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Effect of Protein and Amino Acid Levels on Bone Formation in Diets Varying in Calcium Content[1]

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Abstract: The effect of different dietary levels of amino acids, calcium and phosphorus as influenced by phytase supplementation was evaluated in broiler chickens. The experimental design consisted of a 3 x 4 x 2 factorial arrangement with three levels of digestible lysine (1.10, 1.30 and 1.50%), four levels of calcium (0.50, 0.70, 0.90 and 1.1%) and diets containing 0.35% AP with and without phytase for a total of 24 treatments. Remaining amino acids levels were adjusted with respect to the digestible lysine level using the ideal ratios suggested by Rostagno *et al.* (2005). Each experimental diet was fed to six replicates pens of five male chickens during 21 days. Body weight, FCR, feed intake, bone development (TD), bone mineralization (toe ash), and phosphorus excretion as Total Phosphorus in excreta (TP), Water Soluble Phosphorus in excreta (WSP) and the WSP/TP ratio were evaluated. Birds fed lysine levels higher than 1.1% expressed better body weight in a non-linear trend. Feed intake was decreased by increasing the lysine level while feed conversion improved as lysine level increased. Increasing levels of Ca decreased feed intake, the 1.1% Ca level was detrimental for body weight. Phytase supplementation was effective to alleviate widened-suboptimal Ca:P ratios in terms of feed intake and body weight. The 1.5 % digestible lysine level improved toe ash; however, high levels of lysine were also related to a higher incidence of TD. Ca levels equal or greater than the NRC (1994) recommendation were adequate for optimum bone mineralization. Increasing levels of Ca reduced the incidence and severity of TD. Moreover, Ca levels greater than those suggested by NRC (1994) were adequate to assimilate higher lysine levels without compromising bone development. The higher lysine levels fed reduced TP in excreta but increased the WSP/TP ratio. The supplementation of phytase increased WSP and the WSP/TP ratio. Increasing levels of Ca reduced WSP and the WSP/TP ratio in excreta. Furthermore, high levels of Ca were also effective to overcome the increased WSP and WSP/TP ratio caused by the supplementation of phytase.

Key words: acids, calcium, phosphorus, bone formation

INTRODUCTION

Problems with leg disorders persist in the broiler industry despite continual genetic selection for birds with better skeletal conformation. Although calcium and phosphorus levels tend to be the primary focus of nutritional efforts to reduce the incidence or severity of leg disorders, other nutrients may also interact in this regard. One nutritional aspect that is worthy of attention is the relationship that exists between dietary protein levels and calcium absorption and excretion.

Humans and rats fed high protein diets have increased urinary Ca excretion (Engstrom and DeLuca, 1963; Allen *et al.*, 1979; Kim and Linkswiler, 1979) and may have a negative calcium balance (Johnson *et al.*, 1970; Anand and Linkswiler, 1974). Osteogenesis was reduced by 78% in 28-d old rats fed high protein and normal Ca diets; a diet containing high protein and low Ca resulted in a 98% inhibition in bone formation (Weiss *et al.*, 1981). A high protein diet may not have an effect on bone resorption when Ca and P intakes are adequate (Bell *et al.*, 1975). Stevens and Salmon (1988) reported that tibia ash was reduced in turkey poults fed increasing dietary protein. El-Maraghi *et al.* (1965) reported that when the protein value of the diet was high, diets of low Ca content led to severe mineral osteoporosis in the bones of young rats. In a study conducted in our laboratory (Skinner *et al.*, 1991), increasing the dietary amino acid levels did not affect the linear growth of bones but reduced the calcification, especially when diets contained marginal levels of calcium.

Current trends in the broiler industry are to feed high levels of essential amino acids in broiler starter diets, in an effort to enhance early growth rate and improved breast meat yield. At the same time, dietary calcium and phosphorus levels have been reduced, usually in conjunction with phytase supplementation, in an effort to reduce phosphorus excretion. A recent study from our laboratory (Yan *et al.*, 2006) suggested that while phytase releases a significant amount of phosphorus from plant feedstuffs, little if any calcium is actually released, and suggested that no reduction in Ca level should be implemented with phytase supplementation. Therefore, the practice of reducing both calcium and phosphorus in phytase-supplemented diets may be putting the chicken into a marginal calcium situation that could be exacerbated by the use of high protein-high amino acid diets. The objective of this study was to evaluate the relationship of dietary amino acid levels with dietary calcium and phosphorus levels as influenced by phytase supplementation.

MATERIALS AND METHODS

Diets were formulated by linear programming to contain three different levels of digestible lysine, using the ideal ratios suggested by Rostagno *et al.* (2005). Corn and soybean meal of known protein and moisture content were used as intact sources of crude protein, with L-Lysine HCl, L-Threonine, and MHA-Ca as sources of lysine, threonine, and methionine activity. Total and digestible amino acid values used in formulation were those suggested by a major amino acid producer (Ajinomoto Heartland Lysine LLC, Chicago IL). The digestible lysine levels chosen were 1.10, 1.30, and 1.50% per 2950 ME kcal/kg. This resulted in crude protein levels of 21.04, 24.83, and 28.62%, respectively. A dietary energy level was chosen that would require approximately 1% supplemental poultry oil for the lowest level of lysine. Amino acid requirements were adjusted in relation to dietary energy level. Diets were formulated to contain 0.35% available phosphorus. Space was allocated for varying amounts of ground limestone or washed builder's sand to provide up to 1.10% calcium. Diets were fortified with complete vitamin and trace mineral mixes. Composition of the diets is in Table 1 with the calculated nutrient content in Table 2.

To prepare the experimental diets, the amount of ground limestone and washed builders sand was varied to provide calcium levels of ≈ 0.50[3], 0.70, 0.90 and 1.10%. The dietary treatments consisted of a 3 x 4 x 2 factorial arrangement with three levels of available lysine, four levels of calcium, and diets fed with and without 1200 FTU/kg of phytase supplementation (Ronozyme®, DSM Corporation, Parsippany NJ) for a total of 24 treatments. To avoid potential problems with destruction of phytase, diets were fed in mash form. Each treatment was fed to six replicate pens of five male chickens.

Male chicks of a commercial broiler strain were obtained from a local hatchery where they had been vaccinated in ovo for Marek's disease and had received vaccinations for Newcastle Disease and Infectious Bronchitis post hatch via a coarse spray. Five chicks were placed in each of 144 compartments in electrically heated battery brooders with raised wire floors. Six pens were assigned to each treatment, stratified across tiers in the battery. Test diets and tap water were available for ad libitum consumption. Fluorescent lighting provided 24 hr of light daily. Care and management of birds followed recommended guidelines (FASS, 1999).

Diets 1 through 12 were analyzed for crude protein, calcium, phosphorus, and sodium content. Body weights by pen were determined at 1 and 21 d of age. Any bird that died was removed from the study to alleviate suffering was weighed for adjusting feed conversion. At the conclusion of the study, all birds were killed by CO_2 inhalation. Toes were removed from all

Table 1: Composition (g/kg) of experimental diets

Ingredients	Dig Lysine % per 2950 ME kcal/kg		
	1.10	1.30	1.50
Yellow corn	638.28	526.78	415.26
Poultry oil	9.30	25.92	42.53
Soybean meal	309.30	404.60	499.92
Defluorinated phosphate	12.50	11.91	11.33
Feed grade salt	4.25	4.27	4.30
MHA-84	2.58	3.33	4.08
L-Threonine	0.47	0.50	0.53
L-Lysine Hcl	1.89	1.48	1.06
Vitamin premix[1]	5.00	5.00	5.00
Mintrex P_Se[2]	1.00	1.00	1.00
Variable[3]	15.43	15.21	14.99
TOTAL	1000.00	1000.00	1000.00

[1]Provides per kg of diet: vitamin A (from vitamin A acetate) 7715 IU; cholecalciferol 5511 IU; vitamin E (from dl-alpha-tocopheryl acetate) 16.53 IU; vitamin B_{12} 0.013 mg; riboflavin 6.6 mg; niacin 39 mg; pantothenic acid 10 mg; menadione (from menadione dimethylpyrimidinol) 1.5 mg; folic acid 0.9 mg; choline 1000 mg; thiamin (from thiamin mononitrate) 1.54 mg; pyridoxine (from pyridoxine HCl) 2.76 mg; d-biotin 0.066 mg; ethoxyquin 125 mg.
[2]Provides per kg of diet: Mn (as manganese methionine hydroxy analogue complex) 40 mg; Zn (as zinc methionine hydroxy analogue complex) 40 mg; Cu (as copper methionine hydroxy analogue complex) 20 mg; Se (as selenium yeast) 0.3 mg.
[3]Variable amounts of ground limestone or washed builders sand.

Table 2: Calculated nutrient content of experimental diets. Values in bold italic are at minimum specified level

Nutrient	Digestible Lysine % per 2950 ME kcal/lb		
	1.10	1.30	1.50
Crude protein	21.04	24.83	28.62
Total P	0.59	0.62	0.64
Available P	0.35	0.35	0.35
Sodium	0.25	0.25	0.25
Chloride	0.32	0.32	0.31
ME kcal/kg	3063.58	3063.58	3063.58
Total amino acids %			
Methionine	0.58	0.69	0.80
Lysine	1.26	1.49	1.72
Tryptophan	0.25	0.30	0.35
Threonine	0.84	0.99	1.14
Isoleucine	0.85	1.03	1.20
Histidine	0.56	0.65	0.74
Valine	0.95	1.12	1.29
Leucine	1.83	2.06	2.32
Argininine	1.36	1.66	1.97
TSAA	0.95	1.11	1.27
Gly+Ser	1.96	2.34	2.73
Phe+Tyr	1.84	2.20	2.55
Digestible amino acids %			
Lysine	1.14	1.35	1.56
Methionine	0.50	0.61	0.71
TSAA	0.81	0.96	1.11
Tryptophan	0.21	0.26	0.31
Threonine	0.74	0.88	1.01
Arginine	1.26	1.54	1.82
Valine	0.86	1.01	1.17
Isoleucine	0.78	0.94	1.10
Leucine	1.70	1.92	2.15
Histidine	0.50	0.58	0.67
Phenylalanine	0.92	1.10	1.27

birds and ashed as described by Yan *et al.* (2005). Tibiae were taken from each bird for evaluation of incidence and severity of tibial dyschondroplasia using the scoring system of Edwards and Veltman (1983).

At 19 days of age excreta samples were collected on aluminum foil. Samples were collected from the center of the excreta collection pans to avoid contamination with spilled feed. The excreta samples were frozen, freeze-dried, and ground. Total phosphorus content was determined by Inductively Coupled Plasma Spectroscopy following HNO_3 digestion. The water-soluble P (WSP) was determined by the method of Self-Davis *et al.* (2000). The ratio of WSP/TP was calculated from these data.

Pens means served as the experimental unit for statistical analysis. Data were subject to analysis of variance using the general linear model procedure of SAS (1991). The model included main effects of lysine level, calcium level, phytase supplementation, and all two-way and three-way interactions. Percentage data were converted to arc sine prior to analysis. Data are presented as natural numbers. Significant differences among or between means were separated by repeated t-tests using the least square means option of SAS software. All statements of significance are based on $P \leq 0.05$.

RESULTS AND DISCUSSION

The ANOVA results showing the probability of various dietary factors on live performance, bone development and phosphorous excretion is shown in Table 3. The influence of the various main effects on live performance is shown in Table 4. Body weight was significantly affected by the dietary calcium level. The body weight of birds fed 1.10 % Ca was significantly lower than that of birds fed any other level of calcium evaluated. There was no statistical difference in body weight among birds fed diets containing 0.50%, 0.70% and 0.9% Ca. The negative effect obtained with the 1.10% Ca level can be due to a hypercalcaemia condition as suggested by Hurwitz *et al.* (1995). This result is also in agreement with Williams *et al.* (2000) and Al-Masri (1995). The latter justified the response on a reduced P utilization due to a negative effect of high calcium levels on the phytin hydrolysis. This result also supports our previous study where different AP and Ca levels were fed, concluding that both the total Ca level and the Ca:P ratio should be considered as criteria for calcium adequacy rather that only the Ca:P ratio (Coto *et al.*, 2008).

Moreover, there was a significant effect of the lysine level on body weight. Birds fed the 1.3 % digestible lysine level had a significantly higher body weight than obtained with the 1.1% lysine level. The body weight response obtained when feeding 1.5 % digestible lysine was not statistically different to that obtained with either 1.1% or 1.3 %. This result suggests that the 1.1% digestible Lys level was not adequate to fulfill the chick requirement for body weight whereas the 1.5% Lys level was higher than the optimum. Further studies need to evaluate a closer range of lysine values to more closely pinpoint the lysine requirement.

Vazquez and Pesti (1997) conducted a statistical analysis of published research on lysine requirement of broiler chicks for maximum gain and feed efficiency. Using values for total lysine, they estimated the total lysine requirement of the starting chick (0-3 wk) to be 1.04 ± 0.02% for gain and 1.10 ± 0.04% for FCR using the "ascending line with plateau" (ALP) model and 1.21 ± 0.06 for gain and 1.32 ± 0.10% for FCR using the "ascending quadratic with plateau" (AQP) model. The authors stated that the AQP model may be more realistic and adequate than the ALP model for the prediction theoretically, but the statistical fits of the two models were similar. If one uses a conservative value of 88% for relative lysine digestibility in corn-soy diets, this would translate to approximately 0.92% for gain and 0.97% for FCR using the ALP model and 1.07% for gain and 1.16% for FCR using the AQP model. A summary of recent research reports on digestible lysine needs for broilers during the first 3 weeks of age can be found in Table 5. It is apparent that there is a lack of unanimity among the various reports.

There was no significant effect of phytase supplementation on body weight. This in contrast to what described by Yan *et al.* (2001), Waldroup *et al.* (2000), and Scheideler and Ferket (2000) and suggests that 0.35% available phosphorus was adequate for body weight gain during this period.

There was a significant two-way interaction between Ca level and phytase supplementation (Fig. 1). Birds fed the highest Ca level (1.1%) had a significantly lower body weight when the enzyme was not included in the diet. When phytase was included, the body weight was comparable to that obtained with the remaining calcium levels in combination with the enzyme but lower than the obtained with the 0.5% and 0.7 % Ca level without the enzyme. This result confirms the idea of phytase as an option to alleviate suboptimal Ca:P ratios (Coto *et al.*, 2008), and a higher tolerance to dietary calcium levels as the available phosphorous level is increased (Hurwitz *et al.*, 1995). No other significant two-way interaction was found for body weight. There were no significant three-way interactions among the main effects for body weight.

The influence of various main effects on feed conversion (FCR) is shown in Table 4. The dietary lysine level had a significant influence on the FCR. Birds fed diets with 1.1% lysine had a significantly worsened FCR than birds fed 1.3% and 1.5% digestible lysine. The response was expected since the Lys level for optimum FCR is typically reported to be higher than that required for maximum body weight. There was no significant effect of the calcium level and phytase supplementation on the FCR.

Table 3: ANOVA values (probability > F) for body weight, feed conversion, feed intake, mortality, toe ash, incidence and severity of tibial dyschondroplasia, excreta total P, excreta water soluble P, and excreta ratio of total and water soluble P.

						Tibial Dyschondroplasia			Excreta	
Factor	Body weight	FCR	Feed Intake	Mortality	Toe ash	incidence	severity	Total P	Water Soluble P	WSP/TP
Lys	0.04	<0.001	0.008	0.60	0.002	0.06	0.19	<0.001	0.003	<0.001
Ca	<0.001	0.35	<0.001	0.11	0.006	<0.001	0.002	0.22	<0.001	<0.001
Lys X Ca	0.44	0.10	0.83	0.79	0.604	0.02	0.36	0.76	0.08	0.11
Phy	0.34	0.53	0.47	0.68	0.578	0.29	0.70	0.23	<0.001	<0.001
Lys X Phy	0.97	0.97	0.87	0.30	0.108	0.53	0.82	0.70	0.84	0.81
Ca x Phy	0.005	0.32	0.05	0.91	0.698	0.66	0.93	0.97	0.001	0.002
Lys x Ca x Phy	0.98	0.57	0.96	0.54	0.777	0.94	0.88	0.86	0.52	0.40
CV	6.78	5.59	5.97	3.73	5.49	6.71	3.76	10.18	29.97	30.68

Table 4: Effects of dietary levels of lysine, calcium and phytase supplementation on live performance of broiler chicks abMeans with common superscripts do not differ significantly ($P \leq 0.05$)

Dietary Factor	21 d BW (kg)	0-21 d FCR	0-21 d Feed Intake (kg/bird)	0-21 d Mortality %
Digestible Lysine (%)				
1.1	0.692^{b}	1.555^{a}	1.005^{a}	2.500
1.3	0.717^{a}	1.481^{b}	0.993^{a}	3.333
1.5	0.708^{ab}	1.464^{b}	0.968^{b}	4.167
Calcium level (%)				
0.50	0.727^{a}	1.486	1.012^{a}	5.000
0.70	0.720^{a}	1.504	1.014^{a}	1.667
0.90	0.709^{a}	1.490	0.987^{a}	5.000
1.10	0.667^{b}	1.519	0.941^{b}	1.667
Phytase supplementation				
No	0.702	1.504	0.985	3.056
Yes	0.710	1.495	0.992	3.611

There were no significant three-way interactions among the main effects for feed conversion.

The influence of various main effects on feed intake is shown in Table 4. There was a significant effect of the lysine level on feed intake. Birds fed diets containing 1.5% digestible lysine showed a significantly lower feed consumption than birds fed diets with 1.1% and 1.3% digestible lysine level. Although birds fed 1.1% and 1.3% digestible lysine level had no statistical difference in feed intake, a trend to reduce feed intake as the lysine level increase was maintained. In agreement with our results, Mendes *et al.* (1997) found a lower feed consumption when the lysine level increased. This effect can be explained by the effect of increasing levels of amino acids on satiety as amino acid concentrations are correlated with a reduction in appetite (Halton and Hu, 2004),

In addition, there was a significant effect of the calcium level on feed intake. Birds fed 1.10 % Ca showed a significantly reduced feed intake compared to those birds receiving any of the other calcium levels. This effect possibly due to a hypercalcemia condition, which was possibly exacerbated by the high level of vitamin D (5500 IU/kg) in the diet. There was no significant difference among the remaining Ca levels on feed intake. There was no significant effect of phytase supplementation on feed intake. There was a significant two-way interaction between Ca level and phytase supplementation on feed intake (Fig. 2). Birds fed the highest Ca level (1.1%) had a significant lower feed intake when the enzyme was absent in the diet. At the highest Ca level with the enzyme present, feed intake was comparable to that obtained with the remaining calcium levels accompanied by the enzyme; however, this was lower than that obtained with the 0.5% and 0.7 % Ca levels without the enzyme. This result confirms our previous statement where phytase plays a role in alleviating suboptimal Ca:P ratios (Coto *et al.*, 2008). Phytase increases the available phosphorous level improving the tolerance to high dietary calcium levels (Hurwitz *et al.*, 1995). There were no other significant two-way interactions. There were no significant three-way interactions among the main effects for feed intake.

The influence of various main effects on mortality is shown in Table 4. There were no significant effects of lysine level, calcium level and phytase supplementation on mortality. There were no significant two-way or three-way interactions among the main effects for mortality.

Bone development: The influence of various main effects on toe ash is shown in Table 6. Toe ash was significantly influenced by the lysine level and the calcium level. Toe ash was higher when the 1.5 % digestible lysine level was fed, in agreement with Bonjour (2005) who found in humans that higher levels of protein increase bone mineralization as long as adequate levels of calcium and vitamin D are present. This result is also supported by the fact that lysine is a major constituent of collagen which provides oriented support during the mineralization process (Rath *et al.*,

Table 5: Summary of trials to estimate digestible lysine requirements of broilers up to 3 weeks of age

Age (d)	Sex	Estimate	Criteria	Authors
8-21	M	<1.01	BW (same for fast and slow growing)	Han and Baker, 1991
		<1.21	FCR (same for fast and slow growing)	
1-21	M	1.05	BW gain	Silva Conhalato *et al.* 1999
		1.03	FCR	
		1.08	Protein deposition rate	
1-21	M	1.02	Performance at 29.1 C	Borges *et al.* 2002
1-21	M	1.09	Performance and carcass	Barbosa *et al.*, 2002
1-18	M	1.07-1.11	Growth and carcass	Kidd and Fancher, 2001
1-21	M	1.01/1.10	Body weight/FCR Exp. 1	Garcia and Batal, 2005
		0.99/0.94	Body weight/FCR Exp. 2	
1-21	M	1.183	Weight gain and feed conversion	Costa *et al.*, 2001
	F	1.129		
12-22	M	1.10	Development requirements	Takeara, 2006
		1.25	Body chemical composition	
1-21	M	1.14	Conventional diet	Valerio *et al.* 2003
		1.22	Maintain relationship of amino acids	
1-21	M	1.20	Optimal performance	Zhang *et al.* 2006
6-21	M	1.075/1.179	Growth/FCR	Zaghari *et al.* 2002
	F	1.049/1.149		
1-21	M	1.27	Weight gain	Rodrigues *et al.* 2008
1-21	M	1.143	Body weight and feed intake	Franco *et al.* 2005
1-21	M	1.14	Conventional diet	Lana *et al* 2005
		1.17	Maintaining amino acid balance	
1-21	M	1.13	Digestibility data from diets previously used to estimate total lysine needs	Barboza *et al.* 2000
1-14	M/F	1.05	No difference between sexes.	Schutte and Smink, 1998
14-28		1.00		
0-14	M	1.24	BW, FCR, breast muscle	Labadan *et al.* 2001
14-28		1.11		
1-7	M	1.286	BW, Feed intake, FCR, carcass	Goulart *et al.* 2008
8-21		1.057		

2000). Moreover, it has been found that higher levels of protein improve the intestinal calcium absorption (Kerstetter *et al.*, 2006). There was no statistical difference for the remaining two levels of lysine. On the other hand, toe ash was higher in birds fed the 0.9 % dietary calcium level. At 0.7 % Ca level toe ash response was comparable to the obtained with 0.90 % Ca but not different to the obtained for the remaining Ca levels (0.5% and 1.1%). These results support our previous report (Coto *et al.*, 2008) where dietary calcium levels equal or greater than those suggested by NRC (1994) are needed for optimum bone mineralization.

The phytase supplementation had no significant effect on toe ash in the present study. This in agreement to the findings of Scheideler and Ferket (2000), Sohail and Roland (1999) and Waldroup *et al.* (2000). It is in contrast to the reported by Yan *et al.* (2001; 2003), Carlos and Edwards (1998), Catala *et al.* (2006) and Viveros *et al.* (2002). However, the response to phytase will depend upon the level of available P in the basal diet. In the present study there was apparently sufficient P to support toe ash without need for further supplementation or enhancement with phytase. There were no significant two-way or three-way interactions among the main effects for toe ash.

The influence of various main effects on the incidence of TD is shown in Table 6. TD incidence (the percentage of birds with a TD score greater than 0) was significantly influenced by the lysine level and the calcium level. TD incidence was higher in chicks fed the diet with 1.3 % digestible lysine. TD incidence obtained with 1.1 % digestible lysine level was statistically lower than the obtained with 1.3 % digestible lysine but not different to that observed with 1.5% digestible lysine. This result in agreement with Weiss *et al.* (1981) who pointed out that higher levels of protein induce TD. Moreover, increasing levels of dietary Ca reduced the incidence of TD, having a better response when the 0.9% dietary calcium level was fed. At the highest level of Ca (1.1%) the improvement was not that marked, with the TD incidence comparable to that obtained with both 0.5% and 0.9% Ca. This result in agreement with the report of Whitehead *et al.* (2004) who observed higher TD incidence in diets with low calcium levels. No effect was obtained for the phytase supplementation. There was a significant two-way interaction between Lys level and Ca level (Fig. 3). At the two lowest Ca levels, the incidence of TD was higher at the 1.3% digestible lysine level. When the two highest levels of calcium were fed a reduction in the incidence of TD was obtained regardless of the lysine level. This result suggests that birds fed Ca levels slightly higher than the NRC (1994) recommendation can assimilate higher levels of lysine without compromising bone development. There were no other significant two-way interactions for TD incidence. There were no significant three-way interactions among the main effects for TD incidence. The influence of various main effects on the severity of

Table 6: Effects of dietary levels of lysine, calcium and phytase supplementation on toe ash, incidence and severity of TD, total and water-soluble P in excreta, and ratio of water soluble to total P in excreta

	Toe ash (%)	TD Incidence[1] (%)	TD Severity[2] (%)	Excreta Total P %	Water-soluble P (ppm)	WSP/TP
Digestible Lysine (%)						
1.1	11.57[b]	8.22[b]	1.35	1.268[a]	2184.78[b]	17.62[c]
1.3	11.74[b]	16.35[a]	4.37	1.152[b]	2449.91[a]	21.83[b]
1.5	12.09[a]	12.52[ab]	3.28	1.082[c]	2648.55[a]	24.57[a]
Calcium (%)						
0.50	11.54[b]	24.58[a]	7.84[a]	1.155	4749.91[a]	40.98[a]
0.70	11.85[ab]	13.75[b]	3.61[b]	1.143	1705.81[b]	24.46[b]
0.90	12.11[a]	2.52[c]	0.55[b]	1.175	1396.51[c]	12.47[c]
1.10	11.71[b]	8.61[bc]	0.00[b]	1.198	858.75[d]	7.45[d]
Phytase						
No	11.77	13.81	2.74	1.180	2165.53[b]	18.81[b]
Yes	11.83	10.91	3.26	1.156	2689.96[a]	23.87[a]

[abc]Means with common superscripts do not differ significantly ($P \leq 0.05$).
[1]Percent of birds with TD score greater than 0 based on scoring system of Edwards and Veltmann (1983).
[2]Percent of birds with TD score of 4 based on scoring system of Edwards and Veltmann (1983).

TD (percentage of birds with a TD score of 4) is shown in Table 6. The TD severity was significantly influenced by the calcium level. Increasing the dietary calcium level significantly reduced the severity of TD. This result is consistent to what was observed in a previous study conducted in our lab (Coto *et al.*, 2008) No other significant effect was found for Lysine level and phytase supplementation. There were no significant two-way interactions among lysine level, calcium level and phytase supplementation for TD severity. There were no significant three-way interactions among the main effects for TD severity.

Phosphorous in excreta: The influence of various main effects on the total phosphorous in excreta (TP) is shown in Table 6. TP was significantly influenced by the dietary lysine level. This effect may be due to the phosphorus metabolic role on protein synthesis which increases the requirement of the mineral by protein increasing levels; therefore, reducing the amount of phosphorus excreted (Underwood and Suttle, 1999). Another perspective is that increasing dietary levels of protein stimulate the absorption of calcium requiring more phosphorus to maintain an adequate Ca:P ratio (Kerstetter *et al.*, 2006).

No significant effect on total excreta phosphorus was obtained for the calcium level and the phytase supplementation. There were no significant two-way interactions among lysine level, calcium level and phytase supplementation for TP. There were no significant three-way interactions among the main effects for TP. The influence of various main effects on the water soluble phosphorous in excreta (WSP) is shown in Table 6. WSP was significantly influenced by the lysine, dietary calcium level and phytase supplementation. Birds fed 1.1% digestible lysine had a significantly lower WSP than birds fed 1.3% and 1.5 % digestible lysine. It is important to consider that both amino acids and phosphorous are target of complexation by the phytate molecule. Thus, higher concentrations of amino acids increase the possibility to find them bound to the phytate molecule while phosphorous is more likely to be free. Moreover, increasing levels of dietary calcium reduced significantly the concentration of WSP in excreta. This effect due to a reduction in the solubility of phosphorus by forming complexes with calcium that are stable (Coto *et al.*, 2007). Furthermore, the addition of phytase significantly increased the WSP in excreta. This in agreement with Coto *et al.* (2007). This effect is explained by the release of the phosphorus adsorbed by the phytate molecule, which is less likely to be transported in runoff waters, increasing the amount of phosphate in the aqueous phase (Vadas *et al.*, 2004). A significant two-way interaction between calcium level and phytase supplementation was found for WSP (Fig. 4). At the two lowest calcium levels evaluated, the WSP was higher when phytase was present in the diet. At the remaining calcium levels, the addition of phytase rendered no difference on WSP. This result confirms the effect of the phytase on releasing phosphorus bound to the phytate molecule and suggests that the increase in WSP as a result of phytase can be overcome by increasing levels of calcium. There were no other significant two-way interactions. There were no significant three-way interactions among the main effects for WSP. The influence of various main effects on the WSP/TP ratio is shown in Table 6. The WSP/TP ratio was significantly modified by dietary lysine level, calcium level and phytase supplementation. Birds fed increasing lysine levels had a significantly higher WSP/TP ratio. Moreover, a reduced WSP/TP ratio was observed with increasing Ca levels, in agreement with Leytem *et al.* (2007). In addition, the supplementation of phytase increased the WSP/TP ratio significantly. This was in agreement with Coto *et al.* (2007) and Leytem *et al.* (2007) but conversely to the report by Penn *et al.* (2004). A significant two-way interaction between calcium level

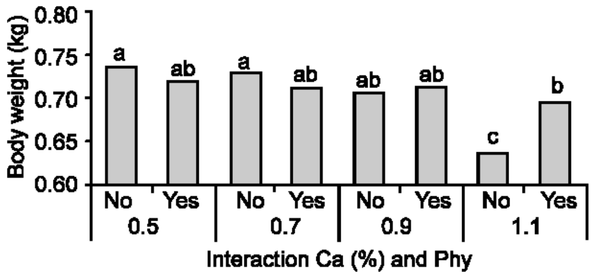

Fig. 1: Effect of the interaction between dietary Ca level and phytase supplementation on body weight.

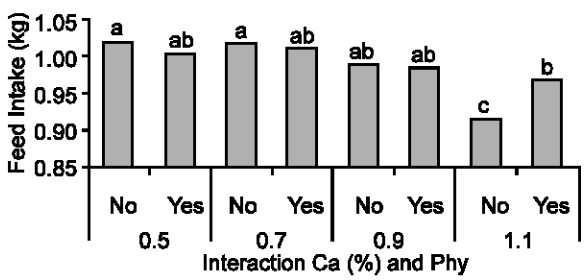

Fig. 2: Effect of the interaction between dietary Ca level and phytase supplementation on feed intake.

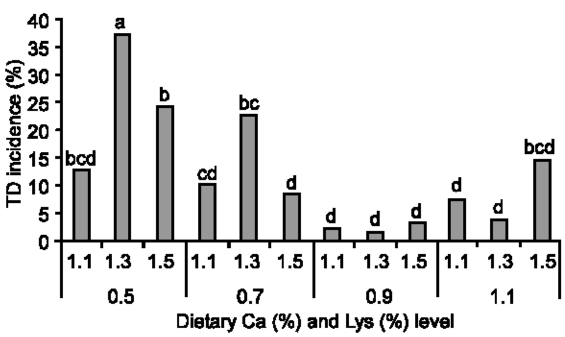

Fig. 3: Effect of the interaction between dietary calcium level and lysine level on the incidence of TD.

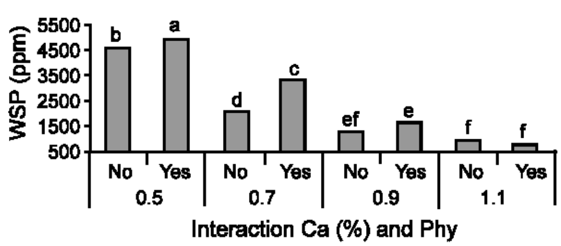

Fig. 4: Effect of the interaction between dietary calcium level and phytase supplementation on the WSP in excreta.

and phytase supplementation was found for WSP (Fig. 5). The WSP/TP ratio was higher at the two lowest calcium levels when phytase was present in the diet. At 0.9% and 1.1% Ca, the addition of phytase generated no difference on the WSP/TP ratio. This result suggests that

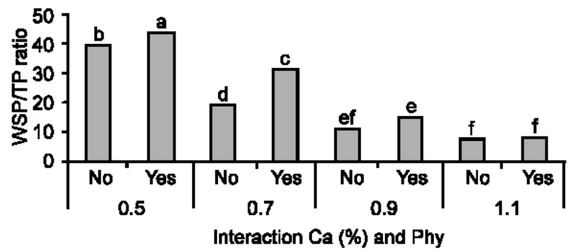

Fig. 5: Effect of the interaction between dietary calcium level and phytase supplementation on the WSP/TP ratio in excreta.

increasing levels of calcium can mitigate the increase of WSP obtained by the addition of phytase. There were no other significant two-way interactions. There were no significant three-way interactions among the main effects for the WSP/TP ratio.

In summary, a high level of dietary calcium such as 0.4% more than the 2:1 Ca:AP ratio affect negatively the body weight response. Birds fed lysine levels higher than 1.1% expressed better body weight in a non-linear trend. Moreover, phytase supplementation helps to sustain the body weight before increasing dietary calcium levels. This effect is more notorious as the Ca:AP ratio is widened. Feed conversion was improved as Lys level increased. Feed intake was decreased by increasing both the lysine and the calcium level. When increasing the calcium level, the phytase addition plays a role in sustaining feed intake.

Increasing levels of Lysine improved toe ash. Ca: AP ratios of 2:1 and 0.2% more than the 2:1 ratio are adequate for the bone mineralization process, whereas wider or narrower ratios worsened the toe ash response. TD incidence was higher by increasing the Lys level in a non-linear behavior. Increasing calcium levels reduced the incidence and the severity of TD. Moreover, increasing dietary calcium levels help to mitigate the effect of lysine level on TD incidence.

Increasing levels of Lysine reduced TP but increased the WSP/TP ratio in excreta. The 1.1% Lysine level expressed the lowest concentration of WSP in excreta. Increasing dietary calcium levels reduced the WSP and the WSP/TP ratio in excreta. Also, phytase supplementation increased the WSP and the WSP/TP ratio in excreta. A higher level of calcium was effective to reduce the increasing WSP and the WSP/TP ratio in excreta due to phytase addition.

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[3]Lowest level possible without calcium supplementation. Ranges from 0.51 to 0.53% of the diet.