,621
3	0.34	0.22	0.0	62.5[a]	51.1[a]	1,566	1,587
4	0.12	0.00	300	61.9[a,b]	49.7[a]	1,530	1,549
5	0.12	0.00	600	61.7[a,b]	50.8[a]	1,604	1,624
6	0.12	0.00	1200	61.5[a,b]	51.1[a]	1,613	1,634
7	0.12	0.00	1800	62.2[a,b]	51.6[a]	1,586	1,606
			SEM	0.42	1.57	33.1	32.9
			P <	0.0162	0.0001	0.60	0.58

[1]Numbers with distinct letter per column were statistically different (P < 0.05).

Table 4. Effect of feeding different amounts of NPP and phytase on eggshell strength, tibia strength, and mineral fraction[1].

Treatment	Dietary NPP %	Inorganic P addition %	Phytase addition FYT/kg	Eggshell resistance to fracture (RF)		Tibia			
				wk 0 g	wk 4 g	RF g	Ca %	P %	Ash %
1	0.12	0.00	0.0	3,286	3,628	17,251[c]	14.3[c]	6.2[c]	38.1[c]
2	0.23	0.11	0.0	3,741	3,809	20,850[a,b]	15.2[b,c]	7.1[b,c]	41.8[b]
3	0.34	0.22	0.0	3,674	3,854	23,448[a]	16.9[a]	8.8[a]	45.4[a]
4	0.12	0.00	300	3,468	3,546	19,782[b,c]	15.1[b,c]	7.3[b]	42.2[b]
5	0.12	0.00	600	3,411	3,580	21,776[a,b]	15.7[a,b]	7.8[a,b]	43.2[a,b]
6	0.12	0.00	1,200	3,693	3,421	23,346[a]	16.1[a,b]	8.0[a,b]	44.3[a,b]
7	0.12	0.00	1,800	3,804	3,624	23,283[a]	15.9[a,b]	8.5[a]	45.6[a]
			SEM	152.04	130.9	1,106.6	0.05	0.39	1.14
			P <	0.18	0.28	0.003	0.03	0.002	0.001

[1]Numbers with distinct letter per column were statistically different (P < 0.05).

allowed the hens to show a performance statistically different ($P < 0.05$), only to the hens fed the lowest NPP intake (112 mg/h/d), for egg production, feed intake, feed efficiency (Table 2), egg weight and egg mass (Table 3). Concerning the tibia data, the hens fed 0.12% NPP + 1,200 and 1,800 FYT/kg showed a statistically higher ($P < 0.003$) RF than the hens fed 0.12% NPP + 300 FYT/kg, but the same RF than the hens fed 0.34% NPP with no phytase addition. For tibia Ca, P, and Ash content, the hens fed 0.12% NPP + 600; 1,200 and 1,800 FYT/kg showed the same performance as the hens fed 0.34% NPP with no phytase addition, but the hens fed 0.12% NPP + 300 FYT/kg showed lower ($P < 0.05$) deposit of these minerals than the hens fed 0.34% NPP with no phytase addition. The regression analysis for the NPP with no phytase addition treatments is shown in Table 5, with a significant linear effect for all the variables analyzed, going from $P < 0.005$ for feed intake to $P < 0.0001$ for Ca, P, and ash content in tibia, being the quadratic effect non-significant ($P > 0.05$) for any of the variables analyzed. When looking at the R^2 values, a numerical difference between the performance production variables can be seen, with R^2 values from 0.47 for feed intake, to 0.64 for egg mass, and the tibia data, with all the R^2 were equal or above 0.8%; this means that the regression equations from the bone numbers explained better the real data compared to the performance production data equations. When

Table 5. Regression analysis for the inorganic P addition treatments, and the P release values obtained from solving each response point from the phytase addition treatments, with the corresponding regression equations.

							Tibia		
Regression	Egg Production %	Feed Intake g/h/d	Gain:feed g/kg	Egg weight g	Egg mass g/h/d	RF g	Ca %	P %	Ash %
Linear effect $P <$	0.0008	0.005	0.0025	0.0024	0.0003	0.0002	0.0001	0.0001	0.0001
Slope	69.2	37.2	342.2	10.3	50.1	28164.2	11.6	11.6	33.3
Intercept	67.6	93.3	434.9	60.1	40.6	17418.4	14.2	6.1	38.1
R^2	0.59	0.47	0.52	0.52	0.64	0.84	0.80	0.81	0.80
Quadratic effect $P <$	0.30	0.89	0.34	0.53	0.43	0.47	0.23	0.16	0.97
				Phytase P release values					
Phytase FYT/kg									
300	0.183	0.231	0.153	0.170	0.181	0.084	0.077	0.110	0.124
600	0.214	0.233	0.184	0.146	0.203	0.155	0.134	0.151	0.153
1,200	0.225	0.175	0.224	0.133	0.209	0.210	0.167	0.163	0.187
1,800	0.222	0.239	0.203	0.196	0.218	0.208	0.150	0.208	0.226

solving the regression equations to get the NPP equivalence values for each of the phytase levels added to the feeds, it was found a wide range for each of the phytase levels and variables analyzed, going for the 300 FYT/kg from 0.077% for tibia Ca to 0.231% for feed intake, for 600 FYT/kg the range went from 0.134% for tibia Ca to 0.233% for feed intake, for 1,200 FYT/kg the range went from 0.133% for egg weight to 0.225 for egg production, and for 1,800 FYT/kg the range went from 0.150% for tibia Ca to 0.239% for feed intake. Hen weight (Table 3) and eggshell RF (Table 4) at week 1 and week 4 (after 8 wk of adaptation period to the experimental feeds) were not affected by the experimental treatments.

Due to the differences in Ca, P, metabolism between mature laying hens and broilers, the research methods to evaluate phytase activity and P utilization, cannot be performed in the same way, being one of the main differences the time-length to observe the effects of the experimental treatments; whereas in broilers with only 2 or 3 wk of experimental period, the response of the bird to the experimental treatments is enough to observe the effect of NPP intake on broiler productive performance and bone features to reach sound conclusions (Yan et al., 2001; Silversides et al., 2004; Fuente et al., 2009; Avila et al., 2012). In hens, depending on the P intake in mg/h/d, and on the hen strain studied in the trials, the time to start seeing a decrease on egg production varies, being reported a start on egg production decrease at 12 wk on experiment (from 15 to 27 wk of age) on hens fed 78 mg NPP/h/d for Hy Line W 36 (Gordon and Roland, 1997). Working with Dekalb Delta White hens, the age at the experimental period beginning had also an effect on the start of the egg production decrease, reporting a decrease on egg production at 8 wk on trial (from 20 to 28 wk of age) with NPP intakes of 100 mg/h/d (Boling et al., 2000a,b). With 40-wk-old Dekalb Delta White hens, the start of a decrease on egg production observed was as follows: 5, 9, 11, and 13 wk on trial (45, 49, 51, and 53 wk of age), with NPP intakes of 100, 123, 136, and 148 mg/h/d, respectively (Snow et al., 2004). In the work of Keshavarz (2003), comparing 4 laying hen strains, it was reported that Babcock B300, Dekalb Delta White, and Isa-White hens show a decrease on egg production during the first 16 wk of the experimental period (from 20 to 35 wk of age) when fed an average of 245 NPP mg/h/d, compared with the positive control hens fed 446 NPP mg/h/d; whereas, the Hy Line W36 birds showed a decrease on egg production when fed 185 NPP mg/h/d during the second phase of the experimental feeding period (from 36 to 51 wk of age), compared to the control hens fed 442 NPP mg/h/d. In this paper, the exact week where the egg production decrease starts is not mentioned. One of the reasons behind the time needed to show any effect of a low P diet in laying hens, could be the presence of the medullary bone. A unique feature of this bone is its quick turnover process, defined by Parfitt (2002) as the total volume of bone that is both resorbed and formed over a period of time. Bone turnover process is slower in structural bone; so, when the hen consumes all its medullary bone reserves, eggshell formation keeps going, but using the structural bone and because this cannot be formed as quick as the medullary bone, starts a degradation process of the bones ending in fragile bones and even cage layer fatigue and mortality. Cortical bone has a lamellar structure, to fulfill its role as support and protection of the soft hen tissues; whereas, medullary bone has a woven structure, intended to provide a labile Ca source for sell formation (Kerschnitzky et al., 2014). Under normal circumstances, the hen gets the Ca needed for shell formation from the feed and from the medullary bone (Miller, 1992), and this bone is quickly restored by a bone remodeling process (Hudson et al., 1993). However, when hens are fed deficient amounts of P, medullary bone turnover process is disrupted, because the bone resorption process keeps going to provide Ca for shell formation, and depending on the degree of P deficiency bone formation process can be from being slowed down to totally stopped because there is not enough P to form the hydroxyapatite crystals of calcium phosphate. The P released from the bone resorption to the blood circulation creates a momentary P excess in the blood and it is eliminated by the kidneys; so, when Ca blood levels increase from the feed intake, there will be not enough P for bone for-

mation, as a summary, P dietary deficiency drives to a hen skeleton bone degradation. Besides its role as being a part of the bone structure, P is an essential constituent of organic compounds involved in almost all body metabolic pathways (Scott et al., 1982), as part of the phosphate molecule, it is involved in energy metabolism as part of the ATP molecule, the energy currency of the living cells, also the phosphate molecule is a component of the nucleotides, the building blocks of nucleic acids, phospholipids, components of cell membrane and phosphorylated proteins, involved in acid–base balance (Proszkowiec-Weglarz and Angel, 2013); so, P deficiency creates skeleton problems, as well as, disruption of energy metabolism and protein synthesis. So, even if the bone is driven towards degradation, when feeding a P deficient diet to the hen, the levels of this mineral needed to run the hen's metabolism are fulfilled, this is from the P released from bone resorption, allowing the hen to keep going with egg production; however, in a period of time length associated with the degree of P deficiency level, the hen starts a decrease on egg production, as early as 8 wk (from 20 to 28 wk of age), when they were fed 100 mg NPP/h/d for Dekalb Delta White hens (Boling et al., 2000a,b), or 12 wk (from 15 to 27 wk of age) when they were fed 78 mg NPP/h/d for Hy Line W 36 hens (Gordon and Roland, 1997), reporting even a shorter period to start the decrease on egg production in older hens, 5 wk (from 40 to 45 wk of age) for Dekalb Delta White hens fed 100 mg NPP/h/d (Snow et al., 2004). Reaching a high mortality and a strong decrease on egg production, that these treatments were taken out from the trials by 21 or 23 wk (from 20 to 41 or 43 wk of age) of being fed 100 NPP mg/h/d (Boling et al., 2000a,b), or 11 wk for older hens, from week 40 to 51 being fed 100 NPP mg/h/d (Snow et al., 2004). It seems that the P deficiency, when the medullary bone is degraded beyond the point of new bone formation, reaches a P deficiency severe enough to even support the energy and protein synthesis metabolism; so, the hen is no longer able to keep production nor maintain metabolic functions.

Regarding the no effect of NNP intake on eggshell RF (Table 4), the data reported agrees with these results, feeding hens with low levels of NPP, even for long periods of time, never affected the eggshell quality (Boling et al., 2000a,b); in fact, it has been reported that hens fed lower levels of NPP showed better specify gravity than hens fed adequate levels of NPP (Keshavarz, 2000, 2003).

Concerning the effect of P intake on the hen body weight, it takes many weeks to observe a response on this variable; in the publication of Keshavarz (2003), working with Dekalb, Isa White, HLW36, and BabCock B300 strains, it took 31 wk on trial (from 20 to 51 wk of age), in hens fed 200 mg/h/d NPP with no phytase supplementation, to see a lower hen body weight. Working with Dekalb White hens, it took 20 wk on trial (from 20 to 40 wk of age) to show a decrease on hen body weight, in hens fed 100 mg/h/d NPP with no phytase supplementation (Boling et al 2000a); whereas, in the work of Snow et al. (2004), hens fed from 100 to 217 mg/h/d NPP w/o phytase did not show an effect on hen body weight after being on trial by 44 wk (21 to 64 wk of age) no effect on hen final BW. So, the length of the present trail was not enough to see an effect on hen body weight.

Looking at the NPP intakes observed in this trial, 122, 225, and 345 mg/h/d, and at the hens' responses, both the performance and bone breaking strength and mineral content, it seems that the adaptation period of 8 wk before the period of data collection fulfilled the expectations to get the hens at a physiological state sensitive enough to measure the effect of the phytase enzyme on phytate P release. Finding a significant ($P < 0.05$) linear effect response to the 3 NPP diet levels fed to the hens, 0.12, 0.23, and 0.34%, with the following NPP mg/h/d intakes: 112, 225, and 345, respectively, for egg production, feed intake, feed efficiency, egg weight, egg mass, tibia BS, and Ca, P, and ash tibia content (Table 5). As mentioned above, the regression lines R2 values showed a greater variation for the performance variables with R2 values not higher than 0.64; whereas, the bone parameters showed R2 values equal or above 0.80, meaning a better relationship for the bone parameters numbers with their respective regression equation lines.

The former observation is very important to do the interpretation of the NPP phytase equivalence values got from the solving of the regression equations, because the ranges of these values for any of the 4 phytase levels analyzed in this study seem very wide (Table 5); however, based on the information discussed above, it is clear that the main effect of feeding a P deficient diet to the hens happens at bone level. Under normal circumstances, the hen shows a loss of its structural bone during its productive live (Whitehead, 2004); however, with an adequate management and nutrition, this bone loss will not end on skeletal problems nor production performance issues.

So, based on the data discussed, the adequate data to evaluate the phytase P releasing values will be the bone data because when using the hen performance data, we may end overestimating the released P amounts, ending up with weak bone issues leading to hen osteoporosis problems. For example, looking at the P release value for the 300 FYT/kg phytase level (Table 5) for the hen productive performance variables, egg production, feed intake, feed efficiency, egg weight, and egg mass, the values range from 0.153 to 0.231% with an average value of 0.184%, whereas for the tibia data the values range from 0.0.77 to 0.124% with an average value of 0.099%, and this pattern is repeated when looking at all other phytase addition levels, always the hen productive performance data yielded higher P release equivalent values, and this can be explained, as mentioned before, from the fact that feeding a P dietary deficiency drives the hen to a net skeleton bone degradation situation, with enough P in the blood to fulfill the metabolic needs of

P for energy metabolism as a part of the ATP molecule, protein turn over, as component of the nucleotides, the building blocks of nucleic acids, cell membrane integrity as part of the phospholipids, and phosphorylated proteins, involved in acid–base balance, but driving then hen sooner or later, depending on the degree of P deficiency, to skeleton problems.

Then, by looking at all the information discussed above, it is not advisable to use the NPP equivalence values got from the hen productive performance data, because these numbers, even though, will produce adequate hen productive numbers, on the medium long run, the hen skeleton will suffer a net degradation process, leading to the presence of osteopenia, or depending on the degree of NPP deficiency, osteoporosis, resulting in high incidence of broken bones and even the development of cage layer fatigue, conditions totally opposed to the concept of hen wellbeing and welfare.

So, from the tibia data showed in Table 5 the average P equivalents release values for the of *Citrobacter braakii* phytase with a corn-SBM diet, are as follows: 300 FYT/kg = 0.099%, 600 FYT/kg = 0.141%, 1,200 FYT/kg = 0.182%, and 1,800 FYT/kg = 0.198%.

These numbers will assure realistic P release values, that will keep the hen skeleton integrity, to assure hen health and wellbeing.

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