EVALUATION OF PHYTASE, VITAMIN D_3 DERIVATIVES, AND BROILER BREED DIFFERENCES ON NUTRIENT UTILIZATION, BROILER PERFORMANCE, LEG DISORDERS, AND THE EXPRESSION OF INTESTINAL CALBINDIN-28 kd mRNA AND PROTEIN

by

ROBERT BRYANT SHIRLEY

(Under the Direction of Hardy M. Edwards, Jr.)

ABSTRACT

Five experiments were conducted to evaluate the effects of elevated phytase levels [0 to 12,000 units (U)/kg of diet] on broiler chick performance, tibia ash deposition, and the utilization of nutrients in total phosphorus (tP)-deficient diets. The effects of varying levels of phytase alone were evaluated in Experiment 1. Experiments 2 and 3 evaluated the interaction between varying levels phytase and dietary 25-hydroxycholecalciferol or 1a-hydroxycholecalciferol. In Experiments 4 and 5, the interactive effects of varying dietary Ca and phytase were evaluated.

In each of the five phytase experiments, supplementing a tP-deficient broiler diet with 6,000 to 12,000 units of phytase hydrolyzed approximately 80 to 95% of dietary phytate. This not only reduced the P-deficiency of the broiler diets, but subsequently increased the overall Ca and P retention, performance and tibia ash deposition of broiler chicks. In Experiments 3 and 4, the addition of 25-hydroxycholecalciferol and 1a-hydroxycholecalciferol improved broiler performance and the utilization of nutrients at the lower levels of phytase supplementation, however, the addition of 1a-hydroxycholecalciferol at the higher levels of phytase

depressed body weight gain and feed intake, despite graded improvements in phytate degradation and mineral retention values. This suggested that 1a-hydroxycholecalciferol improved Ca absorption to the point that it was anorexigenic. In Experiments 4 and 5, increasing the level of dietary Ca to 1.1% in a tP-deficient diet reduced broiler performance, mineral utilization, and tibia ash deposition when 0 or 1,500 U of phytase was supplemented. Increasing the level of phytase to 6,000 U/kg of diet eliminated the negative effects of high dietary Ca. These data suggest that higher levels of phytase are required to increase the liberation of phytate P. Adjustments in the ratio of dietary Ca to total P, and the level of vitamin D_3 metabolites, however, should be considered when attempting to maximize broiler performance, reduce the amount of excreted P, and improve the overall quality and availability of nutrients in a tP-deficient broiler ration.

INDEX WORDS: Broilers, phytase, cholecalciferol derivatives, phosphorus deficiency, calcium, tibial dyschondroplasia, calbindin-28 kd, triodothyronine

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DEDICATION

This dissertation is dedicated to my wife, Colleen. Her sincerity, patience, strength and encouragement has, and always will, inspire me to do the best that I can. Thank you.

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The faculty, undergraduates and graduate students have all played a special part in my education. I want to thank all of you for the personal and professional help that you've given me for the past three years, the opportunity to run some very spectacular research, and for letting me get to know you. In particular, I want to thank Elaine Foster for her friendship, professional guidance in the laboratory, and her endless efforts to help me understand good laboratory procedures. Finally and most importantly, I want to thank Drs. Edwards, Davis, Compton, Pesti and Brown for their individual and combined guidance and friendship throughout the last three years. Each of you has allowed me to grow and think in many different ways. I am eternally grateful for that.

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CHAPTER 1

LITERATURE REVIEW

Introduction

In plant-based broiler diets, most of the phosphorus (P) that is in the form of phytate [myo-inositol1,2,3,4,5,6 hexakis (dihydrogen phosphate)], is not available, and thus unable to meet the P requirement of the rapidly growing broiler chicken (Nelson et al., 1968). When d-old broiler chicks consume diets that are high in phytate P and low in available P (aP), P-deficiency results within approximately 5 to 6 days (personal observations). Signs of P deficiency in the growing chick include low plasma P (< 5 mg/100 mL), high plasma Ca (> 10 mg/100 mL), and a marked increase in lameness that is due to the degeneration and under-mineralization of hypertrophic chondrocytes within the growth plate (Simbliss, 1967; Long et al., 1984a; Qian et al., 1996b,c). To ensure proper bone development in the broiler chick, broiler producers supplement appropriate amounts of inorganic P in broiler diets to meet the 0.45% aP requirement of young broiler chicks (NRC, 1994). While the dietary supplementation with inorganic P meets the growing broilers' P requirement, it increases the cost of the broiler diet, the overall excretion of P, and the potential for P pollution in geographical areas where the number of broiler complexes and subsequent production of poultry manure is large.

In areas where the application of poultry manure as fertilizer per acre of land is great, the leaching of P through soil and into water tables can result in the premature eutrophication of proximate bodies of water (Correll, 1999; Sharpley, 1999). To reduce the amount of P that is excreted by broilers, the poultry industry has started using dietary phytase. This enzyme dephosphorylates phytate and increases the availability of phytate P for broilers. By improving phytate P utilization,

producers can reduce the amount of inorganic P that is supplemented in broiler rations and the amount of P that is excreted by broiler chicks.

The current recommended level of phytase supplementation in broiler rations is approximately 300 to 600 U/kg of diet. This range was based on the linear relationship between increasing levels of supplemental phytase, the degree to which broiler performance [i.e., body weight gain (BWG)] was improved, and the marginal economic return for each additional level of phytase supplementation. In most phytase studies, increasing the level of phytase to approximately 2,500 U/kg of diet resulted in a subsequent linear improvement in broiler performance (Simons et al., 1990; Mohammed, 1991; Schöner et al., 1991; Huyghebaert et al., 1992; Vogt, 1992a,b; Schöner et al., 1993; Broz et al., 1994; Biehl et al., 1995; Kornegay et al., 1996; Mitchell and Edwards, 1996a,b; Qian et al., 1996a; Sebastian et al., 1996; Qian et al., 1997; Zhang et al., 2000). Phytase levels greater than 600 U/kg, however, generally yielded negligible improvements in broiler performance. On the other hand, analysis of data with the level of phytase expressed on a log-scale-basis, ($\log_{10}$[phytase + 100]) indicates that higher levels of phytase may enhance the degradation and utilization of phytate P in broiler diets. In addition, results from our laboratory (Mitchell and Edwards, 1996a,b) indicate that other dietary nutrients such as Ca and vitamin D_3 metabolites may alter the effectiveness of the phytase enzyme and thus the absorption and utilization of phytate P.

Three studies were conducted to evaluate the effect of higher levels of phytase supplementation, vitamin D_3 metabolites and varying levels of dietary Ca on broiler performance, bone ash deposition and the utilization of dietary nutrients. In an unrelated, fourth study, broilers that were divergently selected for a high or low

incidence of tibial dyschondroplasia were used to evaluate the expression of intestinal calcium binding protein (calbindin-28 kd) at low and high levels of dietary vitamin D_3 supplementation.

Phytic Acid

Myo-inositol1,2,3,4,5,6 hexakis dihydrogen phosphate (phytate) is located within grains and oilseeds. The concentration and location of phytate within seeds is dependent on the age and the species of plant. As a plant ages, the phytate content of a seed increases and associates with protein-rich fractions called globoid bodies. The location and the concentration of these bodies varies within seeds of different origin (O'Dell et al., 1972; Reddy et al., 1982). For instance, the germ of corn contains a higher level of phytate than the aleurone layer (O'Dell and de Boland, 1972, 1976), whereas in the case of soybeans, phytate is not bound to globoid bodies, but is rather disbursed throughout the seed (Tombs, 1967; Lott and Buttrose, 1978; Lott, 1985; Lott and Ockenden, 1986). This variation can affect the phytate content of oilseeds, oilseed meals, grains and grain byproducts that are used in broiler feed. So while whole corn and soybean meal with hulls contain an estimated 0.20 and 0.38% phytate P, respectively, corn gluten meal and soybean meal without hulls contain an estimated 0.36 and 0.40% phytate P, respectively (NRC, 1994).

Regardless of where phytate is stored within a seed, it serves as a reservoir for P and other minerals that can be utilized by a growing seedling. Approximately 50 to 90% of the total P within seeds is bound to phytate (Common, 1940; Nelson et al., 1968; Asada et al., 1969; Reddy, 1982; Ravindran et al., 1995a), making

phytate a significant storage reservoir for P. When P binds to inositol, it binds as an orthophosphate. Collectively, the neighboring orthophosphate groups of phytate create negatively charged zones which that serve as binding sites for mono [Na^{+} and K^{+}] and polyvalent cations [Ca^{++} and Mg^{++}], thereby creating the storage form of phytate that is commonly found within seeds (Cosgrove, 1966; Sobolev, 1966). The solubility of phytate and the affinity of minerals to phytate depends on the cation present and the environmental pH. At a pH of 7.4, Oberleas (1973) determined that the binding affinity of the following minerals to phytate is: $Cu^{+2} > Zn^{+2} > Co^{+2} > Mn^{+2} > Fe^{+3} > Ca^{+2}$. Maenz et al. (1999) reported a similar order of binding affinity. At a pH of 7.0 the order of binding affinity is: $Zn^{+2} > Fe^{+2} > Mn^{+2} > Fe^{+3} > Ca^{+2} > Mg^{+2}$. Maenz et al. (1999) also noted that as the pH increased, so did the binding affinity and precipitating effects of these minerals on phytate.

In addition to binding minerals, phytate may also bind the terminal amine group of protein chains, the amine groups of amino acids such as Arg and Lys, or the carboxyl groups of Glu and Asp through cationic mineral interactions (Cheryan, 1980; Reddy, 1982). If the formation of protein/starch/phytate interactions are significant, then the formation of these complexes may interfere with the activities of proteases and sugar-degrading enzymes within the alimentary tract of the broiler chicken (Cheryan, 1980; Reddy, 1982; Lott and Ockenden, 1986; Thompson, 1986). Thus, any reduction in the activity or efficiency of the latter enzymes may result in a lower utilization of dietary nutrients.

Phytase

The phytase enzyme cleaves orthophosphate groups from phytate, forming several myo-inositol and several phytate intermediates that can contain one to five orthophosphate residues. Depending on the organism, phytase can either be a 3'-phosphohydrolase myo-inositol-hexakisphosphate-3-phosphohydrolase (Ec: 3.1.3.8) that catalyzes the cleavage of 3'-orthophosphate from phytate, or a 6'-phosphohydrolase myo-inositol-hexakisphosphate-6-phosphohydrolase (Ec: 3.1.3.26) that catalyzes the cleavage of 6'-orthophosphate from phytate (Cosgrove, 1966; Cosgrove, 1970; Irving and Cosgrove, 1974).

The 3'-phosphohydrolase phytase was first commercialized under the brand name Natuphos®. This phytase was derived from the molecular insertion of the phytase A gene into a variant of Aspergillus niger (NRRL 3135) (Wodzinski and Ullah, 1996). The activity of this phosphatase enzyme is based on the unit (U), which is defined as the amount of enzyme which liberates 1 micromole inorganic P per minute from 0.0051 mol/L sodium phytate at 37^{o}C and at pH 5.50 over a period of 30 to 60 minutes. Because this phytase product has two pH optima (pH 2.5 and 5.5), this assay tends to underestimate the full activity of phytase. In addition to the underestimation of the phytase preparation, this assay does not take into consideration the activity of other enzymes that may have been produced by the Aspergillus niger (NRRL 3135) micoorganism. This point is particularly important since Newkirk and Classen (1998) reported that Natuphos® containes a considerable amount of acid phosphatase, protease and endo-xylanase enzymes that may synergistically improve the utilization of phytate P, protein and starch when added to P-deficient broiler diets. Depending on the number of units that are added

to low-P broiler rations, the broiler industry may be able to increase the availability of phytate P and lower the amount of supplemental inorganic P and other nutrients that are linked to environmental pollution.

Initial Phytase Studies

Phytase Level Studies

In Ca and P-based nutrition studies, parameters such as BWG, feed intake (FI), bone ash deposition, P retention and phytate disappearance are normally reported because these criteria influence the eventual market value of the broiler chicken. In a series of studies where phytase was supplemented from 0 to 2,500 U/kg of diet, Schöner et al. (1991), Huyghebaert et al. (1992), Vogt (1992a,b), Broz et al. (1994), Denbow et al. (1995), Ravindran et al. (1995b), Kornegay et al. (1996), Yi et al. (1996a,b), Zhang et al. (2000) and Camden et al. (2001) reported improvements in broiler performance and the utilization of dietary nutrients. In the initial study of Simons et al. (1990), the addition of 0 to 2,000 U of dietary phytase resulted in 14-d-old BWG values that ranged from 234 to 359 g/chick, and 4-wk BWG values ranged from 788 to 1,125 g/chick. In the second experiment of the same study, the addition of phytase from 0 to1,500 U/kg of diet resulted in 24-d-old BWG values that ranged from 338 to 733 g/chick. While phytase greater than 375 U/kg in the first experiment elicited no further increase in BWG, increasing the level of dietary phytase to 1,500 U/kg of diet in the second experiment resulted in a curvilinear increase in BWG. Using higher levels of dietary phytase, Zhang et al. (2000) reported a similar increase in 5-wk BWG from 1,242 to 1,476 g/chick and an

increase in P retention from 49 to 57% when a P-deficient broiler diet was supplemented with 0 to 2,500 U/kg of phytase.

Vogt (1992a,b) reported that increasing the level of dietary phytase increased the availability and subsequent retention of P from P-deficient broiler diets, thereby resulting in a concurrent increase in BWG, FI and tibia ash deposition. Supplementing 0 to 1,600 U of phytase, Vogt (1992a) observed an improvement in percent tibia ash from 38.8 to 44.6%. In a second study, phytase supplementation from 0 to 1,600 U/kg of diet increased the percent tibia ash from 40.8 to 45.2%, respectively. While dietary phytase levels greater than 750 U/kg of diet had no further affect on bone ash deposition in the first experiment, percent tibia ash responded to the addition of 1,600 U of phytase. In two P studies, Qian et al. (1996b,c) examined histological sections from the proximal tibiotaursus of broiler chicks and determined that P is required for the proper mineralization of chondrocytes and development of tibiae.

In the studies of Schöner et al. (1991, 1993) and Huyghebaert et al. (1992), the addition of phytase to P-deficient broiler diets resulted in curvilinear improvements in P retention and phytate degradation. While Huyghebaert et al. (1992) observed an increase in P retention from 48 to 62% when 0 to 500 U of phytase was supplemented to a P-deficient diet, no additional increase in P retention was observed when phytase was supplemented at a level of 1,000 U/kg of diet. In two other studies, Schöner et al. (1991, 1993) reported a similar increase in P retention. When phytase was supplemented from 0 to 800 U/kg of diet, P retention increased from 53 to 67% and phytate degradation increased from 8 to 35%, respectively (Schöner et al., 1991). When phytase was supplemented from 0 to 1,500 U/kg of

diet , P retention increased from 49 to 64% and phytate degradation increased from 28 to 50%, respectively (Schöner et al., 1993).

The phytase levels that were used in the studies of Schöner et al. (1991), Huyghebaert et al. (1992), Vogt (1992a,b), Broz et al. (1994), Denbow et al. (1995), Ravindran et al. (1995b), Kornegay et al. (1996), Yi et al. (1996a,b), and Zhang et al. (2000) were regressed against BWG, bone ash or phytase disappearance. Among each of the previously cited studies, the addition of phytase from 0 to 2,500 U of phytase positively affected each of the three parameters. The linear model used shows a decreasing marginal return per unit of phytase when more than 600 U of phytase is supplemented (Figures 1, 2 and 3), however, the data are fairly close together and difficult to interpret. The early vitamin research of Almquist (1953) suggests that the linear conversion of log-dose levels of dietary ingredients is a more appropriate models for the interpretation of data. Despite the variable intercepts from each of the experimental data sets, the log-transformation of the phytase levels ($\log_{10}$([Phytase] + 100)) used within each of the previously cites studies did equalize the spacing between data points (Figures 4, 5 and 6) and removed many of the plateau responses to dietary phytase. When the latter bone ash data are compared with the bone ash data of Hall et al. (2003), it becomes apparent that Schöner et al. (1991), Huyghebaert et al. (1992), Schöner et al. (1993), Zhang et al. (2000) and other researchers had not used enough dietary phytase to maximize phytate degradation, the utilization of dietary P or bone ash deposition. Results from the study of Hall et al. (2003) concluded that increasing the level of phytase from 1,500 to 3,000 or 6,000 U/kg of diet could improved BWG and percent tibia ash further.

Variability in Phytate Disappearance

Phytate P degradation has been reported by several authors, however, the basal level of degradation tends to vary between studies. Results from this and other laboratories indicate that the basal phytate disappearance from a P-deficient broiler diet can range from approximately 3 to 63% (Schöner et al., 1991; Schöner et al., 1993; Roberson and Edwards, 1994; Mitchell and Edwards, 1996a,b; Kasim and Edwards, 2000; Kilburn and Edwards, 2001). Several studies also indicate that the degree of phytate degradation is affected by the level of vitamin D_3, phytase level, Ca level or inorganic P level of the diet, or the source and processing of feed ingredients.

Dietary Factors that Affect te Utilization of Phytate Phosphorus

Dietary Phosphorus and Phytate Utilization

An increase in the level of dietary phytase is often associated with an increase in enzyme activity and broiler performance as more phytate P is liberated and available to meet the P requirement if the broiler chick. Increasing the supplementation of inorganic P in a P-deficient broiler diet is, however, associated with a reduction in the efficiency of the phytase enzyme (Vogt, 1992a,b; Denbow et al., 1995; Kornegay et al., 1996; Mitchell and Edwards, 1996a,b). Mitchell and Edwards (1996a,b) reported a decrease in P retention and phytate disappearance as the level of dietary P increased. For example, when 600 U of phytase was supplemented to a 0.97% Ca, P-deficient diet, increasing the level of dietary P from 0.45 to 0.75% resulted in a 13% increase in P retention and a 40% reduction in

phytate degradation. In a second experiment, P retention and phytate degradation were reduced by 22% and 33%, respectively, when the 600 U of phytase was supplemented to a 0.99% Ca, P-deficient diet.

Dietary Calcium and Phytate Utilization

Increasing the level of dietary Ca beyond dietary requirements or widening the ratio of dietary Ca to P is often associated with a decrease in broiler performance, mineral retention and phytate disappearance (Edwards, 1982; Nelson et al., 1990; Schöner et al., 1993; Mitchell and Edwards, 1996a; Qian et al., 1996a,b; Qian et al., 1997). Qian et al. (1996a, 1997) observed an increase in BWG, Ca retention and P retention (14, 17 and 28%, respectively) when P-deficient broiler chicks and turkey poults diets (0.27% P) were supplemented with 900 U of phytase. At each level of dietary phytase, however, increasing the dietary ratio of Ca:P from 1.1:1 to 2.0:1 reduced BWG, Ca retention and P retention by an average 15, 13 and 10%, respectively, suggesting that higher dietary Ca levels may interfere with phytate P liberation and/or utilization. In an earlier study, Schöner et al. (1993) reported that an increase in dietary Ca from 0.60 to 0.90% resulted in a 51, 18 and 35% reduction in Ca retention, P retention and phytate disappearance, respectively. Mitchell and Edwards (1996a) had also reported a reduction in the latter parameters, however, the reductions were alleviated with the addition of dietary phytase. Using a P-deficient (0.43% P), phytase-free diet, increasing the level of dietary Ca from 0.63 to 0.99% resulted in a 76% reduction in phytate disappearance. Furthermore, when 600 U of phytase was supplemented to the previous diets, the overall disappearance

of phytate increased, however, there was still a depression in phytate disappearance (38%) when dietary Ca levels were increased from 0.63 to 0.99%.

Phosphorus-Sparring Effect of Vitamin D_3 Derivatives

Cholecalciferol (vitamin D_3), 25-hydroxycholecalciferol (25-(OH)D_3), 1''-hydroxycholecalciferol (1''-(OH)D_3), and 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) effectively improve the retention of dietary Ca and P of broiler chick diets. The established order of biopotency between the four previously mentioned metabolites is: 1,25-$(OH)_2D_3$ = 1''-(OH)D_3 > 25-(OH)D_3 > cholecalciferol (Myrtle and Norman, 1971; Norman and Wong, 1972; McNutt and Haussler, 1973; Tanaka and DeLuca, 1974; Boris et al., 1977; Edwards et al., 1992; Edwards, 1993; Soares et al., 1995; Edwards, 2002; Edwards et al., 2002). After cholecalciferol is absorbed through the skin or the intestine, it is obligatorily hydroxylated at the 25 position in the liver (25-(OH)D3), and subsequently transported in the plasma by vitamin D binding protein. When a broiler chick is hypocalcemic, renal 1''-hydroxylase activity is up-regulated through a negative feedback loop involving PTH, thereby increasing the conversion of 25-(OH)D_3 to 1,25-$(OH)_2D_3$. The presence of high plasma 1,25-$(OH)_2D_3$ is not only known to increase the number of Ca channels and transport proteins within the kidney and intestine (Theofan et al., 1987; Meyer et al., 1992; Cai etal., 1993), but also the Na-P transport protein content within the broiler chick's small intestine (Cross et al., 1986; Cross and Peterlick, 1988, 1991; Sechman, et al., 1996). The 1,25-$(OH)_2D_3$ and 1''-(OH)D_3 metabolites affect broiler chick performance and Ca and P metabolism similarly, and are considered two of the

most potent vitamin D_3 metabolites. Unlike 1,25-$(OH)_2D_3$, however, there is very little information regarding the effect of 1"-$(OH)D_3$ on intestinal Ca or P channels or transport proteins. Edwards (1989, 1990) noted similar improvements in BWG, G:F and percent tibia ash when 1,25-$(OH)_2D_3$ or 1"-$(OH)D_3$ were supplemented at 10 µg/kg to a low-Ca, P-adequate broiler diet. In two subsequent studies, Roberson and Edwards (1996) and Edwards et al. (2002) observed a depression in the latter parameters when the level of 1,25-$(OH)_2D_3$ exceeded 5-6 µg/kg of diet. The studies of Edwards (1990) and Biehl et al. (1997a,b) confirmed this possible toxicity as supplemental dietary 1"-$(OH)D_3$ had negligible positive affects upon broiler performance past 5-10 µg/kg.

Previous work regarding the combined supplementation of different levels of dietary phytase and cholecalciferol metabolites is scant. Mohammed et al. (1991) initially reported an increase in phytate degradation from 51 to 59% when the cholecalciferol level of a Ca-adequate (1.00%), P-deficient (0.50%) broiler diet was increased from 12.5 to 1,250 µg/kg of diet[1], respectively. Reducing the dietary Ca level to 0.50% and adding 12.5 µg/kg of diet of supplemental cholecalciferol resulted in a phytate hydrolysis of 65%, while 1,250 µg/kg of diet of supplemental cholecalciferol resulted in a phytate hydrolysis of 77%. Edwards (1993) observed a 29% increase in phytate degradation as the level of phytase approached 600 U/kg of diet. When 10 µg of 1,25-$(OH)_2D_3$ was cosupplemented with 600 U of phytase, the level of phytate degradation was increase to 63%.

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[1]1 : g cholecalciferol = 40 IU of cholecalciferol

The previous improvements in phytate degradation between the studies of Mohammed et al. (1991) and Edwards (1993) indicates that the degree of phytate degradation may not only be dependent on the level of phytase, but the level of Ca and the type and concentration of vitmain D_3 metabolites. For instance, Mitchell and Edwards (1996a) observed a 30% increase in phytate degradation when 5 µg/kg of 1,25-$(OH)_2D_3$ was supplemented to a corn-soybean meal diet that contained 0.87% Ca and 0.45% P. An increase in the level of dietary Ca to 1.07% subsequently reduced phytate disappearance, broiler performance and mineral retention values. In a follow up study, Mitchell and Edwards (1996b) observed a 46, 37 and 67% increase in phytate degradation when a P-deficient diet (1.00% Ca, 0.43% P) was supplemented with 600 U of phytase, 5 µg/kg of 1,25-$(OH)_2D_3$, or the combination, respectively. The latter studies of Mitchell and Edwards (1996a,b) imply that there is a strong relationship between 1,25-$(OH)_2D_3$, Ca absorption and phytate degradation. This association may be linked to the influence that 1,25-$(OH)_2D_3$ may have on intestinal phytase synthesis and/or the absorption of Ca and P as dietary phytase degrades phytate. In order to determine if 1"-$(OH)D_3$ influenced the endogenous production of intestinal phytase, Biehl and Baker (1997a) supplemented either 1,430 U/kg of phytase or 20 µg/kg of 1"-$(OH)D_3$ to a Ca-deficient (0.63%), P-deficient (0.42%) corn-soybean meal diet, and evaluated BWG, FI and tibia ash of sham-operated and cecectomized and chicks. The addition of either phytase or 1"-$(OH)D_3$ increased the BWG, FI and tibia ash deposition of all chicks, while cecectomy tended to reduce the BWG and FI responses when either phytase or 1"-$(OH)D_3$ were supplemented. This suggests that phytate degradation was influenced more by microbial phytase rather than the phytase-stimulating

effects of 1 '' -(OH)D_3. In general, these studies indicate that cholecalciferol or one of its metabolites can further improve the utilization of phytate P, especially when phytase is used and/or the level of dietary Ca is lowered.

Extra-Phytase Enzymes in Broiler Nutrition

Natuphos® phytase was derived from the genetic insertion of the phytase A gene into Aspergillus niger NRRL 3135. Despite the overwhelming production of the phytase A protein, òy»a et al. (1995) and Newkirk and Classen (1998) determined that Natuphos® contained minute quantities of other enzymes such as acid phosphatase, protease and endo-xylanase. Research of Ravindran et al. (1999) and the review of òy»a (2001) suggests that the application of various enzyme cocktails in poultry nutrition can improve the availability of nutrients and the performance of broilers and turkeys when dietary phytase preparations contain other enzymes such as acid phosphatase, acid protease, endo-xylanase $-glucanase, arabinoxylanase, cellulase, hemicellulase, or lipase. In an *in vitro* study, òy»a et al. (1995) demonstrated that acid phosphatase, in the absence of phytase, was unable to hydrolyze phytate. When acid phosphatase and phytase were added together, phytate hydrolysis was greater than when phytase was added alone. òy»a et al. (1996) also observed a dietary Ca and P retention of 48 and 67%, respectively, and a percent tibia ash of 42.7% when a P-deficient corn-soybean meal turkey diet was supplemented with 1,000 U of phytase. When an enzyme cocktail that contained phytase A, phytase B (pH optima = 2.0), pectinase, citric acid, acid protease and ascorbic acid was supplemented to the same diet, the dietary Ca and P retentions

and percent tibia ash were increased to 68, 77 and 45.2%, respectively. Low levels of phytase supplementation does not always result in optimal broiler performance. The results of Hall's study (Hall et al., 2003) and the results from the enzymatic cocktail studies of òy»a et al. (1995, 1996, 2000, 2001) suggest that phytase preparations, such as the Natuphos® product, may contain sufficient quantities of exogenous enzymes, thereby improving the utilization of nutrients when supplemented in broiler diets at high levels.

The Effects of Breed, Age and Feed Processing on Phytate Utilization

Breed

A limited amount of research is available concerning the utilization of P and the degree to which different breeds of chickens use P. In a study by Gardiner (1969), the utilization of three different levels of dietary P was compared between commercial broilers and single comb white leghorns (SCWL) in three, four-week studies. In each experiment, Gardiner observed that the commercial broiler chicks had gained significantly more weight than the SCWL chicks, but that the P retention, plasma P and bone ash deposition of the SCWL chicks was significantly greater. Extending this research, Edwards (1982) reported that the SCWL was able to degrade more dietary phytate than the Athens Canadian Randombred broiler chick (56 versus 37%, respectively). Since the commercial broiler is bred for meat and the SCWL is bred for high egg production, selecting the broiler parent stock for

higher egg production may not only alter the degree to which young broiler chickens use dietary P, but how well the present-day broiler chick can degrade and utilize phytate P.

Age

As a broiler chick ages, the size of its gastrointestinal tract increases, as does the microbial population and the production of endogenous enzymes. An early study of Ashton et al. (1960) reported that 6-wk-old broiler chicks were able to hydrolyze more dietary phytate compared to 4-wk-old broiler chicks. Using SCWL chicks, Edwards et al. (1989) reported an increase in phytate disappearance of 19, 26 and 36% when the SCWL chicks were 7, 14 and 21-d-old, respectively. Simons et al. (1990) determined that as broilers aged from 2-wks-old to 4-wks-old, the addition of more than 375 U of phytase to a P-deficient diet had minimal affect on BWG, suggesting that broilers are able to utilize more phytate P as they get older. This is in agreement with the study of Schöner et al. (1993). In Schöner's study, using 14-d-old broiler chicks, phytate retention values increased as the level of supplemental phytase increased from 0 to 1,500 U/kg of diet. At 40-d of age, the overall hydrolysis of phytate was improved, however, the magnitude of this response was markedly less between each additional level of phytase. Comparing the main effects for the two groups of broilers that were consuming the phytase supplemented diets, Schöner et al. (1993) reported a 37 to 50% increase in phytate degradation as broiler chicks aged from 14 to 40-d-old. Schöner's study also indicates that phytase supplementation may be necessary when older broiler chicks are fed P-deficient broiler diets.

Feed Processing

The form of feed may alter the relative availability of phytate P, energy and protein in tP-deficient diets. The study of Kilburn and Edwards (2001) suggests that decreasing the particle size of corn in a tP-deficient corn-soybean meal diet decreases phytate P disappearance, tCa retention, tP retention and ME values. Kasim and Edwards (2000) reported a similar trend, however, they also observed differences in ME values between diets containing coarse versus finely ground corn when dietary phytase was supplemented at 600 U/kg of diet. The addition of phytase had no affect upon the ME of diets containing coarsely ground corn, whereas the ME of diets containing finely ground corn was improved with the addition of phytase. In addition, using corn-soybean meal diets that were formulated and analyzed to have similar Ca, P and phytate P concentrations, Kasim and Edwards (2000) also observed significant differences in phytate P disappearance, tCa retention, tP retention and ME values when four different cultivars of corn were fed. This suggests that the cultivar of grain or oilseed used in broiler rations may affect the degree of phytate P utilization by the broiler chicken.

Purpose of Research

The purpose of the following studies was to evaluate the effects of elevated levels of dietary phytase [0 to 12,000 units (U)/kg of diet] on broiler chick performance, bone ash deposition, and the utilization of nutrients, when added to tP-deficient broiler diets. Using higher levels of phytase, additional studies evaluated the effects of 25-hydroxycholecalciferol, 1a-hydroxycholecalciferol, and

dietary Ca on broiler performance, bone ash deposition and nutrient utilization from P-deficient broiler diets.

Using two levels of cholecalciferol, the expression of calbindin-28 kd and vitamin D receptor, and the levels of plasma thyroid hormones will be evaluated in two broiler strains that were divergently selected for either a low or high incidence of tibial dyschondroplasia.

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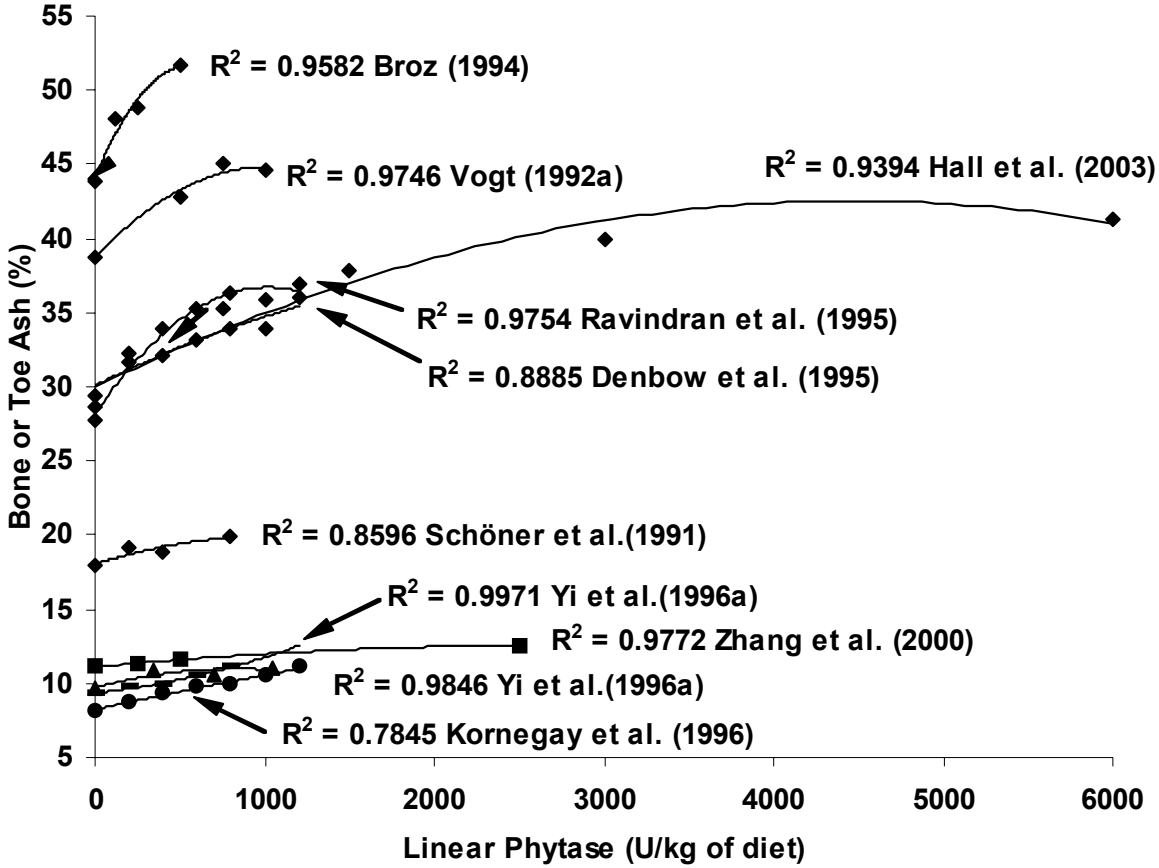
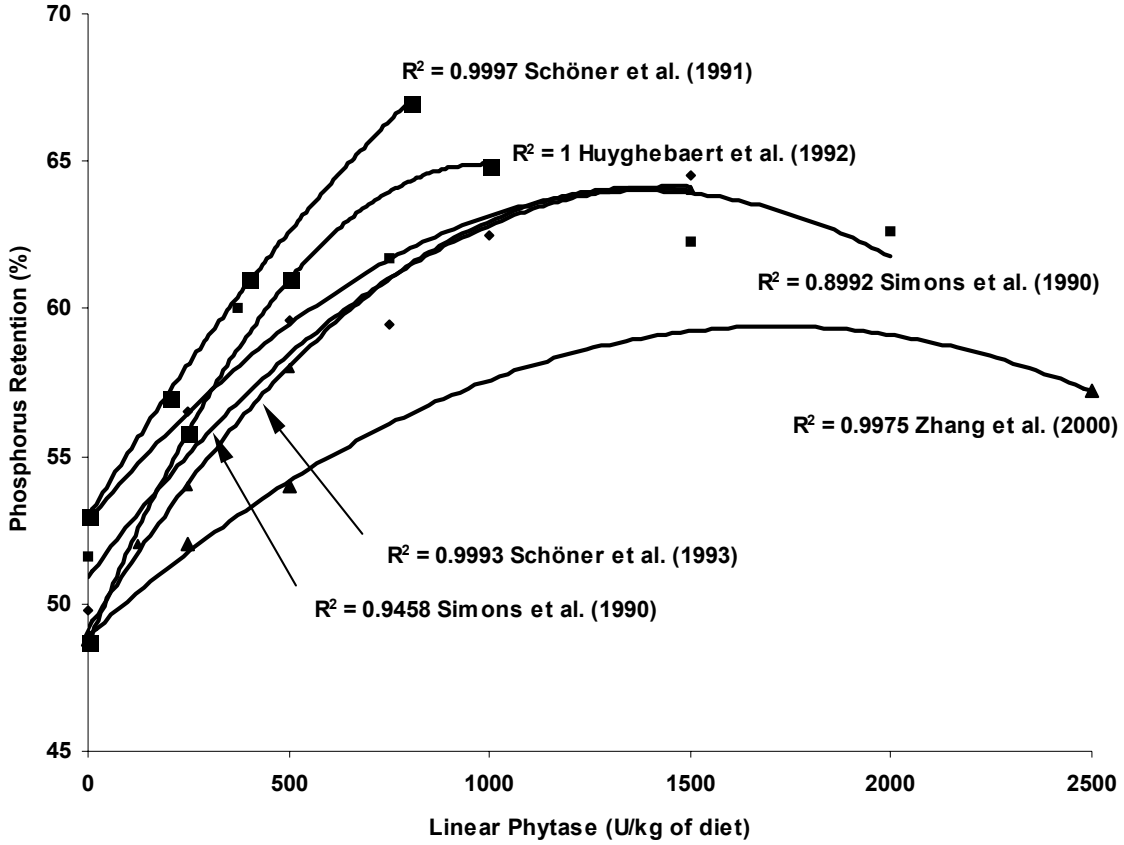
Figures

FIGURE 1.1.



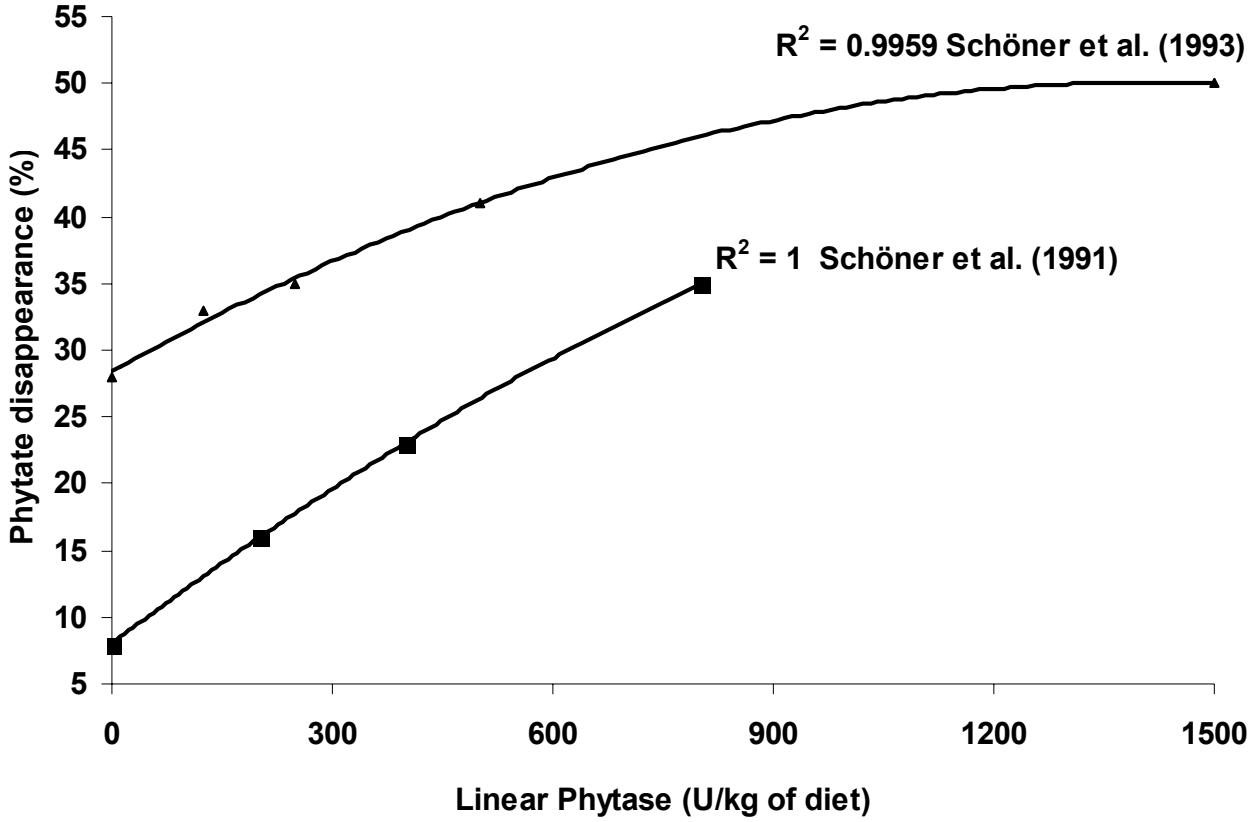
Differences Among Percent Bone Ash Data Reported in the Literature by Various Research Groups. Data represent the regression between bone ash and toe ash values of several authors against various phytase levels. Data from Schöner et al.(1991), Kornegay et al. (1996), Yi et al. (1996 a,b), and Zhang et al. (2000) are toe ash data.

FIGURE 1.2.



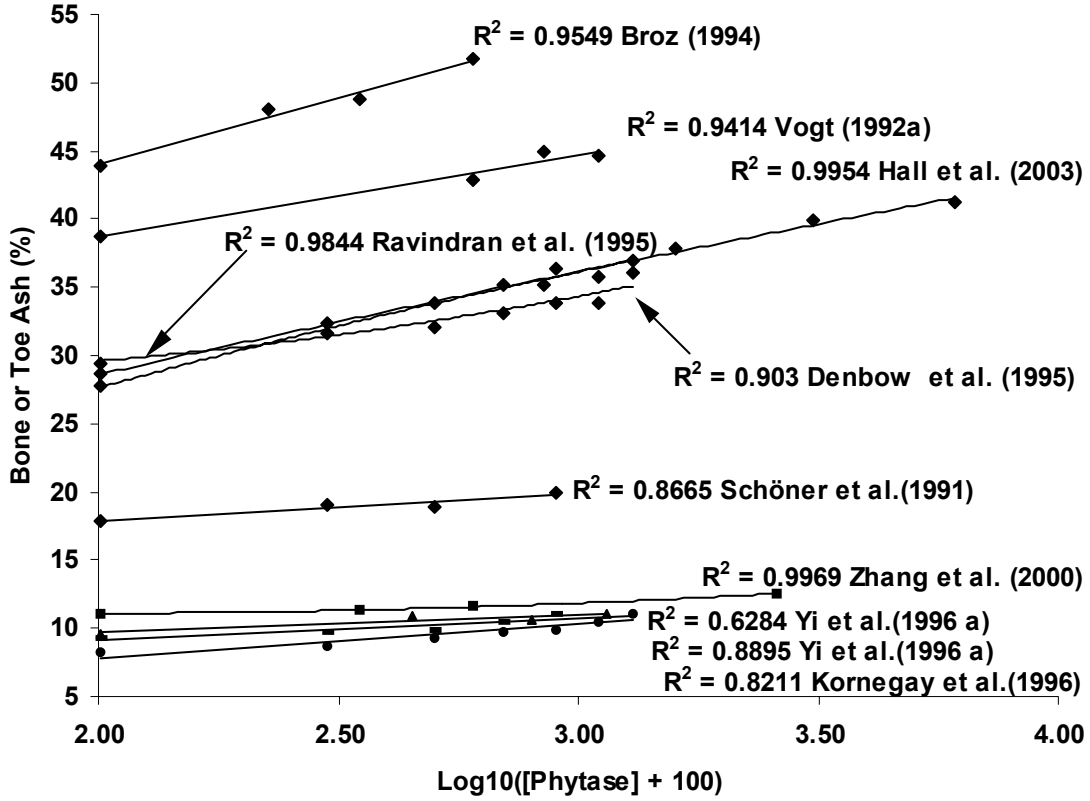
Differences Among Total P Retention Data Reported in the Literature by Various Research Groups. Data represent the regression between dietary P retention values of several authors against various phytase levels.

FIGURE 1.3.



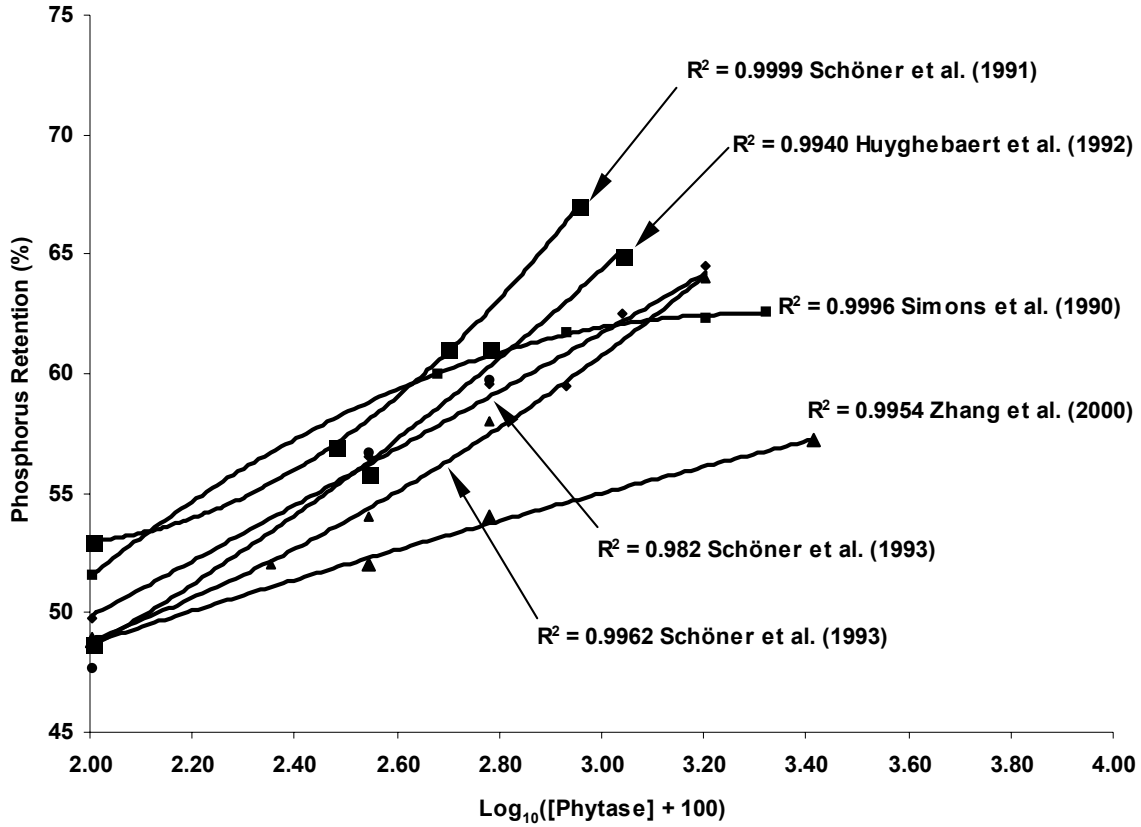
Differences Among Phytate P Disappearance Data Reported in the Literature. Data represent the regression between dietary phytate P degradation values of several authors against various phytase levels.

FIGURE 1.4.



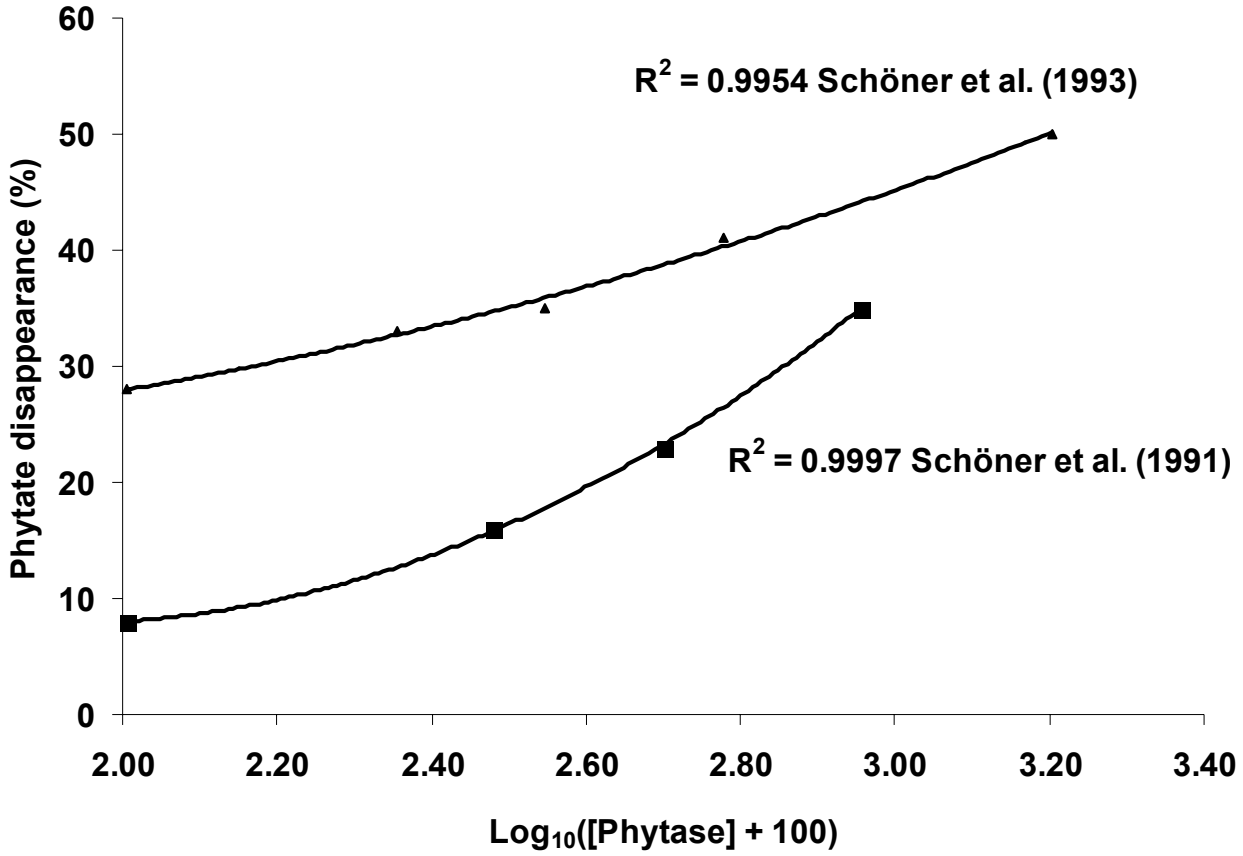
Differences Among Percent Bone Ash Data Reported in the Literature by Various Research Groups. Data represent the regressed bone ash and toe ash values reported by several authors against the $\log_{10}$-transformation of phytase levels ($\log_{10}$([phytase] + 100)). This transformation allows for a more equal spacing of the data points along a linear plot. Data from Schöner et al.(1991), Kornegay et al. (1996), Yi et al. (1996 a,b), and Zhang et al. (2000) are toe ash data.

FIGURE 1.5.



Differences Among Total P Retention Data Reported in the Literature by Various Research Groups. Data represent the regressed total P retention values reported by several authors against the $\log_{10}$-transformation of phytase levels ($\log_{10}$([phytase] + 100)). This transformation allows for a more equal spacing of the data points along a linear plot.

FIGURE 1.6.



Differences Among Phytate P Disappearance Data Reported in the Literature. Data represent the regressed phytate P disappearance values reported by several authors against the $\log_{10}$-transformation of phytase levels ($\log_{10}$([phytase] + 100)). This transformation allows for a more equal spacing of the data points along a linear plot.

CHAPTER 2

GRADED LEVELS OF PHYTASE PAST INDUSTRY STANDARDS IMPROVES BROILER PERFORMANCE[1,2]

[1]Shirley, Robert B. and Hardy M. Edwards, Jr. 2003. Poultry Science. 82:517-690. Reprinted here with permission of publisher.

[2]Experiment B5-01-TD.

Abstract

This study evaluated the overall performance of 0 to 16 d-old, mixed-sex, Cobb x Cobb broiler chicks when dietary phytase levels were supplemented in excess of industry standards. The experimental diet used consisted of a basal corn-soybean meal diet that contained an analyzed CP of 22.2%, a Ca level of 0.88%, a deficient, total P (tP) level of 0.46% [phytate P = 0.272%], and a calculated ME of 3.123 kcal/g diet on an as-is basis. In addition to a positive control diet [0.70% tP], the dietary phytase levels evaluated were: 0, 93.75, 187.5, 350, 750, 1,500, 3,000, 6,000, and 12,000 units (U)/kg diet. Broilers consuming the positive control diet had a respective body weight gain (BWG) and feed intake (FI) of 501 and 594 g/chick, a gain-to-feed (G:F) of 0.843, a plasma phosphorus (plasma P) of 6.9 mg/dL, a 0% incidence of phosphorus rickets (P rickets) , a respective total P (tP) retention and phytate disappearance of 47 and 19%, a tibia ash (TA) of 40%, a tibia ash weight (TA_w) of 0.568 g/tibia, an apparent N retention of 65%, and a nitrogen-corrected, apparent metabolizable energy (AME_n) of 3,232 kcal/kg diet. Supplementing phytase from 0 to 12,000 U significantly increased BWG from 287 to 515 g/chick, FI from 381 to 595 g/chick and G:F from 0.755 to 0.866, plasma P from 2.5 to 7.1 mg/dL, TA from 26 to 41%, TA_w from 0.200 to 0.601 g/tibia, a respective tP retention and phytate disappearance from 51 and 40% to 80 and 95%, an apparent N retention from 58 to 78%, an AME_n from 3,216 to 3,415 kcal/kg diet, and a reduced P rickets from 80 to 3%. Using non-linear regression analysis on log-transformed phytase levels, G:F, apparent N retention, and AME_n responded linearly, with respective R^2 values of 0.76, 0.82, and 0.72, while BWG, FI, plasma P, P rickets, tP retention, phytate disappearance, TA, and TA_w responded quadratically, with

respective R^2 values of 0.93, 0.88, 0.85, 0.84, 0.91, 0.96, 0.96, and 0.98. Few statistically significant differences existed between response data for broilers consuming the positive control diet and diets containing 1,500 to 12,000 U of phytase ($P > 0.05$). This indicates that broilers consuming a tP-deficient corn-soybean meal diet achieved maximal performance when phytase is supplemented to 12,000 U/kg diet; and, that current phytase supplementation levels within the poultry industry may need to be reevaluated.

Key words: broiler performance, dietary phytase supplementation, phytase level

Introduction

Phytase supplementation studies have shown an increase in broiler performance, bone ash, and phytate phosphorus (phytate P) utilization with each additional unit/level of phytase. Evaluating phytase supplementation of tP-deficient broiler diets, Simons et al. (1990), Schöner et al. (1991), Huyghebaert et al. (1992), Vogt (1992), Kornegay et al. (1996), and Zhang et al. (2000 a) determined that tP utilization could be improved in a dose-response manner with graded levels of phytase, thereby reducing total phosphorus (tP) excretion; and, in some cases, equalizing broiler performance to that of broilers consuming rations with a higher available P. Collectively though, research of Simons et al. (1990), Schöner et al. (1991), Huyghebaert et al. (1992) and Zhang et al. (2000 a) indicates that tP utilization may begin to plateau around 500 U when tP-deficient diets are supplemented with up to 2,000 U of phytase, indicating variable marginal returns per unit of supplemental phytase. Comparing the results from the study of Hall et al. (2001) and other unpublished phytase research from this laboratory, increasing the log-dose supplementation of phytase to 6,000 U/kg of diet improves broiler performance, bone ash, and the utilization of phytate P beyond that of any of the previously mentioned studies. Using the log-dose method, a doubling of the previous level of a nutrient or feed additive results in an equal spacing of ingredient levels on log graph paper. Likewise, if the log-dose levels of a nutrient are transformed, an equal spacing of response data between each dietary level occurs when the transformed levels are plotted on standard graph paper. Using the higher log-dose levels of phytase, data from this laboratory suggests that the previously

mentioned authors may not have used enough phytase to obtain a maximal response in broiler performance.

Few studies have addressed the effects of the addition of phytase to broiler diets at levels greater than industry standards. Supplementing phytase to a tP-deficient broiler diet, that consists of a greater proportion of phytate P per unit of tP, should theoretically increase the hydrolysis of dietary phytic acid. The data of Schöner et al. (1991), Schöner et al. (1993), Ravindran et al. (1995 b), Sebastian et al. (1996), and Zhang et al. (2000 a) also suggest that phytase may not only increase phytic acid hydrolysis and improve phytate P utilization, but possibly the overall utilization of dietary nutrients as evidenced by an improvement in broiler performance. Therefore, our hypothesis is that supplementation of phytase up to 12,000 U per kg of a corn-soybean meal diet will improve broiler performance and overall nutrient utilizations when compared to a diet with an adequate level of tP.

Materials and Methods

Experimental Diets

The National Research Council's nutrient values for ingredients (NRC, 1994) was used to formulate the basal diet (Table 1). The basal corn-soybean meal diet used contained a calculated ME of 3.123 kcal/g diet and an analyzed 90% DM, 22.2% CP, 0.88% Ca, and a deficient tP level of 0.46% (phytate P = 0.272%). To the basal diet, phytase [Natuphos 5000 (supplementation was based on the guaranteed value of the phytase product)][3] was added at either 0, 93.75, 187.5, 375,

[3]BASF Corp., Mt. Olive, NJ.

750, 1,500, 3,000, 6,000, or 12,000 U/kg diet. After the experiment was conducted, this product was assayed by BASF using their in-house procedure. The product was assayed to have 6,054 U/g, where one unit (U) is equivalent to one phytase unit (FTU), which is defined as the amount of enzyme which liberates 1 micromole inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at 37°C and at pH 5.50 for 30 to 60 min. In addition, dicalcium phosphate was added to the basal diet to create a positive control diet which contained an analyzed 0.70% tP and no phytase. Sand was used as an inert filler, and was removed from the respective diets as either dicalcium phosphate or phytase was added to the basal diet.

Chick Assay Procedures

On the day of hatch, three-hundred (300), Mareks-vaccinated, mixed-sex, Cobb x Cobb[4] chicks were wing-banded, weighed, and randomly allotted in groups of ten chicks per pen, with three pens per treatment. Throughout the 16-d experiment, chicks were given ad libitum access to water and mash-feed, and brooded in thermostatically controlled Petersime starter batteries[5] with raised wire floors and a 24 h lighting schedule. The starter batteries were kept in an environmentally controlled room (22°C), where fluorescent lights outside and inside the batteries were covered with Arm-a-lite plastic sleeves[6] to eliminate ultraviolet light exposure and possible de novo vitamin D synthesis within the chick. By

[4]Seaboard Farms, Athens, GA.

[5]Petersime Incubator Co., Gettysburg, OH.

[6]Arm-a-lite, Thermoplastic Processes, Sterling, NJ.

eliminating the chicks' exposure to ultraviolet light, a reduction in any additional vitamin D_3-mediated improvement in phytate P utilization was minimized, resulting in a better base-line response to dietary phytase supplementation (Edwards et al, 1994; Elliot and Edwards, 1997).

On d-16, two chicks were randomly selected from each pen, weighed, and a heparinized blood sample was obtained by heart puncture. All remaining chicks were weighed and euthanized via CO_2 asphyxiation. The right tibia of each chick was randomly scored for the incidence of phosphorus rickets (P rickets) (Kasim and Edwards, 2000), while the left tibia of each chick was banded for identification and removed for future percent tibia ash (TA) and tibia ash weight determination [g/tibia (TA_w)]. Excreta was collected from each pen for 24 h between days 15 and 16 of the experiment, dried at 75°C for 36 h, and ground to pass through a 1mm mesh screen.

Sample Analyses

Plasma samples were analyzed for ionized plasma calcium (plasma Ca) (Section N-31)[7] and dialyzable plasma phosphorus (plasma P) (section 7N-46)[8] immediately after the heparinized blood samples were collected and centrifuged for ten minutes at 3,000 x G.

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[7]Section N-31, Technicon Autoanalyzer Methodology, Technicon Corp., Tarrytown, NY.

[8]Section 7N-46, Technicon Autoanalyzer Methodology, Technicon Corp., Tarrytown, NY.

Feed and excreta were analyzed for moisture, chromic oxide (Brisson, 1956), CP [N x 6.25] (Etheridge et al., 1998), gross energy (instructions for 1241 and 1242 Adiabatic Calorimeters, Parr Instrument, Moline, IL), total Ca (tCa) (Hill, 1955), tP (O'Neill and Webb, 1970), and phytate P (Latta and Eskin, 1980). Using the latter values, nutrient utilizations such as the retained Ca:P ratio[9], apparent N retention[10] and nitrogen-corrected, apparent metabolizable energy (AME_n)[11] were calculated using the methods of Edwards and Gillis (1959) and Matterson et al. (1965).

Both TA and TA_w were determined using the left tibia on a dry, fat-free basis (AOAC, 1995), where TA was calculated as a percentage of the ash weight of dry, fat-free tibiae; and, TA_w was calculated as the weight of the tibiae ash, divided by the number of pooled tibiae from a given pen.

Statistical Analyses

All data were analyzed using the General Linear Model procedure of SAS software (1999) and data from the positive control treatment was compared with the data from any one phytase level using Dunnett's Test at an $\alpha = 0.05$. Modifying the non-linear regression analysis procedure of Zhang et al. (2000 b), phytase levels

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[9]Calcium to phosphorus ratios were calculated as: ((%tCa retention x % analyzed dietary Ca on a DM basis)/100)/((%tP retention x % analyzed dietary P on a DM basis)/100)

[10]Apparent N retention of each diet was calculated as: (100- (100 x (% Cr_2O_3 feed/% Cr_2O_3 excreta) x (%N excreta/%N feed)))

[11]Nitrogen-corrected, apparent metabolizable energy (AMEn) of each diet was calculated as: (GE Feed [kcal/g] - ((GE Excreta [kcal/g] x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) + (g N Feed - (g N Excreta x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) x 8.28))) x 1,000

were log-transformed (log_{10}) and regressed against the data of each dependent variable, thereby allowing for the assessment of equally distributed data points along a linear plot (Almquist, 1953). The resulting regression models were then reduced by eliminating non-significant main-effects in order to determine appropriate linear ***[Y = B + M x log$_{10}$(X + 1)]*** and quadratic ***[Y = B + M x log$_{10}$(X + 1) + M' x (log$_{10}$(X + 1))^2]*** prediction equations, where Y is the predicted outcome, # is the intercept, 9 is the linear slope of the equation (change in performance per log-unit of phytase in the diet), 9' is the quadratic slope of the equation, and O is the level of phytase in U/kg diet.

General Results

BWG, FI, GF

Chicks consuming the control diet [0.70% tP] had a BWG of 501 g/chick, a FI of 594 g/chick, and a G:F of 0.843 [g:g] (Table 2). Chicks consuming the basal diet without any phytase or supplemental P, had a BWG of 287 g/chick, a FI of 381 g/chick, and a G:F of 0.755. When 93.75 U of phytase was supplemented , BWG, FI, and G:F values increased to 354 g/chick, 441 g/chick, and 0.805, respectively. Additional supplementation to 750 U further corrected the tP deficiency of the basal diet, as BWG, FI, and G:F increased to 424 g/chick, 505 g/chick, and 0.839, respectively. Increasing levels of phytase to 12,000 U overcame the basal diet's tP deficiency, as BWG, FI, and G:F values became equivalent to the control diet ($P > 0.05$), indicating the overall benefit of higher supplemental phytase levels.

Prevention of a phosphorus deficiency was achieved when12,000 U of phytase was added to the basal diet. Using regression analysis, however, the graded

addition of phytase from 0 to 12,000 U yielded a lower marginal return on performance per unit of additional phytase, as BWG and FI responded quadratically (R^2 = 0.93 and 0.88) and G:F responded linearly (R^2 = 0.76).

Rickets, Tibia Ash, and Plasma Ca and P

Similar to BWG, FI, and G:F, principal improvements in the incidence of P rickets, tibia ash, plasma Ca and plasma P data were indicative of a better phytate P utilization (Table 3). Supplementing the basal diet to 0.70% tP eliminated the incidence of P rickets (0%), increased TA to 40%, TA_w to 0.568 g/tibia, and plasma P to 6.9 mg/dL, and lowered plasma Ca to 11.7 mg/dL. A lack of phytase supplementation to the basal diet elicited a Ca to P imbalance, as plasma Ca increased to 13.3 mg/dL and plasma P decreased to 2.5 mg/dL. This imbalance not only reduced BWG and FI, but also increased P rickets to 80%, and reduced TA and TA_w to 26% and 0.200 g/tibia, respectively. By supplementing the tP-deficient basal diet with a log-dose of phytase to12,000 U/kg diet, plasma Ca was reduced and plasma P increased to11.6 and 7.1 mg/dL, respectively. The latter normalization of plasma Ca and plasma P concentrations were associated with a reduction in the incidence of P rickets to 3.3% and an increase in TA and TA_w to 41% and 0.601 g/tibia, respectively. This essentially equalized the performance of those broilers consuming the higher levels of phytase to that of broilers consuming the control diet (P > 0.05). Overall, plasma Ca, plasma P, P rickets, TA, and TA_w responded quadratically to the addition of phytase, with respective R^2 values of 0.26, 0.85, 0.84, 0.96, and 0.98.

Nutrient Utilizations

Chicks fed the positive control diet had a tCa retention, tP retention, and a retained Ca:P ratio of 51, 47, and 1.37, respectively, followed by a phytate disappearance of 19%, an apparent N retention of 65%, and an AME_n of 3,232 kcal/kg diet (Table 4). With the exception of the tCa retentions and phytate disappearance, nutrient utilizations between the basal diets that were supplemented with 750 U or more of phytase were greater than ($P < 0.05$) or equal to ($P > 0.05$) the positive control diet. When the level of supplemental phytase was graded from 0 to 12,000 U, tCa retention, tP retention, and phytate disappearance were significantly improved approximately 17, 57, and 135%, respectively ($P < 0.0001$), reducing the ratio of retained Ca:P as phytase levels increased. In addition to an increase in mineral utilizations, apparent N retention and AME_n were improved approximately 33 and 6%, respectively. Using regression analysis, tCa retention, tP retention, and phytate disappearance responded quadratically to the graded doses of phytase (R^2 = 0.69, 0.91 and 0.96, respectively), while retained Ca:P, apparent N retention, and AME_n responded linearly (R^2 = 0.74, 0.82, and 0.72, respectively).

Discussion

The results of this study clearly support our initial hypothesis that levels of phytase supplementation above industry standards (12,000 units per kg of diet) are required to make almost all of the phytate P present in a corn-soybean meal diet available (~95%) for maximum utilization by the young broiler chick.

There exists the possibility, however, that microbial breakdown of phytate P may occur in the wet excreta during a 24-h excreta collection, thereby inflating the overall phytate P disappearance values that were calculated using Cr_2O_3 as an indigestible marker. Addressing this concern, Edwards (2002) demonstrated there is very little difference between results obtained by Cr_2O_3 and total collection analysis, as both methods yielded similar phytate disappearance values from excreta, thereby validating our approach. In an earlier study using Cr_2O_3 and diets that contained no phytase or vitamin D derivatives that enhance the utilization of phytate P, Sooncharernying and Edwards (1993) could not detect any enzymatic or microbial degradation of phytate within excreta that was collected on dropping trays over a 32-h period. Extending this work, Escoe (2001) reported that the amount of time excreta are collected on dropping trays (0 to 48-h) has minimal effect on phytate degradation when diets contain phytase, 1,25-dihydroxycholecalciferol, or the combination. Therefore, the calculated phytate disappearance values herein are reported with confidence as phytate P disappearance values also coincided with an increase in tP retention, TA, TA_w, and overall broiler performance as dietary phytase levels increased.

The bone ash, tP retention, and phytate disappearance values from several papers along with the data from this paper are plotted against the $\log_{10}$-transformation [$\log_{10}$([phytase] + 100)] of dietary phytase levels in Figures 1, 2, and 3. Where comparable, the responses in tibia ash data of Vogt (1992), Broz et al. (1994), Denbow et al. (1995), and Ravindran et al. (1995b) are very similar to the responses for the same levels of phytase used in this study. Similar responses in toe ash values to graded levels of phytase are also noted in the papers of Schöner

et al. (1991), Kornegay et al. (1996), and Yi et al. (1996 a,b), and Zhang et al. (2000 a), however, these responses tended to be smaller per unit of additional dietary phytase. In general, an increase in tibia ash data from the previously mentioned investigators paralleled a similar increase in the reported tP retention and phytate disappearance data within the same studies that are presented in Figures 1 and 2. Combined, these tibia ash, phosphorus retention, and phytate P disappearance data further support the data from this present study, for the same criteria, at the lower levels of phytase supplementation. Although the overall response of all three criteria to lower levels of phytase between the previously mentioned researchers and this study are similar, the degree of response differs. These differences are most likely due to differences in the processing of the tibiae for ash analysis, the total Ca and P content of the diets used, the variable levels of supplemental vitamin D, the environment in which the experiment was conducted, the type of chicks used, or a combination thereof. Regardless, Figures 1, 2 and 3 indicate that if greater levels of phytase were fed in the previously mentioned studies, larger responses in tibia ash, tP retention, and phytate disappearance values would have been obtained.

With each additional log-dose of dietary phytase, an improvement in phytate disappearance and tP retention increased plasma P values. Considering the large effects that phytase had on phytate disappearance and tP retention values in this study, higher levels of phytase had little effect on plasma Ca or the tCa retention values from the various diets containing 3,000 U of phytase or less. Higher levels of phytase supplementation, however, tended to increase the retention of dietary Ca as the concentration of plasma Ca decreased. In addition to a marginal increase in the retention of dietary Ca, a larger increase in dietary P retention shifted the ratio

of retained Ca:P from 1.72:1 in the basal diet to 1.28:1 with the addition of 12,000 U of phytase. This shift in tCa to tP retention values and the resulting change in broiler performance, TA data, and plasma Ca and P values may be explained in that the physiological concentrations of Ca within the chick may be more tightly regulated than that of P. For example, chicks consuming the tP-deficient diet had a slightly elevated plasma Ca and a very low plasma P due to a severe P deficiency, however, chicks consuming the phytase-supplemented diets had restored homoeostatic balance between Ca and P as indicated by increasing levels of plasma P and slightly decreasing levels of plasma Ca. The latter relationship between an improved physiologic balance of Ca:P is further substantiated by a decrease in P-rickets and an improvement in TA and TA_W with each additional level of phytase. By increasing the level of dietary phytase in a tP-deficient corn-soybean meal diet, a change in the overall content of calcium to P (Ca:P ratio) in the whole chick occurred. With respect to skeletal development, Qian et al. (1996 a, b) reported on the histology of tibiae from chicks and turkey poults that consumed phytase-supplemented, tp-deficient diets. They observed that higher levels of dietary phytase increased the availability of dietary Ca and P. By increasing the overall availability of Ca and especially P, the initial mineralization and further complex remodeling of bones within the growing chick and turkey poult could normally occur.

Cheryan (1980), Thompson (1986), and Ravindran et al. (1995a) indicate that seeds of different origins (grains versus oilseeds) contain various complex interactions between phytate, minerals, starch and protein. When plant-based diets are ingested, fermented within the crop, and then digested within the gastrointestinal

tract, other complex interactions between dietary phytate, minerals, starch and protein may potentially occur, resulting in lower nutrient utilizations. The effects of phytase on the improvement of Ca and P utilization is well documented, however, it is unclear whether phytase has an effect upon N and AME_n utilization (Kies et al., 2001). In this study though, broilers were able to retain 33% more N and utilize an additional 200 kcal ME/kg diet when 12,000 U of phytase was added to the diet. These improvements were significantly greater than those of the positive control diet ($P < 0.05$), indicating that phytase at the higher levels may be influencing more than mineral utilization. Since body composition data were not taken in this experiment, direct hypotheses into the mechanism behind an improvement in N and AME_n utilization of the phytase-supplemented, tP-deficient diets can not be made without a great deal of speculation. With regard to the phytase used, however, there may be some credence to the ability of phytase to disrupt the various interactions between phytate, minerals, starch and protein, thereby allowing a greater utilization of all nutrients from the diet. By increasing the level of phytase to 12,000 U in the diet, approximately 95% of the phytate P disappeared, potentially removing any anti-nutritional effects phytate may have had in the diet. The increase in phytate hydrolysis and nutrient utilizations may partially be explained by the fact that there are two pH optima at which the BASF product has activity, pH 2.5 and 5.5. The analysis of the BASF phytase took into consideration phytase's activity at pH of 5.5 (5,000 U/g), but not at 2.5. With a slight reduction in the activity of supplemental phytase within corn-soybean meal and wheat-soybean meal diets, Rapp et al. (2001) reported significant phytase activities within the stomach and duodenum of cannulated piglets. This may be similar in chicks, as an overall increase in phytate

hydrolysis within the various parts of the digestive tract may result in an improvement in the overall nutrient digestion within, and absorption from, the small intestine. For example, phytate degradation may initiate within the chick's crop as a phytase-supplemented diet is moistened. As the ingesta passes through the acidic environment of the proventriculus and gizzard [pH of approximately 2 to 2.5] phytate hydrolysis and the acidic/proteolytic digestion of protein would increase. Entering the alkaline environment of the small intestine [pH of approximately 5.5 to 6.5], further hydrolysis of phytate and the digestion of other nutrients within the digesta would occur. Again, by increasing the overall degradation of phytate, complex interactions that may exist between phytate and other nutrients may be reduced, allowing for an increased utilization of all other nutrients. Alternatively, the increase in nutrient utilization at 12,000 U of phytase may just reflect a better physiological Ca:P balance, the quality of the feed ingredients used in the study, or minute quantities of other enzymes (cellulase, xylanase, pectinase, hemicellulase, etc.) that may not have had an effect at the lower levels of phytase supplementation (òy»a et al., 2001), or the combination thereof. The activity of phytase and other potential enzymes within the phytase preparation needs further investigation, especially with regard to the potential increase in enzyme activities within the various parts of the digestive tract of poultry when given supplemental phytase.

Conclusion

Most previous investigators hypothesized that a lower level of phytase was adequate for maximum broiler response, primarily because the lower levels of phytase yielded curvilinear responses. The curvilinear responses that were

observed in Figures 1, 2, and 3 reflect diminishing returns in broiler performance as phytase levels increase. Regressing the same data in a non-transformed model, reduced marginal returns were exacerbated, leading researchers to conclude that most broiler chick responses plateaued at much lower levels of phytase. Data from Hall et al. (2001) and this study suggest that higher levels of phytase can be used to improve the overall utilization of phytate P and possibly other nutrients in a tP-deficient diet, thereby equalizing broiler performance to that of broilers fed diets containing supplemental inorganic phosphorus. The whole effect of phytase on these factors, however, needs further investigation, as data from this study only indicate the potential benefits of higher levels of phytase in broiler chick diets that are deficient in tP.

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Tables and Figures

TABLE 2.1. Composition of the Basal Diet[1,2]

Ingredients	Amount (%)
Corn (8.8% CP)	55.64
Soybean meal (48.5% CP)	36.08
Poultry fat (8.2 kcal/kg)	4.160
Limestone	1.362
Salt (NaCl)	0.450
Dicalcium phosphate	0.377
Vitamin mix[3]	0.250
DL-Methionine	0.186
Cr_2O_3	0.100
Trace mineral mix[4]	0.080
Various	1.315
Total	100.00

[1]All diets contained a calculated ME of 3,123 kcal/kg diet, an analyzed 90.38 ± 0.465 (DM), 22.22 ± 0.347% CP, 0.88 ± 0.056% Ca, and a phytate P level of 0.272 ± 0.0135% ; all phytase-supplemented diets contained a total phosphorus level of 0.46 ± 0.010%; the control diet contained an analyzed total phosphorus level of 0.70%;
[2]Sand was used as an inert ingredient, and removed from the respective diets as either dicalcium phosphate or phytase was added. These ingredients are listed under the heading "Various".
[3]Vitamin mix provided the following (per kilogram of diet):
thiamin•mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B_{12} (*cobalamin*), 12.0 ug; pyridoxine•HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-retinyl acetate, 5,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.
[4]Trace mineral mix provides the following (per kilogram of diet): manganese ($MnSO_4$•H_2O), 60 mg; iron ($FeSO_4$•$7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4$•$5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg.

TABLE 2.2. Effect of High Phytase Levels on 16-d Broiler Performance[1,2]

Phytase		BWG	FI	Gain: feed	Mortality
(U/kg diet)		--------- (g/chick) ---------		(g:g)	---- % ----
0		287*	381*	0.755*	13
93.75		354*	441*	0.805	7
187.5		358*	441*	0.813	10
375		399*	490*	0.814	10
750		424*	505*	0.839	7
1,500		459	548	0.838	0
3,000		481	558	0.861	3
6,000		494	580	0.852	0
12,000		515	595	0.866	0
(+) Control		501	594	0.843	0
	PSEM[3]	11.7	15.2	0.0120	3.5
	MSD[4]	47.7	61.3	0.0478	13.9
ANOVA					
R^2		0.95	0.91	0.79	0.47
Source of variation[5]	df	---------- Probability > F ----------			
Treatment	8	0.0001	0.0001	0.0001	0.1019
Regression Analysis[6, 7]					
R^2		0.93	0.88	0.76	0.35
		---------- Probability > |t| ----------			
Intercept	1	0.0001	0.0001	0.0001	0.0001
Linear [$\log_{10}$ (phytase +1)]	1	0.1416	0.3562	0.0001	0.0011
Quadratic [$\log_{10}$ (phytase +1)]^2	1	0.0003	0.0024	....	....

Continued on the following page.

[1]Average initial chick weight = 46.0 ± 1.11 g/chick; BWG = body weight gain; FI = feed intake

2All diets contained a calculated ME of 3,123 kcal/kg diet, an analyzed 90.38 ± 0.465 (DM), 22.22 ± 0.347% CP, 0.88 ± 0.056% Ca, and a phytate P level of 0.272 ± 0.0135% ; all phytase-supplemented diets contained a total phosphorus level of 0.46 ± 0.010%; the control diet contained an analyzed total phosphorus level of 0.70%; sand was used as an inert ingredient, and removed from the respective diets as either dicalcium phosphate or phytase was added.

[3]PSEM = the calculated pooled standard error of the mean for phytase supplemented diets

[4]MSD = minimal significant difference (" = 0.05) between the control groupand any one phytase group; astrics denote significance

[5]Probability values ($P \geq F$) are only for phytase treatments 0 through 12,000 units

[6]Probability values ($P \geq |t|$) are only for phytase treatments, (computed without control diet)

[7]Prediction equations were based on the phytase supplemented diets:

Body weight gain = $284.3102 + 16.3980 \times \log_{10}(x + 1) + 10.5809 \times (\log_{10}(x + 1))^2$

Feed intake = $378.1857 + 12.8142 \times \log_{10}(x + 1) + 10.7009 \times (\log_{10}(x + 1))^2$

Gain:feed = $0.7516 + 0.0280 \times \log_{10}(x + 1)$

Mortality = $15.0406 - 3.525 \times \log_{10}(x + 1)$

TABLE 2.3. Effect of High Phytase Levels on Plasma Ca and P[1]

Phytase		Plasma Ca	Plasma P	P Rickets	Tibia Ash	Tibia Ash
(U/kg diet)		------- (mg/dL) ------		---------- % ----------		(g/tibia)
0		13.3	2.5*	80.0*	26.0*	0.200*
93.75		13.6	3.0*	93.3*	25.4*	0.240*
187.5		14.1	2.3*	86.7*	26.5*	0.259*
375		14.7*	2.7*	70.0*	28.9*	0.301*
750		14.4	2.8*	70.0*	29.7*	0.335*
1,500		14.1	3.2*	40.0*	34.3*	0.408*
3,000		13.9	4.2*	13.3	36.4*	0.469*
6,000		13.1	5.5*	6.7	38.6	0.544
12,000		11.6	7.1	3.3	40.7	0.601
(+) Control		11.7	6.9	0.0	39.9	0.568
	PSEM[2]	0.73	0.26	8.09	0.51	0.0130
	MSD[3]	2.87	1.38	31.98	2.13	0.0588
ANOVA						
R^2		0.41	0.95	0.90	0.98	0.98
Source of variation[4]	df	---------- Probability > F ----------				
Treatment	8	0.2089	0.0001	0.0001	0.0001	0.0001
Regression Analysis[5, 6]						
R^2		0.26	0.85	0.84	0.96	0.98
		---------- Probability > |t| ----------				
Intercept	1	0.0001	0.0001	0.0001	0.0001	0.0001
Linear [$\log_{10}$ (phytase +1)]	1	0.0267	0.0002	0.0013	0.0001	0.0001
Quadratic [$\log_{10}$ (phytase +1)]^2	1	0.0102	0.0001	0.0001	0.0001	0.0001

Continued on the following page.

[1]All diets contained a calculated ME of 3,123 kcal/kg diet, an analyzed 90.38 ± 0.465 (DM), 22.22 ± 0.347% CP, 0.88 ± 0.056% Ca, and a phytate P level of 0.272 ± 0.0135% ; all phytase-supplemented diets contained a total phosphorus level of 0.46 ± 0.010%; the control diet contained an analyzed total phosphorus level of 0.70%; sand was used as an inert ingredient, and removed from the respective diets as either dicalcium phosphate or phytase was added.
[2]PSEM = the calculated pooled standard error of the mean for phytase supplemented diets
[3]MSD = minimal significant difference (" = 0.05) between the control groupand any one phytase group; astrics denote significance
[4]Probability values ($P \geq F$) are only for phytase treatments 0 through 12,000 units
[5]Probability values ($P \geq |t|$) are only for phytase treatments, (computed without control diet)
[6]Prediction equations were based on the phytase supplemented diets:

Plasma calcium = $13.1170 + 1.5021 \times \log_{10}(x+1) - 0.4102 \times (\log_{10}(x+1))^2$
Plasma phosphorus = $2.6450 - 1.5212 \times \log_{10}(x+1) + 0.6005 \times (\log_{10}(x+1))^2$
Phosphorus Rickets = $81.6865 + 28.8561 \times \log_{10}(x+1) - 12.5915 \times (\log_{10}(x+1))^2$
Tibia ash = $25.8398 - 3.7089 \times \log_{10}(x+1) + 1.8624 \times (\log_{10}(x+1))^2$
Tibia ash weight = $0.2004 - 0.0644 \times \log_{10}(x+1) + 0.0404 \times (\log_{10}(x+1))^2$

TABLE 2.4. Effect of High Phytase Levels on Nutrient Utilization[1,2]

Phytase		Total retention					
		Ca	P	Phytate disappearance	Ca:P[3]	Nitrogen Retention[4]	AME_n[5]
(U/kg diet)		%				%	(kcal/kg)
0		45.6*	51.0	40.3*	1.72*	58.4	3216
93.75		40.8*	50.2	42.3*	1.55	68.4	3321
187.5		42.4*	54.7	51.9*	1.49	69.0	3312
375		42.3*	53.8	49.5*	1.51	68.9	3338*
750		44.1*	60.8*	58.4*	1.38	72.1*	3377*
1,500		42.3*	65.4*	65.2*	1.23	74.5*	3393*
3,000		45.1*	69.0*	73.5*	1.25	73.2*	3368*
6,000		49.5	77.7*	84.9*	1.22	76.9*	3399*
12,000		53.4	79.9*	94.8*	1.28	77.7*	3415*
(+) Control		51.3	47.2	19.3	1.37	64.7	3232
	PSEM[6]	1.42	2.01	2.31	0.049	1.67	23.0
	MSD[7]	5.67	8.01	9.23	0.198	6.80	95.0
ANOVA							
R^2		0.78	0.93	0.97	0.85	0.84	0.76
Source of variation[8]	df	Probability > F					
Treatment	8	0.0001	0.0001	0.0001	0.0001	0.0001	0.0003
Regression Analysis[9, 10]							
R^2		0.69	0.91	0.96	0.74	0.82	0.72
		Probability > |t|					

Continued on the following page.

Intercept	1	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Linear [$\log_{10}$ (phytase +1)]	1	0.0001	0.0005	0.0001	0.0001	0.0001	0.0001
Quadratic [$\log_{10}$ (phytase +1)]^2	1	0.0001	0.0001	0.0001	-----	-----	-----

[1]All values are on a dry matter basis.; tCa = total Ca retention; tP = total P retention.

2All diets contained a calculated ME of 3,123 kcal/kg diet, an analyzed 90.38 ± 0.465 (DM), 22.22 ± 0.347% CP, 0.88 ± 0.056% Ca, and a phytate P level of 0.272 ± 0.0135% ; all phytase-supplemented diets contained a total phosphorus level of 0.46 ± 0.010%; the control diet contained an analyzed total phosphorus level of 0.70%; sand was used as an inert ingredient, and removed from the respective diets as either dicalcium phosphate or phytase was added.

[3]Values were calculated as ((%tCa retention x % analyzed dietary Ca on a DM basis)/100)/((%tP retention x % analyzed dietary P on a DM basis)/100)

[4]Apparent N retention of each diet was calculated as (100- (100 x (% Cr_2O_3 feed/% Cr_2O_3 excreta) x (%N excreta/%N feed)))

[5]Nitrogen-corrected, apparent metabolizable energy (AMEn) of each diet was calculated as: (Gross energy (GE) Feed [kcal/g] - ((GE Excreta [kcal/g] x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) + (g N Feed/g Feed - (g N Excreta/g Excreta x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) x 8.28))) x 1,000

[6]PSEM = the calculated pooled standard error of the mean for phytase supplemented diets

[7]MSD = minimal significant difference (" = 0.05) between the control groupand any one phytase group; astrics denote significance

[8]Probability values ($P \geq F$) are only for phytase treatments 0 through 12,000 units

[9]Probability values ($P \geq |t|$) are only for phytase treatments, (computed without control diet)

[10]Prediction equations were based on the phytase supplemented diets:

Total calcium retention = $45.8369 - 6.5817 \times \log_{10}(x + 1) + 1.9753 \times (\log_{10}(x + 1))^2$

Total phosphorus retention = $50.9229 - 7.2079 \times \log_{10}(x + 1) + 3.6171 \times (\log_{10}(x + 1))^2$

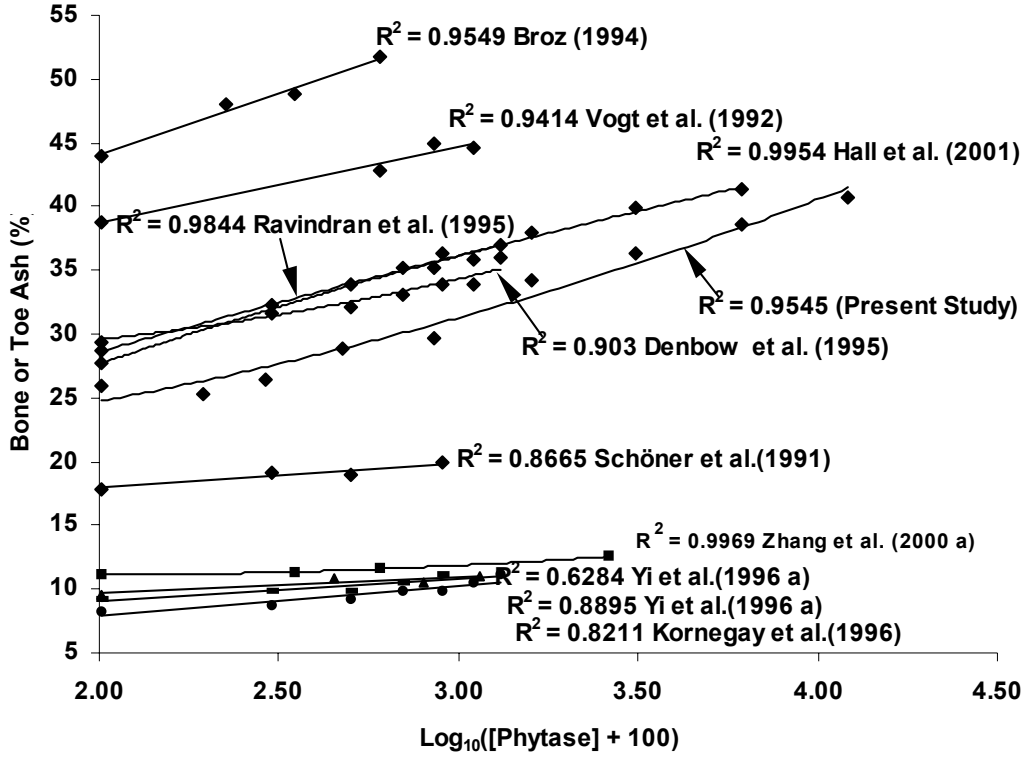
Phytate phosphorus disappearance = $40.5700 - 10.0060 \times \log_{10}(x + 1) + 5.6826 \times (\log_{10}(x + 1))^2$

Ratio of retained Ca:P = $1.7628 - 0.1336 \times \log_{10}(x + 1)$

Appaarent %N retention = $58.3589 + 4.7036 \times \log_{10}(x + 1)$

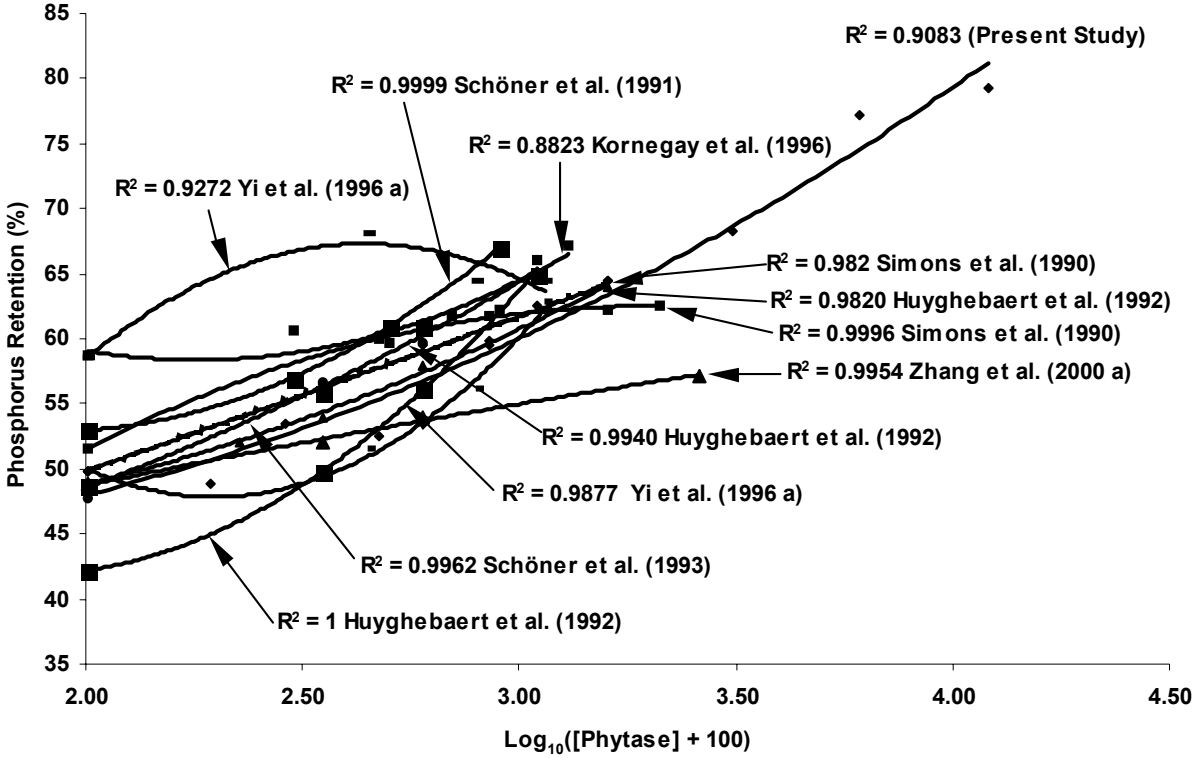
$AME_n = 3217.8092 + 48.6962 \times \log_{10}(x + 1)$

FIGURE 2.1.



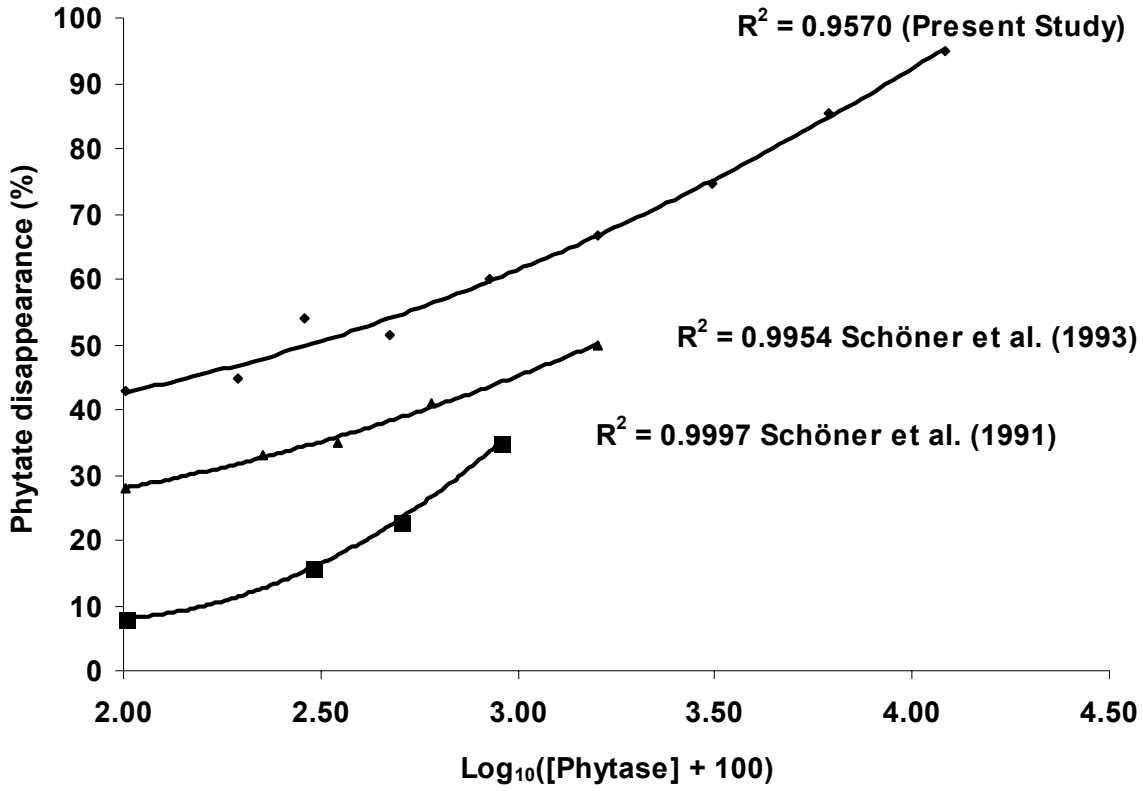
Differences Among Percent Bone Ash Data Reported in the Literature by Various Research Groups. Data represent the regressed bone ash and toe ash values reprted by several authors against the $\log_{10}$-transformation of phytase levels ($\log_{10}$([phytase] + 100)). This transformation allows for a more equal spacing of the data points along a linear plot. Data from Schöner et al.(1991), Kornegay et al. (1996), Yi et al. (1996 a,b), and Zhang et al. (2000a) are toe ash data.

FIGURE 2.2.



Differences Among Total Phosphorus Retention Data Reported in the Literature by Various Research Groups. Data represent the regressed total phosphorus retention values reprted by several authors against the $\log_{10}$-transformation of phytase levels ($\log_{10}$([phytase] + 100)). This transformation allows for a more equal spacing of the data points along a linear plot.

FIGURE 2.3.



Differences Among Phytate P Disappearance Data Reported in the Literature. Data represent the regressed phytate Phosphorus disappearance values reprted by several authors against the log_{10}-transformation of phytase levels (log_{10}([phytase] + 100)). This transformation allows for a more equal spacing of the data points along a linear plot.

CHAPTER 3

THE USE OF 25-HYDROXYCHOLECALCIFEROL OR 1-ALPHA-HYDROXYCHOLECALCIFEROL IN BROILER DIETS THAT CONTAIN A WIDE RANGE OF SUPPLEMENTAL PHYTASE[1,23]

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[1]Shirley, Robert B. and Hardy M. Edwards, Jr.

[2]To be submitted to Poultry Science.

[3]Experiments B7-02-TD and B8-02-TD

Abstract

Two studies evaluated the overall performance of 0 to 16 d-old, straight-run, Ross x Cobb broiler chicks when either dietary 25-hydroxycholecalciferol [25-(OH)D_3 (68.75 ug/kg diet)] (Experiment 1) or 1-alpha-hydroxycholecalciferol [1 " -(OH)D_3 (5 ug/kg diet)] (Experiment 2) were supplemented with log-dose levels of dietary phytase from 0 to 93.75, 187.5, 350, 750, 1,500, 3,000, 6,000 and 12,000 Units (U)/kg diet. The experimental corn-soybean meal basal diets contained an average analyzed CP = 22.83%, a Ca = 0.86%, a deficient, total P (tP) = 0.47%, a phytate P = 0.270% and a calculated ME of 3.123 kcal/g diet.

Using multiple linear regression, supplemental phytase in Experiment 1improved (P # 0.0001) body weight gain (BWG) from 293 to 435 g/chick, feed intake (FI) from 386 to 534 g/chick, plasma P from 2.2 to 7.1 mg/100 mL, tibia ash (TA) from 29 to 40%, tibia ash weight (TA_w)from 0.263 to 0.556 g/tibia, total Ca (tCa) retention from 33 to 63%, tP retention from 59 to 81%, phytate disappearance from 52 to 96%; and, decreased plasma Ca from 12.4 to 10.3 mg/100 mL and the incidence of P rickets from 81 to 1% in a curvilinear manner. The supplementation of 25-(OH)D_3 had no additive affect (P # 0.1494) on BWG, FI, mortality, plasma Ca, tP retention, phytate disappearance or dietary AME, however, phytase by 25-(OH)D_3 interactions (P # 0.0331) were observed for P rickets score, TA, tCa retention, N utilization and dietary AME. These interactions were due to 25-(OH)D_3 improving the latter parameters at the lower levels of phytase. In Experiment 2, phytase linearly increased (P # 0.0021) FI from 475 to 603 g/chick and reduced plasma Ca from 14.3 to 12.0 mg/100 mL, P rickets score from 2 to 0 and tCa retention from 34 to 48%. Additional curvilinear responses were (P # 0.0735) were observed, as

supplemental phytase increased BWG from 359 to 481 g/chick, G:F from 0.755 to 0.797, plasma P from 2.9 to 5.2 mg/100 mL, TA from 30 to 40%, TA_w from 0.343 to 0.679 g/tibia, total Ca retention from 34 to 48%, tP retention from 57 to 77%, phytate disappearance from 56 to 95%, and reduced the incidence of P rickets from 71 to 4% (P # 0.0001). Significant phytase by 1 " -(OH)D_3 interactions (P # 0.0642) were observed for BWG, FI, G:F, mortality, P rickets score, P rickets incidence, TA, TA_w and N utilization. As in Experiment 1, the significant interactions were a direct result of 1 " -(OH)D_3 improving the latter parameters at the lower levels of phytase, however, as the level of phytase increased, 1 " -(OH)D_3 depressed BWG and FI. These data indicate that higher levels of phytase supplementation to a total P-deficient diet can improve broiler performance; and, that 25-(OH)D_3 and 1 " -(OH)D_3 affect broiler performance, tibia deposition and the utilization of nutrients differently.

Key Words: 25-hydroxycholecalciferol, 1 " -hydroxycholecalciferol, broiler performance, dietary phytase supplementation, phytase level

Introduction

The broiler industry currently supplements phytase from 300 to 600 Units (U)/kg diet. This range is primarily based on previous research that evaluated broiler performance at various levels of phytase (Simons et al., 1990; Schöner et al., 1991; Huyghebaert et al., 1992; Vogt, 1992; Broz et al., 1994; Kornegay et al., 1996; Biehl et al., 1995; Zhang et al., 2000a). Results from most of the previously cited research groups indicate that body weight gain, feed intake, tibia ash and the utilization of minerals increases when total P (tP)-deficient diets are supplemented with up to 2,500 U/kg diet of phytase. Results from the study of Hall et al. (2003) and Chapter 2 suggest that phytase supplementation to 6,000 or 12,000 U/kg can greatly improve BWG and tibia ash deposition further, and hydrolyze approximately 95% of the dietary phytate in a tP-deficient, corn-soybean meal diet (0.46%). The latter increase in phytate degradation not only improved phytate P availability and retention, but possibly the utilization other nutrients such as N and ME. The results of Hall et al. (2003) and Chapter 2 indicate that the former research groups did not use enough phytase to obtain maximal broiler performance.

Since the cost of phytase supplementation beyond 600 U/kg diet is economically prohibitive within the broiler industry at the present time, dietary vitamin D_3 metabolites such as cholecalciferol (vitamin D_3), 25-hydroxycholecalciferol (25-(OH)D_3), 1''-hydroxycholecalciferol (1''-(OH)D_3), and 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) have been used experimentally to improve the utilizations of Ca and P within broiler rations (McNutt and Haussler, 1973, Haussler et al., 1973; Boris et al., 1977; Mohammed et al., 1991; Biehl et al., 1995; Mitchell and Edwards, 1996a,b; Biehl and Baker, 1997a,b; Biehl et al.,

1998a,b; Edwards et al., 2002). With regard to broiler performance and mineral utilizations, the established order of biopotency between the four previously mentioned metabolites is: 1,25-$(OH)_2D_3$ = 1 '' -$(OH)D_3$ > 25-$(OH)D_3$ > cholecalciferol (Myrtle and Norman, 1971; McNutt and Haussler, 1973; Norman and Wong, 1972; Boris et al., 1977; Edwards et al., 1992; Edwards, 1993; Soares et al., 1995; Edwards, 2002; Edwards et al., 2002). Of these metabolites, however, cholecalciferol and 25-$(OH)D_3$ are the only two metabolites that are approved for use within the broiler industry of the United States [cholecalciferol at an approximate rate of 25 to 75 :g/kg diet (1,000 to 3,000 IU/kg diet[4]) and 25-$(OH)D_3$ at an approximate rate of 5 to 82 :g/kg diet].

Phytase and vitamin D_3 metabolites have an additive affect on broiler performance and the utilization of nutrients when supplemented in plant-based diets (Roberson and Edwards, 1994; Biehl et al., 1995; Biehl and Baker, 1996; Qian et al., 1997; Mitchel and Edwards, 1996 a, b; Adeola et al., 1998; Aksakal and Bilal, 2002a,b). Research regarding the use of either 25-$(OH)D_3$ or 1 '' -$(OH)D_3$ in tP-deficient diets containing ample cholecalciferol and phytase levels ranging from 0 to 12,000 U/kg of diet, however, does not exist. The following research was designed to test the hypothesize that at each graded log-dose of phytase [up to 12,000 U per kg in a corn-soybean meal diet], the addition of either 25-$(OH)D_3$ or 1 '' -$(OH)D_3$ will positively affect both broiler performance and the overall utilization of nutrients.

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[4]1 :g cholecalciferol = 40 IU of cholecalciferol

Materials and Methods

Experimental Diets

The National Research Council's nutrient values for ingredients (NRC, 1994) was used to formulate the corn-soybean meal basal diets of Experiments 1 and 2 (Table 1). The formulated basal diets in Experiments 1 and 2 contained 1,100 IU of cholecalciferol per kg of diet (27.5 :g/kg diet), and a calculated ME of 3,123 kcal/kg diet. The basal diet of Experiment 1 contained by analysis: 90% DM, 23.0% CP, 0.86% Ca, phytate P of 0.27%, and a deficient tP level of 0.47%. Similarly, the basal diet of Experiment 2 contained by analysis: 91% DM, 22.7% CP, 0.86% Ca, 0.27% phytate P, and a deficient tP level of 0.46%. Both basal diets contained the same soybean meal and phytase that were used in Chapter 2. To preserve the activity of the phytase product, the phytase powder was stored at -20°C between the experiment of Chapter 2 and the two experiments within this chapter. The log-dose supplementation of phytase to the basal diets was based on the guaranteed value of the phytase product [Natuphos 5000][5], which was added at either 0, 93.75, 187.5, 375, 750, 1,500, 3,000, 6,000 or 12,000 U/kg diet. This product was assayed by the University of Georgia's Department of Poultry Science Nutrition Lab to contain 1.11% P and 0.06% Ca. As phytase or 25-(OH)D_3[6] was added to the basal diet in

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[5]BASF Corp., Mt. Olive, NJ. The activity was determined by an in-house BASF assay to be 6,054 U/g. One unit (U) is equivalent to one phytase unit (FTU), and is defined as the amount of enzyme which liberates 1 micromole inorganic phosphorus per minute from 0.0051 mol/L sodium phytate at 37°C and at pH 5.50 for 30 to 60 minutes.

[6]Roche Vitamins Inc., Parsippany, NJ. ROVIMIX Hy"D was the source of 25-hydroxycholecalciferol.

Experiment 1, or as phytase was titrated into the basal diet of Experiment 2, sand was used as an inert filler, and removed from the respective diets (wt/wt). In Experiment 1, 25-(OH)D_3 was supplemented in a powdered form to the basal diet at 68.75 :g/kg diet. The 1"-(OH)D_3 that was used in Experiment 2 was supplied by G. F. Majitech[7] and had a purity of $99%. To supplement the diet with1"-(OH)D_3, 1.382 mg of crystalline 1"-(OH)D_3 was carefully weighed, diluted with 10 mL of 200 proof ethanol, and then diluted with 128.2 mL of propylene glycol to produce a 1"-(OH)D_3 stock solution with a concentration of 10 :g/mL. This solution was added to the basal diet of Experiment 2 as a liquid top-dressing at 5 :g/kg of diet.

Chick Assay Procedures

In each experiment, 624 day-of-hatch, straight-run, Ross x Cobb[8] chicks were wing-banded, weighed, and randomly allotted to each of the 16 treatments in groups of thirteen chicks per pen, with three pens per treatment. Throughout each 16-d experiment, chicks were given free access to water and mash-feed, brooded in thermostatically controlled Petersime starter batteries[9] with raised wire floors, and reared on a 24-h lighting schedule. The starter batteries were kept in an environmentally controlled room (22°C), where fluorescent lights outside and inside

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[7]Majestic Research, Inc. Athens, GA 30601.

[8]Fieldale Farms, Baldwin, GA. Chicks in each experiment were derived from parental stock that were of different ages, yet were nutritionally managed the same.

[9]Petersime Incubator Co., Gettysburg, OH.

the batteries were covered with Arm-a-lite plastic sleeves[10] to eliminate ultraviolet light exposure and any possible *de novo* vitamin D_3 synthesis and vitamin D_3-mediated improvement in phytate P utilization from tP -deficient diets (Edwards et al., 1994; Elliot and Edwards, 1997; Mitchell et al., 1997).

On d-16 of each experiment, one chick was randomly selected from each pen, weighed, and a heparinized blood sample was obtained by cardiac puncture. All remaining chicks were weighed and euthanized via CO_2 asphyxiation. The right tibia of each chick was randomly scored for the incidence of phosphorus rickets (P rickets) (Edwards et al., 1994), while the left tibia of each chick was banded for identification and removed for future percent tibia ash (TA) and tibia ash weight determination [g/tibia (TA_w)], as described in Chapter 2. In addition, excreta was collected from each pen for 24-h between days 15 and 16 of the experiment, dried at 75°C for 36 h, ground to pass through a 1mm mesh screen, and analyzed for nutrient composition.

Sample Analyses

Ionized plasma calcium (plasma Ca) (Section N-31)[11] and dialyzable plasma phosphorus (plasma P) (section 7N-46)[12] analyses were performed immediately after the heparinized blood samples were collected.

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[10]Arm-a-lite, Thermoplastic Processes, Sterling, NJ.

[11]Section N-31, Technicon Autoanalyzer Methodology, Technicon Corp., Tarrytown, NY.

[12]Section 7N-46, Technicon Autoanalyzer Methodology, Technicon Corp., Tarrytown, NY.

Feed and excreta were analyzed for moisture, chromic oxide (Brisson, 1956), CP [N x 6.25] (Etheridge et al., 1998), gross energy (instructions for 1241 and 1242 Adiabatic Calorimeters, Parr Instrument, Moline, IL), total Ca (tCa) (Hill, 1965), tP (O'Neill and Webb, 1970), and phytate P (Latta and Eskin, 1980). These values were then used to calculate nutrient utilizations (Edwards and Gillis, 1959; Matterson et al., 1965).

Statistical Analyses

Analysis of variance for both experiments were completed using the general linear model procedure of SAS (SAS software, 1999). The main effect means for phytase and either vitamin D_3 metabolite within each experiment were separated using Duncan's New Multiple Range Test. A modified, multiple linear regression analysis procedure was used to calculate prediction equations for each of the parameters (Zhang et al., 2000 b and Lyman-Ott and Longnecker, 2001), where vitamin D_3 metabolite and log-transformed ($\log_{10}$) phytase levels within each experiment were regressed against the data of each dependent variable. This equalized the data along a linear plot, thereby yielding a more objective interpretation of the data (Almquist, 1953). The resulting regression models were then reduced by eliminating non-significant main-effects in order to determine an appropriate prediction equation for each of the measured parameters. Depending

on the effects that are significant for a particular dependent variable's model, the following prediction equation will be:

$$Y = B + M \times [D_3 \text{ metabolite}] + M' \times \log_{10}(X + 1) + M'' \times (\log_{10}(X + 1))^2 + M''' \times \log_{10}(X + 1) \times [D_3 \text{ metabolite}],$$

where Y is a parameter's predicted outcome, # is the intercept, 9 is the change in slope due to the presence or absence of either 25-(OH)D_3 (68.75 : g/kg diet) or 1 " -(OH)D_3 (5 : g/kg diet), 9 ' is the linear slope of phytase (change in performance per log-unit of phytase in the diet), 9 '' is the quadratic slope of phytase, 9 ''' is the slope of the interaction between phytase and the presence or absence of either 25-(OH)D_3 or 1 " -(OH)D_3, and O

is the level of phytase in U/kg diet. These prediction equations enable the reader to estimate the amount of phytase and/or the amount of either 25-(OH)D_3 or 1 " -(OH)D_3 to use in a broiler ration for maximal broiler production and mineral utilization.

General Results

Experiment 1

Effect of Phytase

In the presence or absence of 25-(OH)D_3, the log-dose addition of phytase to the basal diet improved (P # 0.0028) BWG, FI and G:F (Table 2), plasma P (Table 3), TA and TA_w (Table 4), and tP retention and phytate disappearance[13] (Table 5).

[13]Total Ca retention, P retention, and phytate P disappearance of each diet was calculated as: (100- (100 x (% Cr_2O_3 feed/% Cr_2O_3 excreta) x (%[Ca, P or phytate P] excreta/%[Ca, P or phytate P] feed)))

Conversely, increasing the level of dietary phytase decreased (P # 0.0168) mortality (Table 2), plasma Ca (Table 3), and P rickets score and P rickets incidence (Table 4). The effects of phytase on N retention[14] and dietary apparent metabolizable energy (AME_n)[15] were variable, yet significant (P # 0.0008) (Table 5). Using multiple regression analysis, all parameters, except tCa retention, responded in a curvilinear manner to phytase (P # 0.0103). Upon further analysis, N utilization and AME responded in a quadratic fashion to phytase (P # 0.0699) [statistical values are not shown in Table 5].

Effect of 25-Hydroxycholecalciferol

There was no effect of 25-$(OH)D_3$ on BWG, FI, G:F, mortality, plasma Ca, tP retention, phytate degradation, or dietary AME. The addition of 25-$(OH)D_3$ to any of the diets containing phytase, however, did improve plasma P (P # 0.0274), TA (P # 0.0001), TA_w (P # 0.0059) and N utilization (P # 0.0855); and, decreased P rickets score (P # 0.0022), P rickets incidence (P # 0.0114) and tCa retention (P # 0.0164).

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[14]Apparent percent nitrogen retention (N) of each diet was calculated as: (100-(100 x (% Cr_2O_3 feed/% Cr_2O_3 excreta) x (%N excreta/%N feed)))

[15]Nitrogen-corrected, apparent metabolizable energy (AME_n) of each diet was calculated as: (GE Feed [kcal/g] - ((GE Excreta [kcal/g] x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) + (g N Feed - (g N Excreta x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) x 8.28))) x 1,000

Interaction Between Phytase and 25-Hydroxycholecalciferol

The effects of 25-(OH)D_3 on broiler performance and the utilization of nutrients was most noticeable at the lower levels of phytase supplementation. With each additional level of dietary phytase, however, the effects of 25-(OH)D_3 became less noticeable. A reduction in P rickets score and an increase in TA with the addition of 25-(OH)D_3 at the lower levels of phytase produced a significant interaction between phytase and 25-(OH)D_3 (P # 0.0331).

Experiment 2

Effect of Phytase

Just as in Experiment 1, the log-dose addition of phytase to the basal diet improved (P # 0.0269) BWG, FI and G:F (Table 6), plasma P (Table 7), TA and TA_w (Table 8), and tCa retention, tP retention and phytate disappearance (Table 9). These increases were followed by a decrease in plasma Ca (P # 0.0168) (Table 7), and P rickets score and P rickets incidence (P # 0.0001) (Table 8). The effect of phytase on N retention and dietary apparent metabolizable energy (AME_n) was again variable, yet highly significant (P # 0.0001) (Table 9). Using multiple regression analysis, responses of tCa, P rickets score and plasma Ca to phytase were linear (P # 0.0021). Responses of BWG, G:F, plasma P, P rickets incidence, TA, TA_w, N utilization, and AME were curvilinear (P # 0.0001). The responses of BWG and FI to phytase were also curvilinear, however, the level of significance was

much lower (P # 0.0735). Further analysis revealed that N utilization and AME also responded in a quadratic fashion to phytase (P # 0.0624) [statistical values are not shown in Table 9].

Effect of 1 " -Hydroxycholecalciferol

The addition of 1 " -(OH)D_3 reduced (P # 0.0096) BWG, FI and the incidence of P rickets. Conversely, the addition of 1 " -(OH)D_3 improved (P # 0.0288) TA, tCa retention, tP retention, phytate degradation and N utilization.

Interaction Between Phytase and 1 " -Hydroxycholecalciferol

Just as in Experiment 1, 1 " -(OH)D_3 had its greatest effects from 0 to 1,500 of phytase. Depending on the parameter measured, the addition of 1 " -(OH)D_3 to diets that contained more than 1,500 U yielded very few additional benefits or had negative affects. For instance, supplementing 1 " -(OH)D_3 to diets containing greater than 3,000 U of phytase improved (P # 0.0533) the P rickets score, the incidence of P rickets and the TA (Table 8) and N retention (P # 0.0637). Supplementing more than 1,500 U of phytase, in the presence of 1 " -(OH)D_3, not only depressed (P # 0.0119) BWG, FI, G:F (Table 6) and TA_w (Table 8), but increased (P # 0.0533) the P rickets score and incidence of P rickets (Table 8).

Discussion

Broiler performance, tibia ash deposition, and mineral utilization data from both experiments support earlier research from this laboratory (Hall et al,. 2003; Chapter

2). Unlike the study in Chapter 2, there was no phytase-mediated improvement in N utilization and AME values in this study. In addition, data from Experiments 1 and 2 suggest that 25-(OH)D_3 and 1"-(OH)D_3 have distinct affects upon broiler performance and nutrient utilizations at lower and higher levels of phytase supplementation. The following discussion will focus on possible factors that may have contributed to differences in broiler performance, N retention, and AME when either phytase, 25-(OH)D_3, or 1"-(OH)D_3 were supplemented.

The results of Chapter 2 and the present two experiments, Ca, tP, and dietary phytate P contents were very similar. In fact, the diets within these studies were mixed using the same bulk stock source of vitamin premix, phytase and soybean meal. The only differences between the two studies was the use of poultry fat versus soybean oil, the flock from which the chicks were obtained, and the source of corn. Theoretically, the anti-nutritional effects of phytate, such as mineral-phytate, protein-phytate, or mineral-protein-phytate interactions, should become less as more phytate is degraded (Cheryan; 1980; Thompson, 1986; Ravindran et al., 1995; Maenz et al., 1999). In Chapter 2 and the two present experiments, the addition of phytase from 0 to 12,000 U/kg of diet resulted in an 94.8, 95.3, and 93.3% reduction in phytate P, respectively. Because many of the ingredients used in Capter 2 and the two experimental diets within this study were from the same bulk stock, the analyzed tP and phytate P content of the three experimental diets were very similar. Increasing phytase from 0 to 12,000 U/kg of diet, it is estimated that the available P content of the three diets was increased to 0.408, 0.423, and 0.393%, respectively. The previous increases in available P among the three experiments resulted in improved tP retentions of 79.9, 81.2, and 74.8%,

respectively. By increasing phytate P disappearance and tP availability, improvements the ratio of Ca to P, BWG, FI, plasma Ca and P, P rickets, TA, and TAw were observed. In addition to the latter dose-response improvements in broiler chick performance and mineral utilizations, we observed a concurrent improvement in N retention and AME_n utilization in Chapter 2. Speculatively, the extra-caloric and extra-nitrogen effect that phytase had in Chapter 2 may have been due to a decrease in the anti-nutritional effects of phytate, such as mineral-phytate, protein-phytate, or mineral-protein-phytate interactions as phytate was degraded (Cheryan; 1980; Thompson, 1986; Ravindran et al., 1995; Maenz et al., 1999). However, this was not the case in Experiments 1 and 2 from this study, as the magnitude of response of N retention and AME_n to phytase was marginal to nonexistant, even though the diets were of a similar composition.

The difference in N retention and AME values, as a function of phytase supplementation, have been reported within other phytase research (Kies, 2001). For instance, Kasim and Edwards (2000) did not observe an improvement in ME when a corn-soybean meal diet was supplemented with 600 U of phytase/kg of diet. Furthermore, Biehl and Baker (1997c), Peter et al. (2000), and Peter and Baker (2001), determined that the utilization of essential amino acids, the order of essential amino acids, and the overall utilization of crude protein is not improved when 1,200 U of phytase is added to corn-peanut, corn gluten meal, soybean meal or casein-based diets. The latter data, in addition to the lack of response of N retention and AME to phytase in this study, contrasts the results of Sebastian et al. (1996), Yi et al. (1996), Ravindran et al. (1999a,b), Ravindran et al. (2000), Camden et al. (2001), and Chapter 2, where a reported increase in either N retention or AME was

observed with the addition of phytase to P-deficient diets. In the studies of Sebastian et al. (1996), Yi et al. (1996), Ravindran et al. (1999a), Ravindran et al. (2000), Camden et al. (2001) and Chapter 2, a 3.4, 9.0, 4.2, 3.2, 3.6, 1.5, and 33.0% increase in N retention was observed, respectively, when dietary phytase levels ranged from 0 to 600, 1,050, 1,200, 660, 400, 1,000, and 12,000 U/kg diet, respectively. In the studies of Ravindran et al. (1999b), Ravindran et al. (2000), and Chapter 2, AME was improved by 147, 57, and 199 kcal/kg diet, respectively, when dietary phytase was supplemented from 0 to 660, 400, and 12,000 U/kg in practical-type, P-deficient broiler diets. These data suggest that there are other factors that affect the N retention and AME of diets that are supplemented with phytase. Discrepancies in N retention and AME between the experiment of Chapter 2 and the present two experiments may therefore be due to other factors, such as: corn cultivar, particle size, and possibly vitamin D_3 source.

Genetically-modified grains and oilseed that contain either a low phytate, high oil, high lysine, or some other desired nutritive traits have been developed (Denbow et al., 1998; Waldroup et al., 2000; Yan et al., 2000; Veum et al., 2002). Differences in the nascent nutritive value among these value-added grains and oilseeds thus, may cause variations in the response of broiler chicks to phytase. For instance, Kasim and Edwards (2000) observed a wide range in nutrient utilization values when chicks were fed corn-soybean meal diets that differed in the cultivar/source of corn. In Kasim and Edwards' study, a common soybean meal source was used in each of five experimental diets that were formulated and analyzed to contain fairly equal Ca, P, phytate P, and ME values. Upon analysis though, the retention coefficient values for Ca ranged from 0.144 to 0.302, P from 0.397 to 0.531, and phytate P

from 0.138 to 0.292. In addition, differences between the highest and lowest ME utilization values between the cultivars of corns was 232 kcal/kg diet (0.971 MJ/kg diet[16]). Kilburn and Edwards (2001) also observed that grinding corn to a coarse versus a fine particle size increased ME values by 48 kcal/kg diet (0.2 MJ/kg diet). Kasim and Edwards (2000) observed a slight increase in ME when corn was coarsely ground, however, the effect was not significant. Between the studies of Kilburn and Edwards (2001) and Kasim and Edwards (2000), it is possible that increasing the surface area of feed particles would increase the relative digestion of nutrients. In general, these two studies indicate that common feed ingredients such as corn may be quite variable in their ability to furnish the mineral, protein, and energy needs of a growing broiler chicken when various sources are fed or processed differently.

Very little information exists regarding the effect of 25-$(OH)D_3$ or 1 '' -$(OH)D_3$ on N retentions and AME values. Of the limited research, Edwards (1993) observed a non-significant increase in ME when a corn-soybean meal diet was supplemented with 75 U of phytase, a slight decrease in ME when 5 µg of 1,25-$(OH)_2D_3$ was supplemented, and a significant decrease in ME when the two were cosupplemented. This similar depression was observed in Experiment 1 and 2 at the lower levels of phytase supplementation, however, as the level of phytase increased to 12,000 U/kg of diet, N retention and AME_n values sporadically improved to the point that a parabolic response was observed [as remarked upon in the results section].

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[16]1,000 calories (1kcal) = 4.184 MJ

In both experiments, improvements in BWG, FI, plasma Ca, plasma P, incidence in P rickets, phytate P disappearance, tCa retention, tP retention, TA and TA_w values were also observed with each additional level of phytase. The addition of phytase increased the ionic concentration and availability of dietary Ca and other minerals within the small intestine at higher levels of phytase (Nelson and Kirby, 1987; Jongbloed et al., 1992), however, the addtion of 1 " -(OH)D_3 increased the absorption of Ca more than P at the higher levels of phytase, thereby resulting in anorexia.

Mohammed et al. (1991), Qian et al. (1997), Adeola et al. (1998), and Aksakal and Bilal (2002 a,b) observed that chicks become anorexic when excessive dietary Ca is supplemented in diets that are adequate in all other nutrients. In diets that are supplemented with higher levels of phytase, is possible that the relative abundance of nutrients such as minerals, proteins, lipids, and starches may have interacted with one another, resulting the formation of Ca-soaps that reduce the ME of a diet (Atteh and Leeson, 1983, 1984; Maenz et al., 1999). The positive effect that 25-(OH)D_3 and 1 " -(OH)D_3 have upon Ca and P absorption may have also made it feasible for Ca and P absorption to be greater than that of other nutrients, resulting in a Ca toxicity that would normally depress feed intake and growth. It is well known that the hydroxylation at the one-position of 25-(OH)D_3 in the kidney is tightly regulated. This may be why Yarger et al. (1995) was unable to report any toxic effects of 25-(OH)D_3 at 69 µg/kg of diet. Since 1 " -(OH)D_3 and 1,25-$(OH)_2D_3$ have similar biopotencies, 1 " -(OH)D_3 may enhance the active absorption of dietary and renal Ca (Tanaka and DeLuka, 1971; Holick et al., 1973; Spencer et al., 1978a,b; Cross et al., 1986; Cross and Peterlik,1988 and 1991; Wassermann et al., 1992; Cai et al., 1993). Comparing

the retained Ca and retained P values in Experiment 2, the biological role of 1"-(OH)D_3 seems to be very similar to that of 1,25-(OH)$_2D_3$. The retention of tP was improved, but the degree of tCa retention was much greater, especially when 1,500 U or more of phytase was supplemented. Comparing the phytase diets that are not supplemented with 1"-(OH)D_3 to those that are, the concurrent increase in the retained Ca to P ratio mirrors the plateau in BWG, FI and G:F. By increasing the availability of Ca through high levels of phytase, 1"-(OH)D_3 seems to enhance dietary Ca absorption to the point that Ca becomes an anorexic agent.

Conclusion

Both experiments offer an insight into the effects that dietary 25-(OH)D_3 and 1"-(OH)D_3 have on broiler performance and nutrient utilizations when added to tP-deficient diets that are supplemented with very low to higher-than-industry levels of dietary phytase. While phytase from 0 to 12,000 U/kg diet increased phytate P disappearance, tP retention and overall broiler performance and tibia ash deposition, it failed to improve N retention and AME_n utilization. In addition, 68.75 :g of 25-(OH)D_3 has little affect on most parameters at the higher levels of phytase supplementation, while the addition of 5 :g of 1"-(OH)D_3, at higher phytase levels reduced BWG and FI. Finally, these data indicate differences in broiler performance may solely be the result of a better balance between dietary Ca and P, and that any additional phytase-effect on N retention and AME values may reflect the nutrient quality of ingredients that are used.

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Tables

TABLE 3.1. Composition of the Basal Diet for Experiments 1 and 2

Ingredients	Experiment 1	Experiment 2
	---------- (%) ----------	
Corn (9.05/8.50% CP)[1]	56.54	56.54
Soybean meal (46.99% CP)	36.65	36.65
Soybean oil (8.8 kcal/kg)	3.29	3.29
Limestone	1.81	1.81
Salt (NaCl)	0.45	0.45
Dicalcium phosphate	0.38	0.38
Vitamin mix[2]	0.25	0.25
DL-Methionine	0.19	0.19
Cr_2O_3	0.10	0.10
Trace mineral mix[3]	0.08	0.08
Various[4]	variable	variable
Total	100.00	100.00
Calculated composition		
ME (kcal/kg)	3,123	3,124
CP	22.4	22.1
Ca	0.95	0.95
Total P	0.45	0.45
Phytate P	0.261	0.261
Analyzed composition[5]		
Crude Protein (N x 6.25)	23.0	22.7
Ca	0.86	0.86
Total P	0.47	0.46
Phytate P	0.270	0.269
Na	0.22	0.18
K	1.33	1.34

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[1]The analyzed CP of the corn in Experiment 1 was 9.09% and the CP of corn in Experiment 2 was based on 8.50% CP value within the NRC for poultry.
[2]Vitamin mix provided the following (per kilogram of diet):
thiamin•mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B_{12} (*cobalamin*), 12.0 ug; pyridoxine•HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-retinyl acetate, 5,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.
[3]Trace mineral mix provides the following (per kilogram of diet): manganese ($MnSO_4•H_2O$), 60 mg; iron ($FeSO_4•7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4•5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg.
[4]In Experiment 1, and, sand was replaced from the basal diet on an equal-weight-basis as powdered phytase (Natuphos 5,000 U/g) or as powdered 25-hydroxycholecalciferol [25-(OH)D_3 (ROVIMIX Hy"D)] was supplemented at 68.75 : g/kg diet; and, in Experiment2, sand was replaced from the basal diet on an equal-weight-basis as powdered phytase (Natuphos 5,000 U/g) was added, however, 1 " -hydroxycholecalciferol (1 " -(OH)D_3) was added as a liquid top-dressing at 5 : g/kg of diet.
[5]These values are on an as-is basis.

TABLE 3.2. Effect of High Phytase Levels and 25-Hydroxycholecalciferol on 16-d Broiler Performance, Experiment 1[1,2]

	BWG		FI		Gain:feed		Mortality	
	25-(OH)D_3 (ug/kg diet)							
Phytase	(0)	(68.75)	(0)	(68.75)	(0)	(68.75)	(0)	(68.75)
(U/kg diet)	(g/chick)				(g:g)		%	
0	285	301	377	395	0.755	0.762	12.8	10.1
187.5	331	315	434	414	0.763	0.762	5.6	2.6
375	347	351	452	457	0.768	0.770	2.6	5.1
750	360	368	471	476	0.765	0.773	0.0	0.0
1,500	374	370	479	470	0.780	0.788	2.6	0.0
3,000	387	385	480	484	0.805	0.794	0.0	0.0
6,000	415	415	509	517	0.815	0.803	0.0	5.1
12,000	434	435	546	522	0.796	0.834	2.6	2.6
PSEM[3]	8.3		9.2		0.0081		2.57	
Main effect means								
25-(OH)D_3								
0	367		469		0.781		2.9	
68.75	368		467		0.786		3.2	
PSEM	2.9		3.3		0.0029		0.91	

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Phytase					
0		293[g]	386[f]	0.759[d]	11.5[a]
187.5		323[f]	424[e]	0.763[d]	2.6[b]
375		349[e]	454[d]	0.769[cd]	3.9[b]
750		364[ed]	474[c]	0.769[cd]	0.0[b]
1,500		372[cd]	474[c]	0.784[bc]	1.3[b]
3,000		386[c]	482[c]	0.800[ab]	0.0[b]
6,000		415[b]	513[b]	0.809[a]	2.6[b]
12,000		435[a]	534[a]	0.815[a]	2.6[b]
	PSEM	5.9	6.5	0.0057	1.82
ANOVA					
R^2		0.93	0.92	0.79	0.50
Source of variation	df	---------- Probability > F ----------			
Treatment	15	0.0001	0.0001	0.0001	0.0360
25-(OH)D_3	1	0.7949	0.7380	0.2471	0.8084
Phytase [$\log_{10}$ (phytase +1)]	7	0.0001	0.0001	0.0001	0.0028
25-(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]	7	0.7208	0.3023	0.1113	0.8271
Regression Analysis[4]					
R^2		0.91	0.87	0.67	0.40
		---------- Probability > |t| ----------			
Intercept		0.0001	0.0001	0.0001	0.0001
25-(OH)D_3		-----	-----	-----	-----
Phytase [$\log_{10}$ (phytase +1)]		0.2384	0.6450	0.0097	0.0003
Phytase x phytase [$\log_{10}$ (phytase +1)]^2		0.0001	0.0001	0.0001	0.0168
25-(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]		-----	-----	-----	-----

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[1]Average initial chick weight = 47.4 ± 1.00 g/chick
[2]On an as-is basis, all diets contained a calculated 3,123 kcal/kg diet, an analyzed 22.96 ± 0.22% CP, 0.86 ± 0.036% Ca, 0.47 ± 0.029% total P, a phytin phosphorous level of 0.270 ± 0.007%, a Na of 0.22 ± 0.023%, and a K of 1.33 ± 0.064%; and, sand was replaced from the basal diet on an equal-weight-basis as powdered phytase (Natuphos 5,000 U/g) or as powdered 25-hydroxycholecalciferol [25-(OH)D_3 (ROVIMIX Hy"D)] was supplemented at 68.75 : g/kg diet.
[3]PSEM = the calculated pooled standard error of the mean
[4]Prediction equations:

Body weight gain = $292.9555 - 6.4845 \times \log_{10}(x + 1) + 10.0892 \times (\log_{10}(x + 1))^2$
Feed intake = $386.0063 + 3.0973 \times \log_{10}(x + 1) + 7.9887 \times (\log_{10}(x + 1))^2$
Gain-to-feed = $0.7586 - 0.0155 \times \log_{10}(x + 1) + 0.0074 \times (\log_{10}(x + 1))^2$
% Mortality = $11.5683 - 6.2088 \times \log_{10}(x + 1) + 0.9536 \times (\log_{10}(x + 1))^2$

TABLE 3.3. Effect of High Phytase Levels and 25-Hydroxycholecalciferol on Plasma Values, Experiment 1

		Plasma Ca		Plasma P	
		---------- 25-(OH)D_3 (ug/kg diet) ----------			
Phytase		(0)	(68.75)	(0)	(68.75)
(U/kg diet)		---------- (mg/100 mL) ----------			
0		13.1	11.8	2.0	2.5
187.5		13.1	13.4	2.0	2.4
375		12.9	12.3	3.2	4.5
750		12.7	12.6	3.6	4.5
1,500		11.5	12.5	4.7	3.9
3,000		11.5	11.4	4.2	4.8
6,000		10.7	10.1	6.5	7.6
12,000		10.4	10.3	7.2	6.7
	PSEM[1]	0.56		0.39	
Main effect means					
25-(OH)D_3					
0		12.0		4.2[b]	
68.75		11.8		4.6[a]	
	PSEM	0.20		0.13	
Phytase					
0		12.4[ab]		2.2[c]	
187.5		13.3[a]		2.2[c]	
375		12.6[ab]		3.9[b]	
750		12.6[ab]		4.1[b]	
1,500		12.0[b]		4.3[b]	
3,000		11.5[bc]		4.5[b]	
6,000		10.4[c]		7.0[a]	
12,000		10.3[c]		7.1[a]	
	PSEM	0.40		0.28	
ANOVA					
R^2		0.64		0.91	
Source of variation	df	---------- Probability > F ----------			
Treatment	15	0.0007		0.0001	
25-(OH)D_3	1	0.4989		0.0274	
Phytase [$\log_{10}$ (phytase +1)]	7	0.0001		0.0001	
25-(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]	7	0.6654		0.1077	
Regression Analysis[3]					
R^2		0.56		0.79	
		---------- Probability > |t| ----------			

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Intercept	0.0001	0.0001
25-$(OH)D_3$	-----	0.0847
Phytase [$\log_{10}$ (phytase +1)]	0.0003	0.0032
Phytase x phytase [$\log_{10}$ (phytase +1)]^2	0.0001	0.0001
25-$(OH)D_3$ x phytase [$\log_{10}$ (phytase +1)]	-----	-----

[1]PSEM = the calculated pooled standard error of the mean

[2]Prediction equations:

Plasma calcium = $12.4551 + 1.3977 \times \log_{10}(x + 1) - 0.4866 \times (\log_{10}(x + 1))^2$

Plasma phosphorus = $2.0032 + 0.0065 \times 25\text{-}(OH)D_3 - 1.0726 \times \log_{10}(x + 1) + 0.5624 \times (\log_{10}(x + 1))^2$

TABLE 3.4. Effect of High Phytase Levels and 25-Hydroxycholecalciferol on Tibia Data, Experiment 1

	Score		Incidence					
	P Rickets		P Rickets		Tibia Ash			
	25-(OH)D_3 (ug/kg diet)							
Phytase	(0)	(68.75)	(0)	(68.75)	(0)	(68.75)	(0)	(68.75)
(U/kg diet)			%				(g/tibia)	
0	2.6	1.9	90	71	27.8	30.2	0.247	0.280
187.5	2.5	1.5	90	66	28.5	31.5	0.291	0.303
375	1.6	0.7	66	30	31.5	33.7	0.323	0.344
750	0.7	0.1	31	8	33.8	35.6	0.364	0.392
1,500	0.2	0.1	19	5	35.3	36.3	0.409	0.411
3,000	0.0	0.1	3	8	37.6	37.9	0.440	0.452
6,000	0.0	0.0	0	0	39.1	39.4	0.511	0.524
12,000	0.0	0.0	0	3	39.6	40.0	0.554	0.559
PSEM[1]	0.14		6.0		0.30		0.0112	
Main effect means								
25-(OH)D_3								
0	1.0[a]		37[a]		34.1[b]		0.392[b]	
68.75	0.5[b]		24[b]		35.6[a]		0.408[a]	
PSEM	0.5		2.1		0.11		0.0040	

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Phytase					
0		2.2[a]	81[a]	29.0[g]	0.263[h]
187.5		2.0[a]	78[a]	30.0[f]	0.297[g]
375		1.1[b]	48[b]	32.5[e]	0.333[f]
750		0.4[c]	19[c]	34.7[d]	0.378[e]
1,500		0.2[cd]	12[cd]	35.8[c]	0.410[d]
3,000		0.0[d]	5[d]	37.8[b]	0.446[c]
6,000		0.0[d]	0[d]	39.3[a]	0.518[b]
12,000		0.0[d]	1[d]	39.8[a]	0.556[a]
	PSEM	0.10	4.2	0.21	0.0079
ANOVA					
R^2		0.96	0.94	0.99	0.97
Source of variation	df	Probability > F			
Treatment	15	0.0001	0.0001	0.0001	0.0001
25-(OH)D_3	1	0.0001	0.0001	0.0001	0.0082
Phytase [$\log_{10}$ (phytase +1)]	7	0.0001	0.0001	0.0001	0.0001
25-(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]	7	0.0004	0.0140	0.0001	0.8390
Regression Analysis[2]					
R^2		0.78	0.75	0.94	0.97
		Probability > |t|			
Intercept		0.0001	0.0001	0.0001	0.0001
25-(OH)D_3		0.0022	0.0114	0.0001	0.0059
Phytase [$\log_{10}$ (phytase +1)]		0.1161	0.9605	0.0662	0.0001
Phytase x phytase [$\log_{10}$ (phytase +1)]^2		0.0103	0.0012	0.0001	0.0001
25-(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]		0.0331	-----	0.0133	-----

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[1]PSEM = the calculated pooled standard error of the mean

[2]Prediction equations:

Phosphorus rickets score = $2.8660 - 0.0161 \times 25\text{-}(OH)D_3 - 0.3034 \times \log_{10}(x + 1) - 0.1160 \times (\log_{10}(x + 1))^2 + 0.0036 \times \log_{10}(x + 1) \times 25\text{-}(OH)D_3$

Phosphorus rickets incidence= $90.4053 - 0.1958 \times 25\text{-}(OH)D_3 - 0.3433 \times \log_{10}(x + 1) - 5.7054 \times (\log_{10}(x + 1))^2$

Percent tibia ash = $27.2543 + 0.0462 \times 25\text{-}(OH)D_3 - 0.7799 \times \log_{10}(x + 1) + 0.9909 \times (\log_{10}(x + 1))^2 - 0.0092 \times \log_{10}(x + 1) \times 25\text{-}(OH)D_3$

Tibia ash weight = $0.2544 + 0.0002 \times 25\text{-}(OH)D_3 - 0.0511 \times \log_{10}(x + 1) + 0.0306 \times (\log_{10}(x + 1))^2$

TABLE 3.5. Effect of High Phytase Levels and 25-Hydroxycholecalciferol on Nutrient Utilizations, Experiment 1

	Retention				Phytate		N[1]			
	Ca		P		Disappearance		Utilization		AME_n[2]	
	25-$(OH)D_3$ (ug/kg diet)									
Phytase	(0)	(68.75)	(0)	(68.75)	(0)	(68.75)	(0)	(68.75)	(0)	(68.75)
(U/kg diet)	%								kcal/kg	
0	34.0	32.1	57.3	60.0	49.5	55.4	72.5	72.4	3473	3450
187.5	52.3	31.1	63.4	61.5	61.6	56.6	69.1	72.7	3368	3458
375	51.2	56.2	67.1	69.3	67.9	71.0	68.9	66.4	3369	3327
750	56.9	46.7	70.5	69.7	75.2	74.1	66.2	68.8	3334	3356
1,500	62.3	52.5	73.5	77.6	78.7	82.2	69.3	69.7	3364	3379
3,000	61.5	58.4	79.4	77.6	84.4	84.0	70.8	70.6	3392	3360
6,000	60.9	61.9	77.6	78.4	89.5	90.0	70.6	71.3	3365	3399
12,000	61.8	64.6	81.2	81.2	95.3	96.2	69.8	73.6	3384	3438
PSEM[3]	2.72		1.81		1.74		0.86		19.4	
Main effect means										
25-$(OH)D_3$										
0	55.1[a]		71.3		75.2		69.7[b]		3381[a]	
68.75	50.4[b]		71.9		76.2		70.7[a]		3396[b]	
PSEM	0.96		0.64		0.61		0.30		6.9	

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Phytase						
0		33.0[d]	58.6[e]	52.4[h]	72.5[a]	3462[a]
187.5		41.7[c]	62.5[d]	59.1[g]	70.9[ab]	3413[b]
375		51.8[b]	68.2[c]	69.4[f]	67.7[c]	3348[c]
750		53.7[b]	70.1[c]	74.7[e]	67.5[c]	3345[c]
1,500		57.4[ab]	75.6[b]	80.4[d]	69.5[b]	3371[bc]
3,000		59.9[a]	78.5[ab]	84.2[c]	70.7[ab]	3376[bc]
6,000		61.4[a]	78.0[ab]	89.7[b]	70.9[ab]	3382[bc]
12,000		63.2[a]	81.2[a]	95.8[a]	71.7[a]	3411[b]
	PSEM	1.92	1.28	1.23	0.61	13.7
ANOVA						
R^2		0.89	0.90	0.97	0.74	0.71
Source of variation	df	Probability > F				
Treatment	15	0.0001	0.0001	0.0001	0.0001	0.0001
25-(OH)D_3	1	0.0016	0.4666	0.2891	0.0253	0.1494
Phytase [$\log_{10}$ (phytase +1)]	7	0.0001	0.0001	0.0001	0.0001	0.0001
25-(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]	7	0.0006	0.6493	0.1132	0.0116	0.0226
Regression Analysis[4]						
R^2		0.70	0.83	0.93	0.35	0.36
		Probability > |t|				

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Intercept	0.0001	0.0001	0.0001	0.0001	0.0001
25-$(OH)D_3$	0.0164	-----	-----	0.0855	-----
Phytase [$\log_{10}$ (phytase +1)]	0.0001	0.8460	0.1621	0.0001	0.0001
Phytase x phytase [$\log_{10}$ (phytase +1)]^2	-----	0.0001	0.0001	0.0001	0.0008
25-$(OH)D_3$ x phytase [$\log_{10}$ (phytase +1)]	-----	-----	-----	-----	-----

[1]Apparent N retention of each diet was calculated as (100- (100 x (% Cr_2O_3 feed/% Cr_2O_3 excreta) x (%N excreta/%N feed)))
[2]Nitrogen-corrected, apparent metabolizable energy (AMEn) of each diet was calculated as: (Gross energy (GE) Feed [kcal/g] - ((GE Excreta [kcal/g] x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) + (g N Feed/g Feed - (g N Excreta/g Excreta x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) x 8.28))) x 1,000
[3]PSEM = the calculated pooled standard error of the mean
[4]Prediction equations:

Total calcium retention = $33.2852 - 0.0682 \times 25\text{-}(OH)D_3 + 7.8520 \times \log_{10}(x + 1)$

Total phosphorus retention = $58.2388 - 0.2629 \times \log_{10}(x + 1) + 1.5396 \times (\log_{10}(x + 1))^2$

Phytate phosphorus disappearance = $51.9892 - 2.1047 \times \log_{10}(x + 1) + 3.2340 \times (\log_{10}(x + 1))^2$

Appaarent %N retention = $72.0977 + 0.0147 \times 25\text{-}(OH)D_3 - 3.5398 \times \log_{10}(x + 1) + 0.8101 \times (\log_{10}(x + 1))^2$

$AME_n = 3465.2880 - 73.7645 \times \log_{10}(x + 1) + 14.0207 \times (\log_{10}(x + 1))^2$

TABLE 3.6. Effect of High Phytase Levels and 1 " -Hydroxycholecalciferol on 16-d Broiler Performance, Experiment 2[1]

	BWG		FI		Gain:Feed		Mortality	
	25-(OH)D_3 (ug/kg diet)							
Phytase	(0)	(5)	(0)	(5)	(0)	(5)	(0)	(5)
(U/kg diet)	(g/chick)				(g:g)		%	
0	339	378	458	491	0.740	0.770	12.8	5.1
187.5	386	402	513	521	0.753	0.771	7.7	0.0
375	420	412	546	529	0.770	0.778	5.1	2.6
750	443	453	573	579	0.773	0.782	5.1	2.6
1,500	468	456	591	576	0.791	0.792	2.6	2.6
3,000	486	464	613	584	0.794	0.795	5.1	0.0
6,000	477	427	603	546	0.791	0.782	0.0	5.1
12,000	512	450	643	563	0.796	0.799	2.6	0.0
PSEM[2]	8.1		10.1		0.0078		2.87	
Main effect means								
1 " -(OH)D_3								
0	441[a]		568[a]		0.776		5.1	
5	430[b]		549[b]		0.784		2.2	
PSEM	2.9		3.6		0.0028		1.01	

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Phytase					
0		359[f]	475[d]	0.755[e]	9.0[a]
187.5		394[e]	517[c]	0.762[de]	3.9[ab]
375		416[d]	537[c]	0.774[cd]	3.9[ab]
750		448[c]	576[b]	0.778[bcd]	3.9[ab]
1,500		462[bc]	584[ab]	0.791[ab]	2.6[ab]
3,000		475[ab]	598[a]	0.794[ab]	2.6[ab]
6,000		452[c]	574[b]	0.786[abc]	2.6[ab]
12,000		481[a]	603[a]	0.797[a]	1.3[b]
	PSEM	5.7	7.1	0.0055	2.02
ANOVA					
R^2		0.93	0.91	0.68	0.39
Source of variation	df	Probability > F			
Treatment	15	0.0001	0.0001	0.0002	0.2143
1 '' -(OH)D_3	1	0.0096	0.0008	0.0585	0.0527
Phytase [$\log_{10}$ (phytase +1)]	7	0.0001	0.0001	0.0001	0.2788
1 '' -(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]	7	0.0001	0.0001	0.3690	0.3996
Regression Analysis[3]					
R^2		0.81	0.78	0.58	0.30
		Probability > |t|			
Intercept		0.0001	0.0001	0.0001	0.0001
1 '' -(OH)D_3		0.0009	0.0059	0.0025	0.0123
Phytase [$\log_{10}$ (phytase +1)]		0.0014	0.0001	0.2708	0.0007
Phytase x phytase [$\log_{10}$ (phytase +1)]^2		0.0735	-----	0.0719	-----
1 '' -(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]		0.0001	0.0001	0.0119	0.0642

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[1]Average initial chick weight = 43.1 $\pm$ 0.85 g/chick
[2]PSEM = the calculated pooled standard error of the mean
[3]Prediction equations:

Body Weight Gain = $329.3043 + 10.7356 \times 1''\text{-(OH)}D_3 + 28.7207 \times \log_{10}(x + 1) + 3.5286 \times (\log_{10}(x + 1))^2 - 4.6658 \times \log_{10}(x + 1) \times 1''\text{-(OH)}D_3$

Feed Intake = $442.3805 + 10.0071 \times 1''\text{-(OH)}D_3 + 45.0355 \times \log_{10}(x + 1) - 4.9504 \times \log_{10}(x + 1) \times 1''\text{-(OH)}D_3$

Gain-to-feed = $0.7387 + 0.0062 \times 1''\text{-(OH)}D_3 + 0.0060 \times \log_{10}(x + 1) + 0.0023 \times (\log_{10}(x + 1))^2 - 0.0017 \times \log_{10}(x + 1) \times 1''\text{-OH}D_3$

Mortality = $12.9925 - 1.7390 \times 1''\text{-(OH)}D_3 - 2.8292 \times \log_{10}(x + 1) - 0.4181 \times \log_{10}(x + 1) \times 1''\text{-(OH)}D_3$

TABLE 3.7. Effect of High Phytase Levels and 1"-Hydroxycholecalciferol on Plasma Values, Experiment 2

		Plasma Ca		Plasma P	
		---------- 1"-(OH)D_3 (ug/kg diet) ----------			
Phytase		(0)	(5)	(0)	(5)
(U/kg diet)		---------- (mg/100 mL) ----------			
0		14.1	14.4	2.7	3.1
187.5		11.8	14.2	3.2	2.6
375		12.8	13.0	2.8	2.9
750		12.9	12.6	3.0	3.8
1,500		13.2	14.0	3.5	3.7
3,000		12.1	12.6	4.2	4.6
6,000		11.9	12.1	5.2	6.3
12,000		11.1	12.8	5.1	5.3
	PSEM[1]	0.81		0.45	
Main effect means					
1"-(OH)D_3					
0		12.5		3.7	
5		13.2		4.0	
	PSEM	0.29		0.16	
Phytase					
0		14.3[a]		2.9[d]	
187.5		13.0[ab]		2.9[d]	
375		12.9[ab]		2.8[d]	
750		12.7[ab]		3.4[cd]	
1,500		13.6[ab]		3.6[cd]	
3,000		12.4[b]		4.4[bc]	
6,000		12.0[b]		5.8[a]	
12,000		12.0[b]		5.2[ab]	
	PSEM	0.57		0.32	
ANOVA					
R^2		0.40		0.75	
Source of variation	df	---------- Probability > F ----------			
Treatment	15	0.2070		0.0001	
1"-(OH)D_3	1	0.0915		0.1491	
Phytase [$\log_{10}$ (phytase +1)]	7	0.0929		0.0001	
1"-(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]	7	0.7443		0.7190	
Regression Analysis[2]					
R^2		0.25		0.62	
		---------- Probability > |t| ----------			

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Intercept	0.0001	0.0001
1 '' -(OH)D_3	0.0706	-----
Phytase [$\log_{10}$ (phytase +1)]	0.0014	0.0020
Phytase x phytase [$\log_{10}$ (phytase +1)]^2	-----	0.0001
1 '' -(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]	-----	-----

[1]PSEM = the calculated pooled standard error of the mean

[2]Prediction equations:

Plasma Ca = $14.0116 + 0.1408 \times 1'' \text{-(OH)}D_3 - 0.5432 \times \log_{10}(x + 1)$

Plasma P = $2.8924 \times \log_{10}(x + 1) - 1.0358 + 0.4221 \times (\log_{10}(x + 1))^2$

TABLE 3.8. Effect of High Phytase Levels and 1"-Hydroxycholecalciferol on Tibia Data, Experiment 2

	Score		Incidence					
	P Rickets		P Rickets		Tibia Ash			
	1"-(OH)D_3 (ug/kg diet)							
Phytase	(0)	(5)	(0)	(5)	(0)	(5)	(0)	(5)
(U/kg diet)			%				(g/tibia)	
0	2.3	1.5	83	60	28.5	31.4	0.313	0.372
187.5	2.2	1.1	77	49	29.1	31.9	0.353	0.402
375	1.2	0.7	52	32	32.1	33.7	0.431	0.436
750	0.3	0.0	22	3	33.6	34.5	0.481	0.491
1,500	0.1	0.0	5	3	35.7	36.7	0.530	0.559
3,000	0.0	0.0	3	0	37.4	38.4	0.601	0.584
6,000	0.0	0.2	3	5	38.6	39.4	0.621	0.599
12,000	0.0	0.2	0	8	39.4	39.9	0.698	0.661
PSEM[1]	0.17		6.1		0.48		0.0130	
Main effect means								
1"-(OH)D_3								
0	0.8		30[a]		34.3[b]		0.503	
5	0.5		20[b]		35.7[a]		0.513	
PSEM	0.06		2.1		0.17		0.0046	

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Phytase					
0		1.9[a]	71[a]	30.0[f]	0.343[g]
187.5		1.7[a]	63[a]	30.5[f]	0.378[f]
375		1.0[b]	42[b]	32.9[e]	0.434[e]
750		0.2[c]	12[c]	34.1[d]	0.486[d]
1,500		0.1[c]	4[c]	36.2[c]	0.544[c]
3,000		0.0[c]	1[c]	37.9[b]	0.592[b]
6,000		0.1[c]	4[c]	39.0[a]	0.610[b]
12,000		0.1[c]	4[c]	39.7[a]	0.679[a]
	PSEM	0.12	4.3	0.34	0.0092
ANOVA					
R^2		0.91	0.92	0.97	0.97
Source of variation	df	Probability > F			
Treatment	15	0.0001	0.0001	0.0001	0.0001
1 '' -(OH)D_3	1	0.0018	0.0014	0.0001	0.1534
Phytase [$\log_{10}$ (phytase +1)]	7	0.0001	0.0001	0.0001	0.0001
1 '' -(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]	7	0.0085	0.0397	0.1201	0.0071
Regression Analysis[2]					
R^2		0.67	0.72	0.92	0.95
		Probability > |t|			
Intercept		0.0001	0.0001	0.0001	0.0001
1 '' -(OH)D_3		0.0043	0.0097	0.0003	0.0008
Phytase [$\log_{10}$ (phytase +1)]		0.0001	0.1195	0.0139	0.0076
Phytase x phytase [$\log_{10}$ (phytase +1)]^2		-----	0.0372	0.0001	0.0001
1 '' -(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]		0.0232	0.0533	0.0236	0.0015

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[1]PSEM = the calculated pooled standard error of the mean

[2]Prediction equations:

P Rickets Score = $2.5959 - 0.2114 \times 1'' \text{-}(OH)D_3 - 0.6590 \times \log_{10}(x + 1) + 0.0547 \times \log_{10}(x + 1) \times 1'' \text{-}(OH)D_3$

P Rickets Incidence = $79.2349 - 2.1212 \times 1'' \text{-}(OH)D_3 - 6.7128 \times \log_{10}(x + 1) - 3.3015 \times (\log_{10}(x + 1))^2$

Percent Tibia Ash = $28.2308 + 0.6221 \times 1'' \text{-}(OH)D_3 - 1.1180 \times \log_{10}(x + 1) + 1.0045 \times (\log_{10}(x + 1))^2 - 0.1214 \times \log_{10}(x + 1) \times 1'' \text{-}(OH)D_3$

Tibia Ash Weight = $0.3028 + 0.0146 \times 1'' \text{-}(OH)D_3 - 0.0318 \times \log_{10}(x + 1) + 0.0316 \times (\log_{10}(x + 1))^2 - 0.0046 \times \log_{10}(x + 1) \times 1'' \text{-}(OH)D_3$

TABLE 3.9. Effect of High Phytase Levels and 1"-Hydroxycholecalciferol on Nutrient Utilizations, Experiment 2

	Retention				Phytate		N[1]			
	Ca		P		Disappearance		Utilization		AME_n[2]	
	1"-$(OH)D_3$ (ug/kg diet)									
Phytase	(0)	(5)	(0)	(5)	(0)	(5)	(0)	(5)	(0)	(5)
(U/kg diet)	%								kcal/kg	
0	32.3	35.6	55.8	58.0	53.0	58.0	74.5	72.1	3480	3400
187.5	28.8	46.6	52.8	71.0	49.9	75.5	71.3	66.9	3411	3220
375	49.2	39.7	65.2	65.1	72.8	75.8	63.8	65.6	3218	3209
750	42.8	40.3	65.4	68.6	76.9	77.0	63.0	71.6	3198	3392
1,500	40.2	42.2	67.2	70.3	76.3	82.6	68.6	73.0	3272	3346
3,000	40.0	42.0	69.7	76.5	81.4	85.7	72.0	72.1	3376	3381
6,000	37.4	48.3	73.8	78.3	84.8	92.8	71.8	73.4	3370	3395
12,000	43.2	52.1	74.8	79.7	93.3	97.3	72.1	76.1	3395	3389
PSEM[3]	3.58		3.71		2.41		1.65		39.2	
Main effect means										
1"-$(OH)D_3$										
0	39.2[b]		65.6[b]		73.5[b]		69.6[b]		3340	
5	43.3[a]		70.9[a]		80.6[a]		71.3[a]		3341	
PSEM	1.27		1.31		0.85		0.59		13.9	

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Phytase						
0		33.9[c]	56.9[e]	55.5[g]	73.3[a]	3440[a]
187.5		37.7[bc]	61.9[de]	62.7[f]	69.1[bc]	3316[bc]
375		44.4[ab]	65.1[cd]	74.3[e]	64.7[d]	3214[d]
750		41.6[abc]	67.0[cd]	77.0[de]	67.3[cd]	3295[c]
1,500		41.2[abc]	68.7[bcd]	79.5[cd]	70.8[ab]	3309[bc]
3,000		41.0[abc]	73.1[abc]	83.6[c]	72.1[ab]	3378[abc]
6,000		42.8[ab]	76.0[ab]	88.8[b]	72.6[ab]	3382[abc]
12,000		47.6[a]	77.2[a]	95.3[a]	74.1[a]	3392[ab]
	PSEM	2.53	2.62	1.70	1.17	27.7
ANOVA						
R^2		0.57	0.67	0.94	0.71	0.70
Source of variation	df	Probability > F				
Treatment	15	0.0059	0.0002	0.0001	0.0001	0.0001
1 '' -$(OH)D_3$	1	0.0288	0.0070	0.0001	0.0458	0.9441
Phytase [$\log_{10}$ (phytase +1)]	7	0.0269	0.0001	0.0001	0.0001	0.0001
1 '' -$(OH)D_3$ x phytase [$\log_{10}$ (phytase +1)]	7	0.0236	0.3709	0.0004	0.0142	0.0029
Regression Analysis[4]						
R^2		0.25	0.59	0.84	0.46	0.33
		Probability > |t|				

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Intercept	0.0016	0.0001	0.0001	0.0001	0.0001
1 '' -(OH)D_3	0.0470	0.0044	0.0001	0.2953	-----
Phytase [$\log_{10}$ (phytase +1)]	0.0021	0.7730	0.8722	0.0001	0.0001
Phytase x phytase [$\log_{10}$ (phytase +1)]^2	-----	0.0143	0.0001	0.0001	0.0001
1 '' -(OH)D_3 x phytase [$\log_{10}$ (phytase +1)]	-----	-----	-----	0.0637	-----

[1] N retention of each diet was calculated as (100- (100 x (% Cr_2O_3 feed/% Cr_2O_3 excreta) x (%N excreta/%N feed)))

[2]Nitrogen-corrected, apparent metabolizable energy (AMEn) of each diet was calculated as: (Gross energy (GE) Feed [kcal/g] - ((GE Excreta [kcal/g] x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) + (g N Feed/g Feed - (g N Excreta/g Excreta x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) x 8.28))) x 1,000

[3]PSEM = the calculated pooled standard error of the mean

[4]Prediction equations:

Total calcium retention = $31.5856 + 0.8200 \times 1'' \text{-(OH)}D_3 + 2.7515 \times \log_{10}(x + 1)$

Total phosphorus retention = $54.1066 + 1.0696 \times 1'' \text{-(OH)}D_3 - 0.6981 \times \log_{10}(x + 1) + 1.4672 \times (\log_{10}(x + 1))^2$

Phytate phosphorus disappearance = $51.7000 + 1.4074 \times 1'' \text{-(OH)}D_3 - 0.3607 \times \log_{10}(x + 1) + 2.4975 \times (\log_{10}(x + 1))^2$

Appaarent %N retention = $74.6254 - 0.5280 \times 1'' \text{-(OH)}D_3 - 7.4402 \times \log_{10}(x + 1) + 1.7151 \times (\log_{10}(x + 1))^2 + 0.3137 \times \log_{10}(x + 1) \times 1'' \text{- (OH)}D_3$

$AME_n = 3440.2163 - 156.9473 \times \log_{10}(x + 1) + 36.8294 \times (\log_{10}(x + 1))^2$

CHAPTER 4

THE EFFECT OF DIETARY CALCIUM UPON BROILER CHICK PERFORMANCE AND THE UTILIZATION OF NUTRIENTS WHEN HIGH LEVELS OF PHYTASE ARE SUPPLEMENTED[1,2,3]

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[1]Shirley, R. B. and Hardy M. Edwards, Jr.

[2]To be submitted to Poultry Science.

[3]Experiments B5-00-TD and B11-00-TD

Abstract

The following two studies evaluated the effect of varying dietary calcium [0.80, 0.95, 1.00, or 1.10% (Experiment 1) and 0.65, 0.80, 0.95, or 1.10% (Experiment 2)] and phytase levels [0, 1,500, 3,000, and 6,000 units (U)/kg diet] on nutrient utilizations and performance of 0 to 16 d-old, straight-run, Ross x Ross broiler chicks. On an as-is basis, the experimental corn-soybean-poultry-by-product meal basal diet of Experiment 1 contained an analyzed CP of 23.3%, a deficient, total P (tP) level of 0.47% [phytate P = 0.26%], and a calculated ME of 3,155 kcal/kg diet; and the experimental corn-soybean meal basal diet of Experiment 2 contained an average CP of 21.8%, a deficient, tP level of 0.39% [phytate P = 0.26%], and a calculated ME of 3,123 kcal/kg diet.

In Experiment 1, increasing the level of total Ca from 0.80 to 1.10% increased the total Ca (tCa) retention from 48.0 to 51.8% and reduced tP retention from 67.4 to 63.0%, depressed body weight gain (BWG) from 363 to 346 g/chick, feed intake (FI) from 482 to 458g/chick, tibia ash (TA) from 34.4 to 33.6%, and tibia ash weight (TA_w) from 0.421 to 0.400 g/tibia. Supplementing phytase from 0 to 6,000 U/kg of diet increased (P # 0.0001) the disappearance of phytate from 12.6 to 78.6%, which allowed for 19 and 53% increases in tCa and tP retention, respectively. Reducing the level of phytate and increasing the P availability of the basal diet improved (P # 0.0001) BWG from 259 to 400 g/chick, FI from 357 to 517 g/chick, TA from 25.8 to 38.9%, and TA_w from 0.200 and 0.541 g/tibia.

In Experiment 2, increasing the total level of dietary Ca from 0.65 to 1.10% depressed (P #0.0632) BWG from 238 to 346 g/chick, FI from 452 to 409 g/chick, TA from 34.5 to 32.8%, TA_w from 0.370 to 0.310 g/tibia, tCa retention from 65.2 to

49.1%, tP retention from 67.8 to 62.6% and phytate P from 73.8 to 66.5%. Despite a depression in tCa retention, tP retention and phytate disappearance, plasma Ca levels were elevated (P #0.0018) from 8.18 to 10.06 mg/100 mL and plasma P levels were reduced from 3.22 to 2.20 mg/100 mL. The addition of 0 to 6,000 U of phytase improved (P #0.0018) tCa retention from 48.1 to 61.1%, tP retention from 53.0 to 78.3% and phytate disappearance from 49.8 to 85.1%. Again, the higher available P content of the basal diet improved (P # 0.0004) BWG from 238 to 346 g/chick, FI from347 to 464 g/chick, TA from 25.3 to 38.3%, and TA_w from 0.193 to 0.421 g/tibia. In both Experiments, increasing the level of dietary Ca to 1.10% had the greatest negative effect on most broiler response and nutrient utilization data when 0 or 1,500 U of phytase was supplemented to the basal diet. Any further effects of high dietary Ca were ameliorated when 3,000 or 6,000 U of phytase were supplemented.

KEY WORDS: Dietary calcium levels, dietary phytase supplementation, phytase level, broiler performance

Introduction

In seeds, it is estimated that approximately 50 to 80% of the total P (tP) is bound in a phytate complex [myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate]. Within this complex, neighboring orthophosphates create a negative charge that chelates other nutrients such as Cu^{+2}, Zn^{+2}, Co^{+2}, Fe^{+2}, Mn^{+2}, Fe^{+3}, Ca^{+2} and Mg^{+2}, or the free amino or carboxyl termini of proteins and amino acids (Cheryan, 1980; Reddy, 1982; Thompson, 1986). As a seed matures, the phytate content of the seed increases, suggesting that the physiological function of phytate is to store copious amounts of P and other minerals in a fairly unavailable/insoluble form prior to the eventual germination and growth of the seed. This compound is relatively resistant to hydrolysis within the gastrointestinal tract of young poultry and is thus considered an antinutritional factor due to its ability to bind other nutrients besides P. Therefore, to make P and other minerals of plant-based rations more available and reduce the amount of P that is excreted by broiler chicks, the broiler industry supplements between 300 and 600 U of phytase/kg of feed. Data from this laboritory (Roberson and Edwards, 1994; Mitchell and Edwards, 1996a,b; Kasim and Edwards, 2000) suggests that phytate degradation can vary from approximately 10 to 73% when 600 U of dietary phytase is supplemented to a tP-deficient broiler diet that contains an adequate dietary Ca (NRC, 1994). This extreme range of phytate degradation may be due to the source of dietary ingredients, broiler strain, and possibly dietary vitamin D_3 supplementation, or the ratio of dietary Ca to available P (aP) (Schöner et al., 1993; Mitchell and Edwards,1996a,b; Qian et al., 1996; Yi et al., 1996; Qian et al., 1997).

By supplementing higher levels of phytase [12,000 U/kg of feed] to a tP-deficient diet, a 95% reduction in phytate P, a 53% increase in apparent tCa retention, and an 80% increase in tP retention was observed in Chapters 2 and 3. Although this level of supplementation is not economical at the present time, it does suggest that almost all of the dietary phytate P can be made available to the growing broiler chick. If the availability of phytate P can be maximized at these higher levels of phytase, the level of Ca may need to be adjusted so that a Ca imbalance and subsequent depression in broiler performance does not occur.

Little is known about the effects of dietary Ca on broiler performance and the utilization of nutrients when phytase levels exceed 1,500 U/kg of diet. Based on previous phytase, Ca and P research of Nelson et al. (1990), Mohammed et al. (1991), Schöner et al. (1991, 1993), Mitchell and Edwards (1996a,b) and Qian et al. (1996, 1997), it is hypothesized that supplementing 0 to 6,000 U of phytase to a tP-deficient broiler diet will reduce, but not eliminate the negative effects of increasing levels of dietary Ca.

Materials and Methods

Experimental Diets

The National Research Council's nutrient values for ingredients (NRC, 1994) were used to formulate the corn-soybean-poultry-by-product meal basal diet of Experiment 1 and the corn-soybean meal diet of Experiment 2 (Table 1). The phytase product that was used in Experiments 1 and 2 of the present study was also used in the study of Hall et al. (2003) and Chapters 2 and 3. So that the enzymatic

activity of the phytase was maintained between each of the studies, the phytase was stored at -20°C. The log-dose supplementation of phytase to the respective treatments was based on the guaranteed value of the powdered phytase product [Natuphos 5000][4], which was added at either 0, 1,500, 3,000, or 6,000 U/kg diet. To maintain semi-isocaloric and isonitrogenous diets, sand was used substituted on an equal-weight basis in with either limestone, phytase or dicalcium phosphate in Experiments 1 and 2.

Chick Assay Procedures

In each experiment, 600, d-of-hatch, straight-run, Ross x Ross[5] chicks were wing-banded, weighed, and randomly allotted to each of the 20 treatments in groups of 10 chicks per pen, with 3 pens per treatment. Throughout each 16-d experiment, chicks were given free access to water and mash diets, were brooded in thermostatically controlled Petersime starter batteries[6] with raised wire floors, and were reared on a 24-h lighting schedule. The starter batteries were kept in an environmentally controlled room (22°C). Fluorescent lights outside and inside the batteries were covered with Arm-a-lite plastic sleeves[7] to eliminate ultraviolet light

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[4]BASF Corp., Mt. Olive, NJ. The activity was determined by an in-house BASF assay to be 6,100 U/g. One unit (U) is equivalent to one phytase unit (FTU), and is defined as the amount of enzyme which liberates 1 micromole inorganic phosphorus per minute from 5.1 mMol sodium phytate at 37°C and at pH 5.50 for 30 to 60 minutes.

[5]Seaboard Farms, Athens, GA.

[6]Petersime Incubator Co., Gettysburg, OH.

[7]Arm-a-lite, Thermoplastic Processes, Sterling, NJ.

exposure and any additional vitamin D_3-mediated improvement in phytate P utilization from tP -deficient diets that would have otherwise resulted in a better base-line response from either dietary phytase or vitamin D_3 metabolite supplementation (Edwards et al., 1994; Elliot and Edwards, 1997; Mitchell et al., 1997).

On d-16 of each experiment, one chick was randomly selected from each of the three pens within each of the dietary treatments (n = 3), weighed, and a heparinized blood sample was taken by cardiac puncture. All remaining chicks were then weighed and euthanized via CO_2 asphyxiation. The right tibia of each chick was randomly scored for the incidence of phosphorus rickets (P rickets) (Edwards et al., 1994), while the left tibia of each chick was banded for identification and removed for future percent tibia ash (TA) and tibia ash weight determination [g/tibia (TA_w)]. After a 24-h collection period [between days 15 and 16 of the experiment], an excreta sample was collected from each pen, dried at 75°C for 36-h, ground to pass through a 1mm mesh screen, and analyzed for the composition of nutrients.

Sample Analyses

Plasma samples were collected and refrigerated overnight, and analyzed for total plasma calcium (plasma Ca) (Section N-31)[8] and dialyzable plasma phosphorus (plasma P) (section 7N-46)[9] after being thawed for 2-h at room

[8]Section N-31, Technicon Autoanalyzer Methodology, Technicon Corp., Tarrytown, NY.

[9]Section 7N-46, Technicon Autoanalyzer Methodology, Technicon Corp., Tarrytown, NY.

temperature, vortexed twice for 10 sec, and centrifuged for 10 min at 3,000 x g. Analyzed plasma values in Tables 3 and 7 can be transformed back to a fresh basis using the following prediction eqautions that were derived from Shirley and Edwards (unpublished):

Frozen plasam Ca = - 0.6044 + 0.8979 x (fresh plasma Ca): R^2 = 0.70

Frozen plasma P = 0.0381 + 0.6872 x (fresh plasma P): R^2 = 0.93

Feed and excreta were analyzed for moisture, chromic oxide (Brisson, 1956), CP [N x 6.25] (Etheridge et al., 1998), gross energy (instructions for 1241 and 1242 Adiabatic Calorimeters, Parr Instrument, Moline, IL), total Ca (tCa) (Hill, 1965), tP (O'Neill and Webb, 1970), and phytate P (Latta and Eskin, 1980). These values were then used to calculate nutrient utilizations such as phytate P disappearance[10], nutrient retention[11], and AME_n[12] values (Edwards and Gillis, 1959; Matterson et al., 1965).

The left tibiae were used to determine TA and TA_w on a dry, fat-free basis (AOAC, 1995). Percent tibia ash was calculated as a percentage of the ash weight

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[10]Total Ca retention, P retention, and phytate P disappearance of each diet was calculated as: (100- (100 x (% Cr_2O_3 feed/% Cr_2O_3 excreta) x (%[Ca, P or phytate P] excreta/%[Ca, P or phytate P] feed)))

[11]Apparent percent nitrogen retention (N) of each diet was calculated as: (100- (100 x (% Cr_2O_3 feed/% Cr_2O_3 excreta) x (%N excreta/%N feed)))

[12]Nitrogen-corrected, apparent metabolizable energy (AME_n) of each diet was calculated as: (GE Feed [kcal/g] - ((GE Excreta [kcal/g] x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) + (g N Feed - (g N Excreta x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) x 8.28))) x 1,000

of dry, fat-free tibiae; and, tibia ash weight was calculated as the weight of the tibiae ash, divided by the number of pooled tibiae from a given pen.

Statistical Analyses

The four levels of dietary Ca and phytase were arranged in each experiment as a completely-randomized 4 x 4 factorial arrangement. Analysis of variance for both experiments were completed using the general linear model procedure of SAS (Lyman Ott and Longnecker, 2001; SAS, 1999), wherein the main effect means for phytase and dietary Ca within each experiment were separated using Duncan's New Multiple Range Test. In addition, Dunnett's t-test (Lyman Ott and Longnecker, 2001) was used to compare the mean of each P-adequate diet, at each of the dietary Ca levels, with the mean of each dietary phytase level at the same Ca level [i.e. the BWG of the P-adequate diet that had a dietary Ca level of 0.65% was compared to the BWG of the diet that had 0 U of phytase and a dietary Ca level of 0.65%, then the diet that had a phytase level of 1,500 U and a dietary Ca level of 0.80%, etc.]. The same test was used to compare the P-adequate diets (across all dietary Ca levels) against each of the dietary phytase levels [i.e. the mean BWG of the P-adequate diets that contained 0.65 to 1.10% Ca were compared to the mean BWG of the diet that contained 0.65 to 1.10% Ca and no phytase, then 1,500 U of phytase, etc.]. Multiple linear regression analysis was used to calculate prediction equations for each of the parameters (Lyman Ott and Longnecker, 2001), where phytase and Ca levels within each experiment were regressed against the data of

each dependent variable. Depending on the effects that are significant for a particular dependent variable (P# 0.1000), the following general prediction equation will be:

$$Y = B + M \times (Ca\ level) + M' \times (Ca\ level)^2 + M'' \times (phytase\ level) + M''' \times (phytase\ level)^2 + M'''' \times ((phytase\ level) \times (Ca\ level)).$$

General Results

Experiment 1

Dietary Ca Effect

Increasing the total level of dietary Ca from 0.8 to 1.1% resulted in a significant linear reduction (P # 0.0874) in BWG and FI (Table 2), TA and TA_w (Table 4), and tP retention (Table 5) in Experiment 1. While tCa retention responded in a curvilinear fashion (P # 0.0884). Dietary Ca content had no statistical affect on gain to feed (G:F), % mortality (Table 2), plasma P (Table 3), TD (Table 4), phytate P disappearance, N retention, or AME_n values (Table 5).

Dietary Phytase Effect

Among the four levels of dietary Ca, supplemental phytase from 0 to 6,000 U/kg of diet improved (P # 0.0253) BWG, FI and G:F (Table 2), plasma P (Table 3), TA and TA_w (Table 4), and tCa retention, tP retention and phytate P disappearance (Table 5). The increase in the availability of phytate P decreased (P # 0.0008) the % mortality (Table 2), plasma Ca (Table 3) and P rickets (Table 4), and increased (P # 0.0001) the incidence of TD (Table 4).

The supplementation of 0 to 6,000 U of phytase linearly reduced (P # 0.0135) plasma Ca and linearly increased (P # 0.0001) plasma P. Significant curvilinear responses (P # 0.0253) to increasing levels of phytase were observed for BWG, FI, P rickets, TA, TA_w, tP retention, and phytate P disappearance. Phytase supplementation from 0 to 6,000 U/kg of feed had no affect on N utilization or AME_n values.

Interactions Between Dietary Ca and Phytase

Interactions between dietary Ca and phytase levels affected (P # 0.0707) FI and the concentration of plasma Ca. For instance, as the level of dietary Ca increased, plasma Ca levels increased, however, as the level of dietary phytase increased, the concentration of plasma Ca was reduced. The opposite was observed for FI.

The relative difference between the diets that contained 0.66% P and each of the phytase-supplemented diets at any one dietary Ca level is based on the minimum significant difference from Dunnett's t-test (" = 0.05). Increasing the level of dietary Ca from 0.80 to 1.10% depressed all parameters at each level of phytase. The greatest negative effects of increasing levels of Ca were upon BWG, FI, P rickets, TA, TA_w and tP retention when 0 or 1,500 U of phytase was supplemented. Phytate disappearance values for diets that contained no phytase were similar, however, increasing the level of phytase improved phytate degradation when compared to the tP-adequate or deficient groups. Increasing the level of dietary Ca to 1.1% did reduce the degradation of phytate, however, the values remained greater than either of the phytase-free diets.

Experiment 2

Dietary Ca Effect

The supplementation of dietary Ca from 0.65 to 1.10% linearly reduced (P # 0.0155) BWG, FI and G:F (Table 6), plasma P (Table 7), TD, TA and TA_w (Table 8) and tCa retention (Table 9), and elevated plasma Ca (P # 0.0018). A curvilinear reduction (P # 0.0787) in tP retention and phytate disappearance was observed (Table 9) as the level of dietary Ca increased. Unlike Experiment 1, a reduction in BWG, FI, TAw and phytate disappearance, and very little change in other parameters was observed when the level of dietary Ca increased to 1.10% in diets that contained 0.67% P.

Dietary Phytase Effect

Increasing the level of dietary phytase from 0 to 6,000 U/kg improved (P # 0.0833) BWG, FI, G:F and mortality (Table 6), plasma P (Table 7), P rickets, TD, TA and TA_w (Table 8), tCa retention, tP retention, phytate disappearance (Table 9), and reduced plasma Ca in a curvilinear manner (Table 9). Supplemental phytase had no affect on plasma Ca (Table 7), N utilization or AME_n values (Table 9).

Interactions Between Dietary Ca and Phytase

Of the parameters measured, interactions (P # 0.0340) between dietary Ca and phytase were noted for G:F and tCa retention. At each level of supplemental

phytase, elevating the level of dietary Ca reduced G:F and tCa retention, however, the negative affects of high Ca became less with each additional level of supplemental phytase.

As in Experiment 1, increasing the dietary Ca level to 1.10% dietary Ca depressed BWG and FI (Table 6), P rickets, TA and TA_w (Table 8) and phytate P disappearance values (Table 9) at each level of phytase when compared to diets that had 0.67% P and no phytase. The effect of high levels of dietary Ca were again greatest when 0 to 1,500 U of phytase was supplemented; and, regardless of phytase supplementation, phytate P disappearance values were greater than that of diets containing adequate tP and no phytase at any one dietary Ca level. Chicks consuming diets that had 0.67% tP had a higher BWG and FI (Table 6), plasma P (Table 7), TD, TA and TA_w (Table 8) and tCa retention (Table 9), and a lower P rickets and phytate P disappearance (Table 9) when compared to tP-deficient diets that contained 0 to 1,500 U of phytase.

Discussion

The two experiments within this study were designed to test the combined effect of increasing levels of dietary Ca and higher levels of supplemental phytase on broiler performance and the utilization of nutrients. In both Experiments, dietary concentration of CP, Ca and tP were not what they were calculated to be. The degree of variation in the diet analyses cannot be fully explained since feed formulation and mixing procedures were followed according to general lab procedures. Therefore, the higher CP of Experiment 1, the approximate 0.10% difference between the dietary Ca levels within each experiment, and the extremely

low tP of the basal diet in Experiment 2 suggests that: the diets were not adequately sampled for analysis, there were general mixing errors, the dietary ingredients used contained levels of nutrients that were different than NRC values (NRC, 1994), and/or the manual-scales used to weigh out the limestone and dicalcium phosphate were not accurately calibrated. Despite the differences in dietary CP, Ca and P, data from Experiments 1 and 2 give valuable insight into the effects of high Ca levels within diets that contain levels of phytase that are not supplemented within the broiler industry.

Studies without the supplementation of dietary phytase suggest that there is a negative relationship between increasing levels of dietary Ca and BWG, FI, tibia ash deposition and P retention (Nelson et al., 1990; Schöner et al., 1993; Rennie et al., 1995; Mitchell and Edwards, 1996a,b). In the absence of supplemental phytase, Schöner et al. (1993) observed that increasing the dietary Ca level from 0.6 to 0.9% reduced BWG, FI, tCa retention, tP retention and the hydrolysis of phytate. Supplementing phytase from 0 to 1,500 U/ kg of diet, Schöner et al. (1993) also reported an improvement in each of the latter parameters, however, increasing the level of dietary Ca in the presence of phytase negatively affected BWG, FI, tCa retention, tP retention and the degradation of phytate. Mitchell and Edwards (1996a) corroborate the findings of Schöner et al. (1993) in two studies. By increasing the level of dietary Ca from 0.63 to 0.99% in a tP-deficient diet (0.43%), BWG, G:F, TA, tP retention, and phytate disappearance were reduced by 26, 11, 8, 6, and 57%, respectively. Just as in the study of Schöner et al. (1993), the addition of dietary phytase (600 U/kg of diet) alleviated the effects of higher levels of dietary Ca in tP-deficient diets and improved all of the latter parameters. Even

with the additon of dietary phytase however, increasing the levels of dietary Ca reduced broiler performance and the utilization of dietary nutrients. These reductions imply that Ca is either inhibiting the liberation of phytate P or the absorption of dietary P. Maenz et al. (1999) suggests that the chelation of Ca to phytate results in an insoluble Ca-phytate complex within at pH values that are similar to those that are found within the gastrointestinal tract (pH = 5.0 to 6.5). In the case of higher phytate degredation and the lower negative effects of dietary Ca at higher levels of phytase supplementation, the supplementation of phytase reduced the binding of Ca to phytate within the gastrointestinal tract. As it was discussed in Chapter 2, the phytase that was used had two pH optima of 2.5 and 5.5. Prior to the ingesta entering the small intestine, it is concievable that a significant amount of phytate degredation occured at the higher levels of phytase supplementation. Given the moistening of feed within the crop and subsequent acidification of the ingesta within the proventriculus (pH of approximately 2 to 2.5), phytate within the feed would have been subjected to the degradative effects of phytase at the higher levels of phytase supplementation. Providing that a large proportion of the phytate P was liberated, ionic Ca and P could readilly be absorbed from the intestinal lumen, thereby reducing the negative effects of high dietary Ca. Compared to diets that had 3,000 or 6,000 U of phytase, the previous theory is partially supported by the slightly lower phytate degradation and broiler performance at 1,500 U of phytase, and the progressive decrease in phytate degradation at the higher levels of dietary Ca.

Comparing Experiments 1 and 2, differences in the utilization of dietary N and energy were observed. Even though the average-initial-chick-weight was greater

in Experiment 2, the increase in G:F values of chicks in Experiment 1 resulted in greater dietary N reterntion and energy utilization when compared to Experiment 2. These higher utilization values may be due to the higher CP and aP levels of diet the diets in Experiment 1. Regardless of any difference between Experiments 1 and 2, the N utilization and dietary AME results of the two experiments within this study are are in contrast to the phytase-mediated responses of Chapter 2. Hypothetically, as phytate degradation increases, the interactions between phytate, minerals, proteins and starch should decrease, resulting in a higher utilization of dietary N and energy (Cheryan,1980; Reddy, 1982; Thompson, 1986). Since an increase in the level of dietary Ca to 1.10% or the level of phytase to 6,000 U/kg of diet had no affect upon N retention or energy utilization, there may be other unidentified dietary factors than phytate that affect N and energy utilization of broiler diets. Factors such as the initial nutritional status or health of the broiler chick, feed particle size or the source of dietary ingredients can affect the how well dietary nutrients are utilized, and thus the overall performance of growing broiler chicks (Biehl and Baker, 1997; Kasim and Edwards, 2000; Peter et al. (2000); Kilburn and Edwards, 2001; Peter and Baker (2001).

Conclusion

In both experiments, a comparison among the dietary phytase treatments across each of the dietary Ca levels revealed that performance and P utilization data of chicks consuming the basal diet became progressively worse as dietary Ca levels approached 1.00%. Results of the two experiments, however, suggest that phytase levels of 3,000 to 6,000 U/kg of feed can liberate approximately 79 to 85% of the

phytate P in a corn-soybean meal-based diet, and negate the affects of moderate to high levels of dietary Ca. The fact that the analyzed dietary Ca levels of each experiment were not what they were supposed to be does not detract from this message. Nevertheless, these trials should be rerun so that the optimal supplementary dietary Ca and phytase levels can be determined for maximal broiler performance and nutrient utilizations.

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Tables

TABLE 4.1. Composition of the Basal Diet for Experiments 1 and 2.

	Experiment 1					Experiment 2				
Ca level (%)	0.80	0.90	1.00	1.10	0.80[4]	0.65	0.80	0.95	1.10	0.65[5]
Ingredients	(%)									
Corn (8.5% CP)	57.284	57.284	57.284	57.284	57.284	56.538	56.538	56.538	56.538	56.538
Soybean meal (48.5% CP)	31.707	31.707	31.707	31.707	31.707	35.055	35.055	35.055	35.055	35.055
Poultry by-product meal (60.0% CP)	3.000	3.000	3.000	3.000	3.000	-----	-----	-----	-----	-----
Poultry fat (8.2 kcal/g)	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000	4.000
Salt (NaCl)	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450
Limestone	1.479	1.743	2.006	2.269	0.860	0.443	0.443	0.443	0.443	0.443
Vitamin mix[1]	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250
Dicalcium phosphate	0.225	0.225	0.225	0.225	1.294	0.398	0.398	0.398	0.398	1.702
DL-Methionine	0.184	0.184	0.184	0.184	0.184	0.198	0.198	0.198	0.198	0.198
Cr_2O_3	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100
Trace mineral mix[2]	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080
Phytase[3]	variable	variable	variable	variable	-----	variable	variable	variable	variable	-----
Sand	variable	variable	variable	variable	variable	variable	variable	variable	variable	variable
Total	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000

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Calculated composition										
ME (kcal/kg)	3,155	3,155	3,155	3,155	3,155	3,123	3,123	3,123	3,123	3,123
CP	22.2	22.2	22.2	22.2	22.2	21.9	21.9	21.9	21.9	21.9
Ca	0.80	0.90	1.00	1.10	0.80	0.65	0.80	0.95	1.10	0.65
Total P	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.69
Phytate P	0.233	0.233	0.233	0.233	0.233	0.244	0.244	0.244	0.244	0.244
Analyzed composition										
Crude Protein (N x 6.25)	23.3	23.3	23.3	23.3	23.3	21.8	21.8	21.8	21.8	21.8
Ca	0.89	0.93	0.96	0.99	0.89	0.65	0.74	0.83	0.96	0.65
Total P	0.47	0.47	0.47	0.47	0.66	0.39	0.39	0.39	0.39	0.67
Phytate P	0.264	0.264	0.264	0.264	0.264	0.256	0.256	0.256	0.256	0.256

[1]Trace mineral mix provides the following (per kilogram of diet): manganese ($MnSO_4 \cdot H_2O$), 60 mg; iron ($FeSO_4 \cdot 7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4 \cdot 5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg.
[2]Vitamin mix provided the following (per kilogram of diet):
thiamin•mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B_{12} (*cobalamin*), 12.0 ug; pyridoxine•HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-retinyl acetate, 5,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.
[3]Phytase percentages for 0, 1,500, 3,000 and 6,000 U/kg of diet were 0, 0.03, 0.06, and 0.12%, respectively. Sand was substitued on an equal weight basis as phytase was added to the respective diets.
[4]The diets that contain no phytase and a high total P contain either 0.80, 0.90, 1.00, or 1.10% dietary Ca. Sand was substitued on an equal weight basis as limestone was added to the respective diets.
[5]The diets that contain no phytase and a high total P contain either 0.65, 0.80, 0.95 or 1.10% dietary Ca. Sand was substitued on an equal weight basis as limestone was added to the respective diets.

TABLE 4.2. Effect of Various Calcium and Phytase Levels on 16-d Broiler Performance, Experiment 1[1,2]

Calcium	Phytase	Total P	BWG	FI	Gain:Feed	Mortality
(%)	(U/kg diet)	(%)	-------- (g/chick) --------		(g:)	----- % -----
0.8	0	0.47	286*	394*	0.725	6.7
0.9			271*	375*	0.721*	10.0
1.0			239*	327*	0.731	23.3*
1.1			240*	331*	0.725	20.0
0.8	1,500	0.47	377	498	0.757	6.7
0.9			370	494	0.748	6.7
1.0			301	434	0.693	0.0
1.1			349	466	0.748	0.0
0.8	3,000	0.47	389	515	0.754	6.7
0.9			377	508	0.741	3.3
1.0			392	497	0.789	3.3
1.1			393	515	0.763	3.3
0.8	6,000	0.47	402	522	0.770	3.3
0.9			408	520	0.786	0.0
1.0			390	509	0.764	3.3
1.1			400	519	0.770	3.3
0.8	0	0.66	388	500	0.777	3.3
0.9			404	526	0.768	3.3
1.0			402	518	0.776	0.0
1.1			390	522	0.746	6.7
	PSEM[3]		19.3	19.7	0.0166	4.22
Main effect means[5]						
Calcium (% of diet)						
0.8		0.47	363[a]	482[a]	0.751	5.8
0.9			356[ab]	474[a]	0.749	5.0
1.0			331[b]	442[b]	0.741	7.5
1.1			346[ab]	458[ab]	0.752	6.7
	PSEM[4]		10.2	10.2	0.0092	2.12
Phytase (FTU/ kg diet)						
0		0.47	259[c†]	357[c†]	0.726[b†]	15.0[a†]
1,500			349[b†]	473[b†]	0.733[b†]	3.3[b]
3,000			388[a]	509[a]	0.762[a]	4.2[b]
6,000			400[a]	517[a]	0.772[a]	2.5[b]
0[6]		0.66	396	516	0.767	3.3
		PSEM[3]	9.7	9.8	0.83	2.11
		PSEM[4]	10.2	10.2	0.0092	2.12

Continued on the following page.

ANOVA[7]					
R^2		0.80	0.84	0.52	0.53
Source of variation	df	---------- Probability > F ----------			
Treatment	15	0.0001	0.0001	0.0240	0.0163
Calcium	3	0.1433	0.0411	0.8077	0.8562
Phytase	3	0.0001	0.0001	0.0023	0.0005
Calcium*Phytase	9	0.6291	0.7701	0.1268	0.2078
Regression Analysis[8, 9]					
R^2		0.73	0.80	0.24	0.29
		---------- Probability > |t| ----------			
Intercept	1	0.0001	0.0001	0.0001	0.0001
Calcium Level	1	0.0874	0.0052	-----	-----
Calcium*Calcium	1	-----	-----	-----	-----
Phytase Level	1	0.0001	0.0405	0.0001	0.0014
Phytase*Phytase	1	0.0001	0.0001	-----	0.0136
Calcium*Phytase	1	-----	0.0707	-----	-----

[1]Average initial chick weight = 36.3 $\pm$ 1.15 g/chick
[2]*significant difference (" = 0.05) between the 0.66% P diet at 0.8, 0.9, 1.0, or 1.1% Ca and any one phytase level at the same Ca level using Dunnett's t-test.
[3]PSEM = the calculated pooled standard error of the mean for all treatements.
[4]PSEM = the calculated pooled standard error of the mean for phytase treatments 0 through 6,000 units.
[5](a-d) Duncan's mean seperation of dietary phytase levels (0 through 6,000 units) or Ca levels with no common superscript significantly differ (" = 0.05).
[6]† significant difference (" = 0.05) between the 0.66% P diet diet and any one phytase concentration across all Ca levels using Dunnett's t-test.
[7]Probability values ($P \geq F$) are only for phytase treatments 0 through 6,000 units.
[8]Probability values ($P \geq |t|$) are only for phytase treatments 0 through 6,000 units.
[9]Prediction equations were based on the calculated Ca content of the diets:

Body Weight Gain = 336.4199 - 78.8588 x Ca + 0.0644 x Plv - 0.000007 x $(Plv)^2$

Feed Intake =554.4359 - 203.0023 x Ca + 0.4325 + 0.0437 x Plv - 0.000009 x Plv^2 + 0.0372 x Ca x Plv

Gain-to-feed = 0.7264 + 0.000008 x Plv

Mortality = 13.8864 - 0.0061 x Plv + 0.00000072 x Plv^2

TABLE 4.3. Effect of Various Calcium and Phytase Levels on Plasma Calcium and Phosphorus, Experiment 1[1]

Calcium	Phytase	Total P	Plasma Ca	Plasma P
(%)	(U/kg diet)	(%)	---------- (mg/100 mL) ----------	
0.8	0	0.47	7.14	3.60*
0.9			7.63*	3.20*
1.0			6.67	3.47
1.1			7.73	3.40
0.8	1,500	0.47	6.46	4.43*
0.9			7.50*	3.92
1.0			6.92	4.95
1.1			7.50	4.38
0.8	3,000	0.47	6.47	5.60
0.9			6.54	5.57
1.0			6.35	5.37
1.1			6.08	4.93
0.8	6,000	0.47	4.69	6.80
0.9			5.90	6.37
1.0			6.27	6.67
1.1			6.99	6.25
0.8	0	0.66	5.62	7.05
0.9			5.82	5.77
1.0			6.22	5.38
1.1			6.19	4.88
		PSEM[2]	0.442	0.519
Main effect means[4]				
Calcium (% of diet)				
0.8		0.47	6.19[b]	5.11
0.9			6.89[ab]	4.76
1.0			6.55[ab]	5.11
1.1			7.07[a]	4.74
		PSEM[3]	0.231	0.272
Phytase (FTU/ kg diet)				
0		0.47	7.29[a†]	3.42[d†]
1,500			7.09[a†]	4.42[c†]
3,000			6.36[b]	5.37[b]
6,000			5.96[b]	6.52[a]
0[5]		0.66	5.96	5.77
		PSEM[2]	0.221	0.260
		PSEM[3]	0.231	0.272

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ANOVA[6]			
R^2		0.57	0.70
Source of variation	df	---------- Probability > F ----------	
Treatment	15	0.0068	0.0001
Calcium	3	0.0535	0.6328
Phytase	3	0.0008	0.0001
Calcium*Phytase	9	0.2644	0.9895
Regression Analysis[7, 8, 9]			
R^2		0.40	0.65
		---------- Probability > |t| ----------	
Intercept	1	0.0001	0.0001
Calcium Level	1	0.8831	-----
Calcium*Calcium	1	-----	-----
Phytase Level	1	0.0135	0.0001
Phytase*Phytase	1	-----	-----
Calcium*Phytase	1	0.0448	-----

[1]*significant difference ('' = 0.05) between the 0.66% P diet at 0.8, 0.9, 1.0, or 1.1% Ca and any one phytase level at the same Ca level using Dunnett's t-test.
[2]PSEM = the calculated pooled standard error of the mean for all treatements.
[3]PSEM = the calculated pooled standard error of the mean for phytase treatments 0 through 6,000 units.
[4](a-d) Duncan's mean seperation of dietary phytase levels (0 through 6,000 units) or Ca levels with no common superscript significantly differ ('' = 0.05).
[5]† significant difference ('' = 0.05) between the 0.66% P diet diet and any one phytase concentration across all Ca levels using Dunnett's t-test.
[6]Probability values ($P \geq F$) are only for phytase treatments 0 through 6,000 units.
[7]Probability values ($P \geq |t|$) are only for phytase treatments 0 through 6,000 units.
[8]Prediction equations were based on the calculated Ca content of the diets:
Plasma Calcium = 7.5197 - 0.2387 x Ca - 0.0012 x Plv + 0.0010 x Ca x Plv
Plasma Phosphorus = 3.5875 + 0.0005 x Plv
[9]The following plasma prediction equations will convert the frozen plasma values back to a fresh-basis:
Plasma Ca = - 0.6044 + 0.8979 x (fresh plasma Ca): R^2 = 0.6962
Plasma P = 0.0381 + 0.6872 x (fresh plasma P): R^2 = 0.9266

TABLE 4.4. Effect of Various Calcium and Phytase Levels on Tibiae Parameters, Experiment 1[1]

			Incidence			
Calcium	Phytase	Total P	P rickets	TD	Tibia Ash	Tibia Ash
(%)	(U/kg diet)	(%)	---------- % ----------			(g/tibia)
0.8	0	0.47	88.9*	0.0	26.5*	0.241*
0.9			74.8*	3.7	26.1*	0.230*
1.0			76.9*	0.0*	25.6*	0.200*
1.1			84.3*	3.7	25.2*	0.210*
0.8	1,500	0.47	14.3	25.6	34.8*	0.423
0.9			17.5	6.7	34.4*	0.417*
1.0			13.3	6.7	35.0*	0.367*
1.1			23.3*	6.7	33.5*	0.379*
0.8	3,000	0.47	7.4	14.8	37.2	0.467
0.9			7.0	18.5	36.9	0.463
1.0			10.0	21.1	36.3	0.464
1.1			17.4*	17.0	37.3	0.477
0.8	6,000	0.47	0.0	26.7	39.2*	0.554
0.9			0.0	24.1	39.4	0.549
1.0			3.3	20.4	38.8	0.528
1.1			3.7	27.8	38.4	0.533*
0.8	0	0.66	7.0	18.5	37.7	0.509
0.9			3.3	23.3	37.5	0.503
1.0			3.3	30.0	37.1	0.477
1.1			0.0	11.1	37.0	0.473
		PSEM[2]	6.26	8.36	0.44	0.0186
Main effect means[4]						
Calcium (% of diet)						
0.8		0.47	27.7	16.8	34.4[a]	0.421[a]
0.9			24.8	13.2	34.2[ab]	0.415[ab]
1.0			25.9	12.0	33.9[ab]	0.390[b]
1.1			32.2	13.8	33.6[b]	0.400[ab]
		PSEM[3]	3.42	3.92	0.24	0.0100
Phytase (FTU/ kg diet)						
0		0.47	81.2[a†]	1.9[c†]	25.8[d†]	0.220[d†]
1,500			17.1[b†]	11.4[bc]	34.4[c†]	0.396[c†]
3,000			10.5[bc]	17.9[ab]	36.9[b]	0.468[b]
6,000			1.8[c]	24.7[a]	38.9[a†]	0.541[a†]
0[5]		0.66	3.4	20.7	37.3	0.490
		PSEM[2]	3.13	4.18	0.22	0.0093

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ANOVA[6]					
R^2		0.91	0.43	0.98	0.95
Source of variation	df	---------- Probability > F ----------			
Treatment	15	0.0001	0.1305	0.0001	0.0001
Calcium	3	0.4533	0.8517	0.0883	0.1258
Phytase	3	0.0001	0.0020	0.0001	0.0001
Calcium*Phytase	9	0.9526	0.8499	0.5226	0.9090
Regression Analysis[7, 8]					
R^2		0.84	0.31	0.96	0.93
		---------- Probability > |t| ----------			
Intercept	1	0.0001	0.1293	0.0001	0.0001
Calcium Level	1	-----	-----	0.0528	0.0487
Calcium*Calcium	1	-----	-----	-----	-----
Phytase Level	1	0.0001	0.0001	0.0001	0.0001
Phytase*Phytase	1	0.0001	-----	0.0001	0.0001
Calcium*Phytase	1	-----	-----	-----	-----

[1]*significant difference ('' = 0.05) between the 0.66% P diet at 0.8, 0.9, 1.0, or 1.1% Ca and any one phytase level at the same Ca level using Dunnett's t-test.
[2]PSEM = the calculated pooled standard error of the mean for all treatements.
[3]PSEM = the calculated pooled standard error of the mean for phytase treatments 0 through 6,000 units.
[4](a-d) Duncan's mean seperation of dietary phytase levels (0 through 6,000 units) or Ca levels with no common superscript significantly differ ('' = 0.05).
[5]† significant difference ('' = 0.05) between the 0.66% P diet diet and any one phytase concentration across all Ca levels using Dunnett's t-test.
[6]Probability values ($P \geq F$) are only for phytase treatments 0 through 6,000 units.
[7]Probability values ($P \geq |t|$) are only for phytase treatments 0 through 6,000 units.
[8]Prediction equations were based on the calculated Ca content of the diets:

Phosphorus Rickets Incidence = $76.6295 - 0.0380 \times Plv + 0.0000043 \times Plv^2$
Tibial Dyschondroplasia Incidence = $4.2994 + 0.0037 \times Plv$
Percent Tibia Ash = $28.9365 - 2.8230 \times Ca + 0.0056 \times Plv - 0.0000006 \times Plv^2$
Tibia Ash Weight = $0.3120 - 0.0898 \times Ca + 0.0001 \times Plv - 0.00000001 \times Plv^2$

TABLE 4.5. Effect of Various Calcium and Phytase Levels on the Utilization of Nutrients, Experiment 1[1]

			Retention		Phytate	N	
Calcium	Phytase	Total P	Ca	P	Disappearance	Utilization	AME_n
(%)	(U/kg diet)	(%)	---------- % ----------		---------- % ----------		kcal/kg
0.8	0	0.47	40.6	54.4	14.8	69.2	3383
0.9			47.0	51.3	2.8	69.1	3410
1.0			49.7	50.3	15.5	67.8	3359
1.1			50.5	47.6	15.0	70.9	3420
0.8	1,500	0.47	46.3	63.0	53.8	69.6	3373
0.9			46.4	63.0	53.1*	69.4	3409
1.0			52.6	64.0	54.8*	66.0	3356
1.1			48.0	60.2	48.1*	71.8	3411
0.8	3,000	0.47	47.2	72.6	66.7*	69.3	3410
0.9			49.1	71.7*	62.9*	71.8	3421
1.0			56.0	60.0	58.9*	71.6	3425
1.1			52.9	68.1*	55.5*	70.3	3409
0.8	6,000	0.47	57.9	79.5*	82.7*	70.1	3429
0.9			54.6	77.1*	81.3*	70.8	3425
1.0			55.0	79.5	76.3*	69.1	3379
1.1			55.8	75.9*	74.1*	70.5	3396
0.8	0	0.66	48.9	63.9	29.0	70.8	3425
0.9			55.1	57.7	5.6	70.7	3416
1.0			59.7	55.7	8.1	72.2	3442
1.1			56.6	54.7	15.6	69.4	3343
		PSEM[2]	4.33	3.71	6.51	1.99	32.5
Main effect means[4]							

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Calcium (% of diet)							
0.8	0.47		48.0	67.4	54.5	69.6	3399
0.9			49.3	65.8	54.3	70.3	3416
1.0			53.3	63.4	54.2	68.6	3380
1.1			51.8	63.0	48.2	70.9	3409
	PSEM[3]		2.19	1.77	2.72	1.062	16.4
Phytase (FTU/ kg diet)							
0	0.47		46.9[bt]	50.9[dt]	12.6[d]	69.3	3393
1,500			48.3[b]	62.6[c]	52.4[ct]	69.2	3387
3,000			51.3[ab]	68.1[bt]	61.2[bt]	70.8	3416
6,000			55.8[a]	78.0[at]	78.6[at]	70.1	3407
0[5]	0.66		55.1	58.0	16.1	70.7	3407
	PSEM[2]		2.17	1.86	3.23	0.993	16.3
	PSEM[3]		2.19	1.77	2.72	1.062	16.4
ANOVA[6]							
R^2			0.35	0.81	0.91	0.19	0.20
Source of variation		df	---------- Probability > F ----------				
Treatment		15	0.3673	0.0001	0.0001	0.9191	0.9076
Calcium		3	0.3199	0.2759	0.2819	0.4805	0.4423
Phytase		3	0.0349	0.0001	0.0001	0.6994	0.5862
Calcium*Phytase		9	0.9223	0.6327	0.7992	0.9246	0.9505
Regression Analysis[7, 8]							
R^2			0.28	0.76	0.86	-----	-----
			---------- Probability > |t| ----------				

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Intercept	1	0.0005	0.0001	0.0001	-----	-----
Calcium Level	1	0.0884	0.0462	-----	-----	-----
Calcium*Calcium	1	-----	-----	-----	-----	-----
Phytase Level	1	0.0013	0.0001	0.0001	-----	-----
Phytase*Phytase	1	-----	0.0253	0.0001	-----	-----
Calcium*Phytase	1	-----	-----	-----	-----	-----

[1]*significant difference (" = 0.05) between the 0.66% P diet at 0.8, 0.9, 1.0, or 1.1% Ca and any one phytase level at the same Ca level using Dunnett's t-test.
[2]PSEM = the calculated pooled standard error of the mean for all treatements.
[3]PSEM = the calculated pooled standard error of the mean for phytase treatments 0 through 6,000 units.
[4](a-d) Duncan's mean seperation of dietary phytase levels (0 through 6,000 units) or Ca levels with no common superscript significantly differ (" = 0.05).
[5]† significant difference (" = 0.05) between the 0.66% P diet diet and any one phytase concentration across all Ca levels using Dunnett's t-test.
[6]Probability values ($P \geq F$) are only for phytase treatments 0 through 6,000 units.
[7]Probability values ($P \geq |t|$) are only for phytase treatments 0 through 6,000 units.
[8]Prediction equations were based on the calculated Ca content of the diets:

Calcium Retention = 31.9364 + 15.4167 x Ca + 0.0015 x Plv

Phosphorus Retention = 66.1091 - 15.5042 x Ca + 0.0073 x Plv - 0.0000005 x Plv^2

Phytate Disappearance =15.5075 + 0.0236 x Plv - 0.000002 x Plv^2

TABLE 4.6. Effect of Various Calcium and Phytase Levels on 16-d Broiler Performance, Experiment 2[1,2]

Calcium	Phytase	Total P	BWG	FI	Gain:Feed	Mortality
(%)	(U/kg diet)	(%)	------- (g/chick) -------		(g:g)	----- % -----
0.65	0	0.39	295*	412*	0.715	6.7
0.80			251	362*	0.694	10.0
0.95			213*	317*	0.670	6.7
1.10			193*	297*	0.649*	20.0
0.65	1,500	0.39	346	468	0.740	0.0
0.80			322	449	0.718	0.0
0.95			344	472	0.728	3.3
1.10			326	450	0.726	0.0
0.65	3,000	0.39	332	447	0.743	6.7
0.80			303	434	0.688	0.0
0.95			331	453	0.731	3.3
1.10			314	438	0.718	3.3
0.65	6,000	0.39	352	479	0.735	0.0
0.80			366	481	0.760	3.3
0.95			325	442	0.733	3.3
1.10			341	453	0.752	0.0
0.65	0	0.67	345	467	0.740	3.3
0.80			337	457	0.738	3.3
0.95			339	472	0.717	10.0
1.10			320	440	0.727	6.7
		PSEM[3]	15.9	17.5	0.0136	3.58
Main effect means[5]						
Calcium (% of diet)						
0.65		0.39	331[a]	452[a]	0.733[a]	3.3
0.80			310[ab]	432[ab]	0.715[ab]	3.3
0.95			303[b]	421[b]	0.715[ab]	4.2
1.10			294[b]	409[b]	0.711[b]	5.8
		PSEM[4]	8.5	9.24	0.0070	1.56
Phytase (FTU/ kg diet)						
0		0.39	238[c†]	347[b†]	0.681[c†]	10.8[a]
1,500			335[ab]	460[a]	0.728[ab]	0.8[b]
3,000			320[b]	443[a]	0.720[b]	3.3[b]
6,000			346[a]	464[a]	0.745[a]	1.7[b]
0[6]		0.67	335	459	0.731	5.83
		PSEM[3]	7.9	8.8	0.0068	1.79
		PSEM[4]	8.5	9.2	0.0070	1.56

Continued on the following page.

ANOVA[7]					
R^2		0.80	0.81	0.68	0.57
Source of variation	df	---------- Probability > F ----------			
Treatment	15	0.0001	0.0001	0.0001	0.0068
Calcium	3	0.0238	0.0206	0.1341	0.6379
Phytase	3	0.0001	0.0001	0.0001	0.0003
Calcium*Phytase	9	0.0861	0.0881	0.0576	0.1428
Regression Analysis[8, 9]					
R^2		0.57	0.58	0.46	0.25
		---------- Probability > |t| ----------			
Intercept	1	0.0001	0.0001	0.0001	0.0001
Calcium Level	1	0.0145	0.0120	0.0062	-----
Calcium*Calcium	1	-----	-----	-----	-----
Phytase Level	1	0.0001	0.0001	0.9742	0.0023
Phytase*Phytase	1	0.0004	0.0001	0.0833	0.0156
Calcium*Phytase	1	-----	-----	0.0340	-----

[1]Average initial chick weight = 41.8 $\pm$ 1.06 g/chick

[2]*significant difference (" = 0.05) between the 0.67% P diet at 0.65, 0.8, 0.95, or 1.1% Ca and any one phytase level at the same Ca level using Dunnett's t-test.

[4]PSEM = the calculated pooled standard error of the mean for all treatements.

[3]PSEM = the calculated pooled standard error of the mean for phytase treatments 0 through 6,000 units.

[5](a-d) Duncan's mean seperation of dietary phytase levels (0 through 6,000 units) or Ca levels with no common superscript significantly differ (" = 0.05).

[6]† significant difference (" = 0.05) between the 0.67% P diet diet and any one phytase concentration across all Ca levels using Dunnett's t-test.

[7]Probability values ($P \geq F$) are only for phytase treatments 0 through 6,000 units.

[8]Probability values ($P \geq |t|$) are only for phytase treatments 0 through 6,000 units.

[9]Prediction equations were based on the calculated Ca content of the diets:

Body Weight Gain = 318.8427 - 80.5455 x Ca + 0.0463 x Plv - 0.000005 x $(Plv)^2$

Feed Intake = 438.9640 - 91.2893 x Ca + 0.0553 x Plv - 0.000006 x Plv^2

Gain-to-feed = 0.7783 - 0.1040 x Ca - 0.0000004 x Plv - 0.000000002 x Plv + 0.00002 x Ca x Plv

Mortality = 9.6288 - 0.0045 x Plv + 0.0000006 x Plv^2

TABLE 4.7. Effect of Various Calcium and Phytase Levels on Plasma Calcium and Phosphorus, Experiment 2[1]

Calcium	Phytase	Total P	Plasma Ca	Plasma P
(%)	(U/kg diet)	(%)	---------- (mg/100 mL) ----------	
0.65	0	0.39	8.69	1.58*
0.80			11.50*	1.72*
0.95			8.49	1.58*
1.10			11.37	1.65*
0.65	1,500	0.39	8.20	3.73
0.80			8.98	2.78
0.95			9.28	1.97*
1.10			9.25	1.95
0.65	3,000	0.39	8.24	3.23
0.80			8.72	4.47
0.95			9.88	1.97*
1.10			9.90	2.13
0.65	6,000	0.39	7.59	4.34
0.80			8.90	3.57
0.95			9.97*	2.69*
1.10			9.70	3.08
0.65	0	0.67	8.28	4.55
0.80			8.77	4.43
0.95			7.78	3.93
1.10			8.55	2.89
		PSEM[2]	0.375	6.58
Main effect means[4]				
Calcium (% of diet)				
0.65		0.39	8.18[b]	3.22[a]
0.80			9.52[a]	3.13[a]
0.95			9.40[a]	2.05[b]
1.10			10.06[a]	2.20[b]
		PSEM[3]	0.333	0.179
Phytase (FTU/ kg diet)				
0		0.39	10.01[a†]	1.63[c†]
1,500			8.93[b]	2.61[b†]
3,000			9.18[ab]	2.95[ab†]
6,000			9.04[ab]	3.42[a]
0[5]		0.67	8.35	3.95
		PSEM[2]	0.321	0.190
		PSEM[3]	0.333	0.179

Continued on the following page.

ANOVA[6]			
R^2		0.55	0.78
Source of variation	df	---------- Probability > F ----------	
Treatment	15	0.0113	0.0001
Calcium	3	0.0031	0.0001
Phytase	3	0.1089	0.0001
Calcium*Phytase	9	0.1240	0.0157
Regression Analysis[7, 8, 9]			
R^2		0.19	0.55
		---------- Probability > |t| ----------	
Intercept	1	0.0001	0.0001
Calcium Level	1	0.0018	0.0001
Calcium*Calcium	1	-----	-----
Phytase Level	1	-----	0.0001
Phytase*Phytase	1	-----	0.0493
Calcium*Phytase	1	-----	-----

[1]*significant difference ('' = 0.05) between the 0.67% P diet at 0.65, 0.8, 0.95, or 1.1% Ca and any one phytase level at the same Ca level using Dunnett's t-test.
[2]PSEM = the calculated pooled standard error of the mean for all treatements.
[3]PSEM = the calculated pooled standard error of the mean for phytase treatments 0 through 6,000 units.
[4](a-d) Duncan's mean seperation of dietary phytase levels (0 through 6,000 units) or Ca levels with no common superscript significantly differ ('' = 0.05).
[5]† significant difference ('' = 0.05) between the 0.67% P diet diet and any one phytase concentration across all Ca levels using Dunnett's t-test.
[6]Probability values ($P \geq F$) are only for phytase treatments 0 through 6,000 units.
[7]Probability values ($P \geq |t|$) are only for phytase treatments 0 through 6,000 units.
[8]Prediction equations were based on the calculated Ca content of the diets:
Plasma Calcium = 6.0844 + 3.6644
Plasma Phosphorus = 4.0904 - 2.7556 x Ca + 0.0006 x Plv - 0.00000006 x Plv^2
[9]The folloing plasma prediction equations will convert the forzen plasma values back to a fresh-basis:
Plasma Ca = - 0.6044 + 0.8979 x (fresh plasma Ca): R^2 = 0.6962
Plasma P = 0.0381 + 0.6872 x (fresh plasma P): R^2 = 0.9266

TABLE 4.8. Effect of Various Calcium and Phytase Levels on Tibiae Parameters, Experiment 2[1]

			Incidence			
Calcium	Phytase	Total P	P rickets	TD	Tibia Ash	Tibia Ash
(%)	(U/kg diet)	(%)	---------- % ----------			(g/tibia)
0.65	0	0.39	92.6*	10.4*	27.6*	0.250*
0.80			88.9*	11.1*	25.1*	0.199*
0.95			96.3*	3.7	25.1*	0.176*
1.10			87.8*	0.0	23.3*	0.146*
0.65	1,500	0.39	20.0*	26.7	34.8v	0.363
0.80			13.3	13.3*	34.9*	0.344
0.95			38.5*	3.3	35.0*	0.357
1.10			33.3	7.7	34.1*	0.337*
0.65	3,000	0.39	7.5	28.3	36.7	0.391
0.80			16.7	13.3*	37.5	0.374
0.95			10.7	20.0	36.9	0.386
1.10			10.4	13.7	36.2	0.358
0.65	6,000	0.39	3.3	20.0	38.9	0.475
0.80			3.3	23.7*	38.5	0.460
0.95			0.0	17.0	38.6	0.399
1.10			3.3	10.0	37.5	0.401
0.65	0	0.67	3.3	48.4	37.5	0.428
0.80			0.0	55.6	38.6	0.420
0.95			0.0	22.4	38.1	0.423
1.10			0.0	10.0	39.1	0.412
		PSEM[2]	6.58	6.74	0.72	0.0206
Main effect means[4]						
Calcium (% of diet)						
0.65		0.39	30.9	21.3a	34.5a	0.370a
0.80			30.6	15.4ab	34.0a	0.344ab
0.95			36.4	11.0b	33.9a	0.329bc
1.10			33.7	7.6b	32.8b	0.310c
		PSEM[3]	3.66	3.24	0.37	0.0102
Phytase (FTU/ kg diet)						
0		0.39	91.4a†	6.3b†	25.3d†	0.193c†
1,500			26.3b†	12.5ab†	34.7c†	0.350b†
3,000			11.3c	17.7a†	36.8b†	0.378b†
6,000			2.5c	18.8a†	38.3a	0.434a
0[5]		0.67	0.83	34.2	38.3	0.421
		PSEM[2]	3.29	3.37	0.36	0.0103
		PSEM[3]	3.66	3.24	0.37	0.0102

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ANOVA[6]					
R^2		0.92	0.44	0.96	0.91
Source of variation	df	---------- Probability > F ----------			
Treatment	1	0.0001	0.1106	0.0001	0.0001
Calcium	5	0.6442	0.0309	0.0234	0.0024
Phytase	3	0.0001	0.0392	0.0001	0.0001
Calcium*Phytase	3	0.5689	0.7753	0.4028	0.4195
	9				
Regression Analysis[7, 8]					
R^2		0.87	0.34	0.91	0.85
		---------- Probability > |t| ----------			
Intercept	1	0.0001	0.0003	0.0001	0.0001
Calcium Level	1	-----	0.0015	0.0155	0.0005
Calcium*Calcium	1	-----	-----	-----	-----
Phytase Level	1	0.0001	0.0158	0.0001	0.0001
Phytase*Phytase	1	0.0001	0.0741	0.0001	0.0001
Calcium*Phytase	1	-----	-----	-----	-----

[1]*significant difference (" = 0.05) between the 0.67% P diet at 0.65, 0.8, 0.95, or 1.1% Ca and any one phytase level at the same Ca level using Dunnett's t-test.
[2]PSEM = the calculated pooled standard error of the mean for phytase treatments 0 through 6,000 units.
[3]PSEM = the calculated pooled standard error of the mean for all treatements.
[4](a-d) Duncan's mean seperation of dietary phytase levels (0 through 6,000 units) or Ca levels with no common superscript significantly differ (" = 0.05).
[5]† significant difference (" = 0.05) between the 0.67% P diet diet and any one phytase concentration across all Ca levels using Dunnett's t-test.
[6]Probability values ($P \geq F$) are only for phytase treatments 0 through 6,000 units.
[7]Probability values ($P \geq |t|$) are only for phytase treatments 0 through 6,000 units.
[8]Prediction equations were based on the calculated Ca content of the diets:

Phosphorus Rickets Incidence = $87.8770 - 0.0417 \times Plv + 0.000005 \times Plv^2$

Tibial Dyschondroplasia Incidence = $32.5085 - 30.4012 \times Ca + 0.0061 \times Plv + 0.0000007 \times Plv^2$

Percent Tibia Ash = $28.8318 - 3.0060 \times Plv - 0.0000007 \times Plv^2$

Tibia Ash Weight = $0.3160 - 0.1285 \times Ca + 0.00009 \times Plv - 0.000000009 \times Plv^2$

TABLE 4.9. Effect of Various Calcium and Phytase Levels on the Utilization of Nutrients, Experiment 2[1]

			Retention		Phytate	N	
Calcium	Phytase	Total P	Ca	P	Disappearance	Utilization	AME_n
(%)	(U/kg diet)	(%)	---------- % ----------		---------- % ----------		kcal/kg
0.65	0	0.39	53.4*	56.9	53.1*	63.7	3133
0.80			45.8	54.6	58.6*	64.2	3149
0.95			46.3	53.3	46.7*	62.6	3135
1.10			47.0	47.3	40.7	62.1	3182
0.65	1,500	0.39	62.6	65.5	74.7*	62.2	3092
0.80			57.5	63.7*	70.4*	58.6	3005
0.95			51.2	63.2	72.6*	61.8	3070
1.10			47.1	60.2	67.7*	61.1	3088
0.65	3,000	0.39	69.6	70.8*	81.7*	64.8	3127
0.80			66.1	73.5*	82.3*	62.0	3141
0.95			58.0	75.9*	78.9*	65.4*	3167
1.10			52.0	69.5*	77.4*	61.8	3107
0.65	6,000	0.39	75.1	78.0*	85.5*	63.7	3114
0.80			62.5	81.3*	87.3*	64.0	3123
0.95			56.5	80.4*	87.3*	64.1	3092
1.10			50.3	73.3*	80.1*	63.8	3089
0.65	0	0.67	66.9	50.4	42.5	63.5	3111
0.80			57.2	51.5	33.2	63.5	3051
0.95			54.1	51.7	30.6	60.5	3041
1.10			51.2	52.6	31.0	63.4	3100
		PSEM[2]	2.71	3.53	2.93	1.65	42.7
Main effect means[4]							

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Calcium (% of diet)						
0.65	0.39	65.2[a]	67.8	73.8[a]	63.6	3117
0.80		58.0[b]	68.3	74.6[a]	62.2	3105
0.95		53.0[c]	68.2	71.4[a]	63.5	3116
1.10		49.1[c]	62.6	66.5[b]	62.2	3117
	PSEM[3]	1.40	1.87	1.45	0.85	22.3
Phytase (FTU/ kg diet)						
0	0.39	48.1[c†]	53.0[d]	49.8[d†]	63.2[ab]	3150[a†]
1,500		54.6[b]	63.1[c†]	71.4[c†]	61.0[b]	3064[b]
3,000		61.4[a]	72.4[b†]	80.1[b†]	63.5[ab]	3136[a]
6,000		61.1[a]	78.3[a†]	85.1[a†]	63.9[a]	3105[ab]
0[5]	0.67	57.3	51.5	34.3	62.7	3076
	PSEM[2]	1.36	1.76	1.46	0.82	21.4
	PSEM[3]	1.40	1.87	1.45	0.85	22.3
ANOVA[6]						
R^2		0.82	0.78	0.92	0.31	0.29
Source of variation	df	Probability > F				
Treatment	15	0.0001	0.0001	0.0001	0.5251	0.5816
Calcium	3	0.0001	0.1079	0.0017	0.4949	0.9775
Phytase	3	0.0001	0.0001	0.0001	0.0902	0.0490
Calcium*Phytase	9	0.1114	0.9730	0.2850	0.8461	0.8722
Regression Analysis[7, 8]						
R^2		0.79	0.76	0.89	-----	-----
		Probability > |t|				

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Intercept	1	0.0001	0.6686	0.4805	-----	-----
Calcium Level	1	0.0037	0.1091	0.1131	-----	-----
Calcium*Calcium	1	-----	0.0787	0.0632	-----	-----
Phytase Level	1	0.0001	0.0001	0.0001	-----	-----
Phytase*Phytase	1	0.0002	0.0018	0.0001	-----	-----
Calcium*Phytase	1	0.0011	-----	-----	-----	-----

[1]*significant difference (" = 0.05) between the 0.67% P diet at 0.65, 0.8, 0.95, or 1.1% Ca and any one phytase level at the same Ca level using Dunnett's t-test.
[2]PSEM = the calculated pooled standard error of the mean for all treatements.
[3]PSEM = the calculated pooled standard error of the mean for phytase treatments 0 through 6,000 units.
[4](a-d) Duncan's mean seperation of dietary phytase levels (0 through 6,000 units) or Ca levels with no common superscript significantly differ (" = 0.05).
[5]† significant difference (" = 0.05) between the 0.67% P diet diet and any one phytase concentration across all Ca levels using Dunnett's t-test.
[6]Probability values ($P \geq F$) are only for phytase treatments 0 through 6,000 units.
[7]Probability values ($P \geq |t|$) are only for phytase treatments 0 through 6,000 units.
[8]Prediction equations were based on the calculated Ca content of the diets:

Calcium Retention = $64.3230 - 18.9891 \times Ca + 0.0118 \times Plv \times 0.0000007 \times Plv^2 - 0.0063 \times Ca \times Plv$

Phosphorus Retention = $12.0965 + 107.7058 \times Ca - 67.5507 \times Ca^2 + 0.0085 \times Plv - 0.0000007x\ Plv^2$

Phytate Disappearance = $17.8891 + 95.3977 \times Ca - 64.0943 \times Ca^2 + 0.0150 \times Plv - 0.000001 \times Plv^2$

CHAPTER 5

THE EXPRESSION OF CALBINDIN IN CHICKS THAT ARE DIVERGENTLY SELECTED FOR EITHER A LOW OR HIGH INCIDENCE OF TIBIAL DYSCHONDROPLASIA[1,2,3]

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[1]Shirley, Robert B., Adam J. Davis, Mark M. Compton and Wallace D. Berry

[2]Submitted to the Journal of Nurtrition for review June 3, 2003.

[3]Experiments B6-01-TD, B6-01-TDR, B10-02-TD

Abstract

Three experiments were conducted using broiler chicks that were divergently selected for either a low or high incidence of tibial dyschondroplasia (LTD and HTD, respectively) to determine if the expression of intestinal calbindin-28 kd mRNA and protein differed between the two strains. In addition, the levels of intestinal vitamin D receptor mRNA and plasma thyroid hormone concentrations were also examined. In Experiment 1, LTD and HTD chicks were fed a corn-soybean meal diet that was adequate in all nutrients except cholecalciferol (D_3), which was titrated to 5 or 40 µg/kg diet in a completely randomized 2 x 2 factorial arrangement. At 4 and 8-d of age, HTD chicks fed 5 µg D_3/kg diet had a lower ($P<0.05$) expression level of calbindin-28 kd than the LTD chicks fed the same diet. At both time periods, intestinal expression of the calbindin-28 kd protein was equivalent for the LTD chicks fed 5 µg of D_3 and both strains of chicks fed 40 µg of D_3. HTD chicks fed 5 µg of D_3 had the lowest expression of calbindin-28 kd protein. The expression of vitamin D receptor mRNA did not differ for the broiler strains at either level of D_3 supplementation. In Experiment 2, there was no significant difference in the expression of calbindin-28 kd mRNA and vitamin D receptor mRNA between d-of-hatch LTD, HTD, and commercial broiler chicks. Experiment 3 was similar in design to the first experiment except that the birds were fed for 18-d. Calbindin-28 kd and vitamin D receptor mRNA expression levels at 18-d were similar to those observed in Experiment 1. Plasma triiodothyronine and free-triiodothyronine concentrations

were greater for LTD chicks, regardless of dietary D_3 supplementation levels. These results suggest that divergent selection of broilers for LTD or HTD alters their physiological response to lower levels of dietary D_3.

KEY WORDS: calbindin, vitamin D receptor, thyroid hormones, broiler chicks.

Introduction

In a normally developing long bone such as the tibiotarsus, chondrocytes of the epiphyseal growth plate are organized in columns. The resting chondrocytes of the proximal epiphysis divide into proliferating cells that progressively mature into proliferating, prehypertrophic, and hypertrophic chondrocytes that eventually develop into endochondral/metaphyseal bone (1, 2). Alterations in this normal process can lead to several bone diseases such as Ca rickets, P rickets or tibial dyschondroplasia (TD[4]). The characteristic feature of a TD lesion is the presence of a non-mineralized, amorphous area of avascular cartilage near the metaphysis where hypertrophic chondrocytes have an arrested development, possibly leading to premature apoptotic cell death (2-4).

Tibial dyschondroplasia is a crippling bone disease that is often observed in rapidly growing poultry. Poultry that develop TD have a characteristic bowlegged and unstable gait, are less likely to consume feed and water or move away from more aggressive birds, and have reduced growth rates and a higher incidence of morbidity and mortality. As reviewed by Orth and Cook (3), the complex etiology of TD remains unclear, however, several factors may be involved such as genetic selection for growth rate, exposure to aflatoxin, and feeding diets which are either cholecalciferol-deficient, contain excessive chloride or have an imbalance of dietary Ca and P.

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[4]Abbreviation Key: GAPDH = glyceraldehyde-3-phosphate dehdrogenase; HTD = high incidence of tibial dyschondroplasia; LTD = low incidence of tibial dyscondroplasia; TA = tibia ash percent; TD = tibial dyschondroplasia; T_4 = thyroxine; T_3 = 3, 5, 3' - triiodothyronine; 1,25-$(OH)_2D_3$ = 1,25-dihydroxycholecalciferol; cholecalciferol = vitamin D_3

Increasing the ratio of dietary Ca:P decreases the incidence and severity of TD in broiler chicks (5-11). Likewise, dietary supplementation with cholecalciferol, 1-" -hydroxycholecalciferol and 1,25-dihydroxycholecalciferol can also lower the incidence and severity of TD (7-12) by increasing Ca and P absorption and reabsorption from the intestine and the kidney, respectively. An increase in active transport of Ca across epithelial cells of the duodenum has been linked, in part, to a Ca/vitamin D_3-dependent protein, calbindin-28 kd (13-17). Intestinal expression of calbindin- 28 is enhanced by D_3 and 1,25-dihydroxycholecalciferol (13-17). In addition, other investigators (13-16) have reported that an increase in the expression of calbindin-28 kd caused by 1,25-dihydroxycholecalciferol can be further enhanced by the addition of thyroid hormone (thyroxine and 3, 5, 3'-triiodothyronine). These results suggest that an increase in calbindin-28 kd synthesis within the duodenum may be linked to differences in the serum concentration of either 1,25-dihydroxycholecalciferol or thyroid hormone.

Through genetic selection of commercial broiler chicks, Wong-Valle et al. (18) established two genetic lines of broiler chicks that have either a low (LTD) or high (HTD) incidence of TD. The heritability of TD in the HTD line was 0.437 (18). Although the LTD and HTD strains have been used in several studies to investigate dietary factors that may either alleviate or exacerbate TD (19, 20), there has been limited investigation into the potential biochemical differences between these two strains of broilers. Therefore, three experiments were conducted to test two hypotheses: that the expression of intestinal calbindin-28 kd mRNA, calbindin-28

kd protein and vitamin D receptor mRNA differs between broiler chicks divergently selected for either low or high incidence of TD, and that plasma thyroid hormone concentrations between the strains reflect differences in the incidence of TD.

Materials and Methods

Experiment 1. The National Research Council's nutrient values for ingredients (21), were used to formulate a corn-soybean meal basal diet (Table 1). The basal diet met or exceeded the National Research Council's nutrient requirements, except for vitamin D_3, for poultry from 0 to 21 d-of-age. Crystalline D_3 (250 mg/vial, Sigma Chemical Co., St. Louis, MO, USA) was carefully diluted with 10 mL of ethanol, and then diluted with 190 mL of propylene glycol to produce a D_3 stock solution with a concentration of 1 mg/mL. This solution was then added to the basal diet as a liquid top-dressing at either 5 µg D_3/kg of diet (200 IU/kg diet) to create treatment 1 or 40 µg D_3/kg diet to create treatment 2. In a completely-randomized 2 x 2 factorial arrangement, d-of-hatch, mixed-sex chicks from each TD line (Auburn University, Auburn, AL) were wing-banded, weighed, and randomly allotted to each of the two dietary treatments. Each treatment was replicated six times, and each replicate pen contained four birds. Throughout the experiment, chicks were given free access to water and mash-feed, brooded in thermostatically controlled Petersime (Petersime Incubator Co., Gettysburg, OH, USA) starter batteries with raised wire floors, and reared on a 24-h lighting schedule. The starter batteries were kept in an environmentally controlled room (22°C), where fluorescent lights outside and inside

the batteries were covered with Arm-a-Lite® plastic sleeves (Arm-a-Lite® Thermoplastic Processes, Sterling, NJ, USA) to eliminate ultraviolet light exposure and any possible de novo vitamin D_3 synthesis by the chick (10, 20, 22).

On d-4 and 8 of Experiment 1, two randomly selected chicks from each pen were killed by cervical dislocation. The proximal duodenum from each bird was immediately excised to obtain epithelial cells for future calbindin-28 kd mRNA and vitamin D receptor mRNA Northern blot analyses and calbindin-28 kd Western blot analysis. The Institutional Animal Care and Use Committee of the University of Georgia approved all animal procedures.

Experiment 2. This experiment was conducted to determine if differences in calbindin-28 kd mRNA expression existed between the LTD and HTD broiler lines at the time of hatch. Intestinal scrapings from 24 chicks per strain were collected from the LTD and HTD strains as well as from a Ross 308 commercial broiler strain (control). Intestinal cells were pooled from 2 birds of each line (n=12) for RNA extraction and future Northern blot analysis for calbindin-28 kd and the vitamin D receptor.

Experiment 3. This experiment was done to determine if chicks from the LTD and HTD lines differed in their plasma thyroid hormone concentrations. The experimental design for this experiment was very similar to Experiment 1, except that the birds were maintained on the dietary treatments for 18 days. Furthermore, in addition to utilizing chicks from the LTD and HTD lines, chicks from a commercial broiler strain (Ross 308) were used as a control. Each treatment contained 4 pens

of 6 birds per pen in a completely randomized 3 x 2 factorial arrangement. On d-18, chicks were weighed and a heparinized blood sample was obtained by cardiac puncture from each bird. Chicks were then killed by cervical dislocation. Intestinal scrapings from the proximal duodenum of two randomly selected chicks from each pen of the LTD and HTD lines were collected and pooled for RNA extraction. After blood and tissue samples were taken, the right tibia of each chick was then scored for the incidence and severity of cholecalciferol-dependent rickets (23) and TD (5), while the left tibia of each chick was banded for identification and removed for future percent tibia ash (TA) determination. TA was determined on a dry, fat-free basis (24).

Plasma Analysis and Radioimmunoassays. Heparinized blood samples were centrifuged for ten minutes at 3,000 x g. Plasma from each sample was separated into two volumes. One volume was used for an immediate determination of ionized plasma calcium (Section N-31, Technicon Autoanalyzer Methodology, Technicon Corp., Tarrytown, NY.). The other volume was frozen at -20°C for future thyroid hormone assays.

Plasma concentrations of T_4, T_3, and free-T_3 were determined by RIA using kits from ICN Pharmaceuticals, Diagnostic Division (Costa Mesa, CA). The RIA were performed following the manufacturers instructions. The R^2 value for each of the standard curves was greater than 0.99.

SDS-PAGE and Western Immunoblot analysis. The intestinal cells scraped from the proximal portion of the duodenum were briefly sonicated in a lysis buffer

containing protease inhibitors (25). The cell lysate was centrifuged at 15,000 x g for 15 minutes at 4°C, and the supernatant fraction was recovered. For each sample, 40 µg of cell lysate protein was electrophoretically separated using a 12.5% sodium dodecyl sulfate polyacrylamide gel and electrophoretically blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA) using procedures previously described (25).

Immunostaining was performed using procedures previously described (25). The PVDF membranes were incubated at room temperature for two hours with a 1:1,250 dilution of mouse, anti-chicken calbindin-28 kd monoclonal antibody (Sigma Chemical Co., St. Louis, MO). Following a washing step, the membranes were incubated at room temperature for 1.5 hours with a 1:8,000 dilution of goat, anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO). Following a washing step, the membranes were treated with a fluorogenic substrate (CDP-Star, Applied Biosystems, Bedford, MA 01730, USA) and autoradiography was used to visualize calbindin-28 kd expression.

The relative level of calbindin-28 kd expression was determined densitometrically for the samples of each blot by calculating the signal intensity for each sample relative to the strongest calbindin-28 kd signal which was assigned a value of 1. The densitometry procedures followed those previously described (26). To normalize protein loading and transfer, the membranes were stripped after the calbindin-28 kd immunostaining and then immunostained with a goat, anti-Sea Urchin monoclonal "-tubulin antibody (Sigma Chemical Co., St. Louis, MO). The

membranes were stripped at 50°C for 30 min with gentle agitation in a buffer containing 0.1 mol/L 2-meraptoethanol, 0.07 mol/L sodium dodecyl sulphate and 0.0625 mol/L Tris-HCl pH 6.7.

RNA Extraction and Northern Blot Analyses. Total RNA was extracted from intestinal cells collected from two birds for each sample using a guanidine isothiocynate/phenol-chloroform method (27). Total RNA (25 µg/sample) was run on an agarose/formaldehyde gel and then transferred to a nylon membrane as previously described (26). Chicken calbindin-28 kd (28), vitamin D receptor [a generous gift from Dr. H. DeLuca, University of Wisconsin-Madison, (29)] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (26) cDNA clones were prepared and labeled with ^{32}P for Northern blot analysis as previously described (26). The order of the hybridizations was calbindin-28 kd, GAPDH and the vitamin D receptor. Blots were stripped of the previously hybridized probe before being hybridized with the subsequent probe as previously described (30). Hybridization and densitometry procedures also followed those described previously (26). For each experimental duration, there were two Northern blots with the replicate samples from each treatment split evenly between the two blots. The two blots were hybridized at the same time and exposed to the same film. Relative mRNA expression for calbindin-28 kd or vitamin D receptor was determined by densitometric scanning as described for the quantification of calbindin-28 kd protein. Before calculation of relative calbindin-28 kd and vitamin D receptor mRNA levels, GAPDH mRNA expression was used to correct the calbindin-28 kd and vitamin D receptor values for equality of RNA loading and transfer for each blot.

Statistical Analyses. Analysis of variance for the three experiments was completed using the General Linear Model procedure of SAS (31). Tukey's multiple-comparison procedure (32) was used to detect significant differences among the diets and strain of birds for the Northern and Western Immunoblot data. Significant differences between pen means among the broiler strains for all other measured parameters were detected using Duncan's Multiple Range Test (33). Differences were considered significant when P-values were <0.05.

Results

Experiment 1. There were no differences in feed consumption and body weight gain for either the 4-d or 8-d experimental period (data not shown) between the TD strains at either dietary level of D_3 supplementation .

When 5 µg of D_3 was supplemented to the diet, LTD chicks had a greater expression of calbindin-28 kd mRNA (Figures 1 and 2) and protein (Figures 3 and 4) than the HTD broiler chicks for both the 4 and 8-d experimental durations. Chicks fed 40 µg of D_3 had greater intestinal expression of calbindin-28 kd mRNA than those fed 5 µg of D_3. Intestinal expression of calbindin-28 kd protein, however was equal for chicks fed 40 µg of D_3, and the LTD chicks fed 5 µg of D_3 (Figure 3).

After quantification by densitometry and correction for RNA loading and transfer with GAPDH, the mean ± SEM relative vitamin D receptor mRNA values at 4-d were 0.83 ± 0.08 and 0.68 ± 0.05 for the LTD chicks and 0.73 ± 0.05 and 0.77 ± 0.06 for the HTD chicks fed 5 and 40 µg of D_3/kg diet, respectively. At 8-d, the relative vitamin D receptor mRNA values were 0.86 ± 0.03 and 0.81 ± 0.05 for the LTD chciks and 0.80 ± 0.04 and 0.91 ± 0.04 for the HTD chicks fed 5 and 40 µg of

D_3/kg diet, respectively. There were no differences in the relative expression of the mRNA for the vitamin D receptor between the LTD and HTD strains or between the two dietary levels of D_3 (Figure 2).

Experiment 2. After quantification by densitometry and correction for RNA loading and transfer with GAPDH, the mean ± SEM relative calbindin-28 kd mRNA values at day-of-hatch were 0.569 ± 0.08, 0.444 ± 0.09 and 0.458 ± 0.11 for the Ross 308 (control) LTD and HTD strains, respectively. The mean relative vitamin D receptor mRNA expression values were 0.68 ± 0.08, 0.91 ± 0.05 and 0.70 ± 0.07 for the Ross 308 (control) LTD and HTD strains, respectively. There were no differences in the mRNA expression of either calbindin-28 kd or the vitamin D receptor between the three strains of broilers .

Experiment 3. Compared to commercial broiler chicks, LTD and HTD broiler chicks consuming the basal diet supplemented with either 5 or 40 µg of D_3 had lower body weight gain values (Table 2). Plasma calcium concentrations were greater in chicks fed the basal diet that was supplemented with 40 µg of D_3 than in chicks fed the basal diet that was supplemented with 5 µg of D_3 (Table 2).

As expected, chicks from the HTD strain exhibited a higher incidence and severity of TD than the LTD chicks (Table 3). Furthermore, chicks fed a vitamin D_3 deficient diet (5 µg D_3/kg diet) had an increased incidence and severity of TD and D_3 rickets, and a lower percentage of tibia ash than chicks fed a diet adequate in vitamin D_3 (40 µg D_3/kg diet; Table 3).

The plasma concentrations of T_3 and free T_3 were not different for the control and HTD strains, but the concentrations of both forms of T_3 were significantly elevated in the LTD strain compared to the other two strains of birds, regardless of the dietary D_3 concentration (Table 4). Plasma concentrations of T_4 tended to decrease as the dietary supplementation of D_3 increased (Table 4).

The expression pattern of the mRNA for calbindin-28 kd and the vitamin D receptor between the LTD and HTD chicks in this experiment was the same as it was for Experiment 1 (data not shown).

Discussion

Since the mid 1990's, the LTD and HTD lines have been maintained as two separate flocks and no further genetic selection for TD incidence has occurred. Even though there has been an absence of genetic selection pressure for TD in these lines, they still exhibit a significant difference in the incidence and severity of TD while their relative growth rate remains equal. The HTD and LTD lines were originally selected from Ross genetic stock. The closest available genetic representative of this original stock is the Ross 308 broiler that was utilized in Experiments 2 and 3. Clearly, the LTD line no longer has a lower incidence or severity of TD compared to the Ross 308 line and both the LTD and HTD broilers grow at a slower rate than the Ross 308 broilers.

When the LTD and HTD chicks were fed a deficient level of dietary vitamin D_3 (5 µg D_3/kg diet), the LTD chicks expressed a higher level of the calbindin-28 kd transcript at 4, 8 and 18 days of age. This increased level of calbindin-28 kd expression in the LTD chicks was associated with a decrease in the severity of TD

at 18 days of age compared to the HTD birds. The differential response of the two genetic lines to dietary vitamin D_3 appears to be related to a developmental process, since the endogenous levels of calbindin expression in day-old chicks is similar between the two strains. It is conceivable that the higher calbindin-28 kd mRNA expression seen in the LTD chicks may be related to the elevated plasma levels of T_3 present in this strain.

Cross et al. (13) and Cross and Peterlik (14, 15) reported that although T_3 alone did not increase chick intestinal calcium absorption, when increasing levels of 1,25-$(OH)_2D_3$ were supplemented to chick intestinal segments alone or in the presence of a constant level of T_3, intestinal calcium absorption was always greater when T_3 was co-supplemented at each level of 1,25-$(OH)_2D_3$. In a subsequent study, Sechman et al. (16) reported that exogenous administration of T_3 alone had no effect on calbindin-28 kd expression, whereas the administration of 1,25-$(OH)_2D_3$ increased the in vivo expression of chick intestinal calbindin-28 kd protein. When T_3 and 1,25-$(OH)_2D_3$ were co-injected, Sechman et al. (16) observed a synergistic increase in intestinal calbindin-28 kd expression beyond what was observed for chicks solely injected with 1,25-$(OH)_2D_3$.

In the current study the significantly higher plasma levels of T_3 and free T_3 found in the LTD chicks compared to the HTD chicks, may account for the higher intestinal levels of calbindin-28 kd mRNA and protein expression detected in these chicks. It is hypothesized that the increased level of calbindin-28 kd expression detected in the LTD chicks protected them from TD when dietary vitamin D_3 was marginal. Since the HTD chicks had plasma T_3 and free- T_3 concentrations equivalent to those of the Ross 308 chicks, it is unlikely that T_3 is directly involved

in the higher incidence of TD observed in these birds. There were no differences in the intestinal mRNA expression of the vitamin D receptor between the LTD and HTD lines. Berry et al. (34) reported that commercial broiler chicks exhibiting TD had reduced levels of the vitamin D receptor in the hypertrophic chondrocytes of the tibia, and that the receptors had a lower affinity for 1,25-$(OH)_2D_3$. Additionally, levels of vitamin D receptor were found to be significantly lower in the duodenal mucosa of chicks with a high incidence of TD compared to chicks with a low incidence of TD (35). The lack of difference in vitamin D receptor mRNA expression in the LTD and HTD lines used in the current research agrees with a previous report which demonstrated that vitamin D receptor protein expression was not different between the pedigreed LTD and HTD lines (19).

In summary, when the LTD and HTD lines of broilers were initially established it is very likely that the LTD line was actually being selected for increased levels of plasma T_3 and free-T_3. Thus, the high levels of T_3 would enhance the expression of calbindin-28 kd and make the LTD broilers less susceptible to marginal dietary levels of vitamin D_3. The high levels of T_3 are also likely to have positive effects on the chondrocytes of the developing tibia. However, it is unclear why the HTD line of broilers still has an elevated incidence of TD even when they are fed a vitamin D adequate diet. The HTD birds do not seem to have an endogenous deficiency in plasma thyroid hormone levels or in the intestinal expression of vitamin D receptor and calbindin-28 kd. Further research is needed to determine why the incidence and severity of TD is so high in the HTD line of broilers since it could provide valuable insight into the etiology of this disease that has a significant monetary impact on the broiler industry.

Conclusions

Log-dose supplementation of phytase to 12,000 U/kg of diet liberated approximately 95% of the phytate P within P-deficient diets. This degradation resulted in BWG, G:F, tibia ash deposition and mineral retentions that were equivalent to broiler diets that had an adequate aP and no phytase. These data support the hypothesis that higher levels of phytase then previously employed are required to achieve maximal broiler performance.

The addition of 5 µg of 25-(OH)D_3 or 1 " -(OH)D_3 improves broiler performance and mineral utilizations when phytase was supplemented from 0 to 187.5 U/kg of diet. At each additional level of phytase supplementation, 25-(OH)D_3 had virtually no additional affect on broiler performance or mineral retention data. The addition of 1 " -(OH)D_3 to diets containing more than 3,000 U of phytase resulted in a depression in BWG and FI, even though improvements in phytate disappearance and mineral retention values were comparable to diets that contained only phytase. The depression in broiler performance in the presence of higher mineral retentions, especially Ca, suggests that 1 " -(OH)D_3 increased the absorption of dietary Ca and P to the point that the chicks become anorexic.

It is well documented that dietary Ca is transported actively through the influence of vitamin D_3 metabolites, and passively as a function of intestinal concentration. In addition Ca can precipitate phytate and form insoluble Ca-P salts with ionic P within the gastrointestinal tract. Based on two experiments, the graded addition of dietary Ca depressed broiler performance, phytate degradation and the retention of minerals. Increasing the level of phytase to 1,500, 3,000 and 6,000 U/kg of diet reduced the negative effects of high Ca on the later parameters,

indicating that high levels of phytase may degrade sufficient amounts of phytate to overcome the potential Ca-phytate interactions that otherwise lead to insoluble Ca-phytate and severe P-deficiency when the level of dietary Ca is high.

Among the three phytase studies, the affect of phytase on the utilization of dietary N and AME was extremely variable. Factors such as particle size, source of soybean meal or corn, or the initial nutritional status of the broiler chick may have affected either parameter.

The expression of calbindin-28 kd mRNA and preotein was higher in LTD chicks that consumed 5 µg of dietary cholecalciferol. Increasing the level of dietary cholecalciferol to 40 µg/kg of diet, equalized the expression of calbindin mRNA and protein between the two broiler strains. Despite an increase in calbindin-28 kd expression, the HTD chicks retained a high incidence of TD. In addition, the LTD strain had a higher plasma T_3 and free-T_3 levels than the HTD broiler strain. Since T_3 enhances calbindin expression in the presence of low 1,25-$(OH)_2D_3$, the higher calbindin-28 kd expression was mediated by T_3 when 5 µg of cholecalciferol was supplemented. Since the increase in calbindin-28 kd expression at 40 µg of cholecalciferol supplementation did not reduce the incidence of TD in the HTD broiler strain, it is likely that the intestinal expression of calbindin-28 kd is not linked to the development of TD.

Acknowledgments

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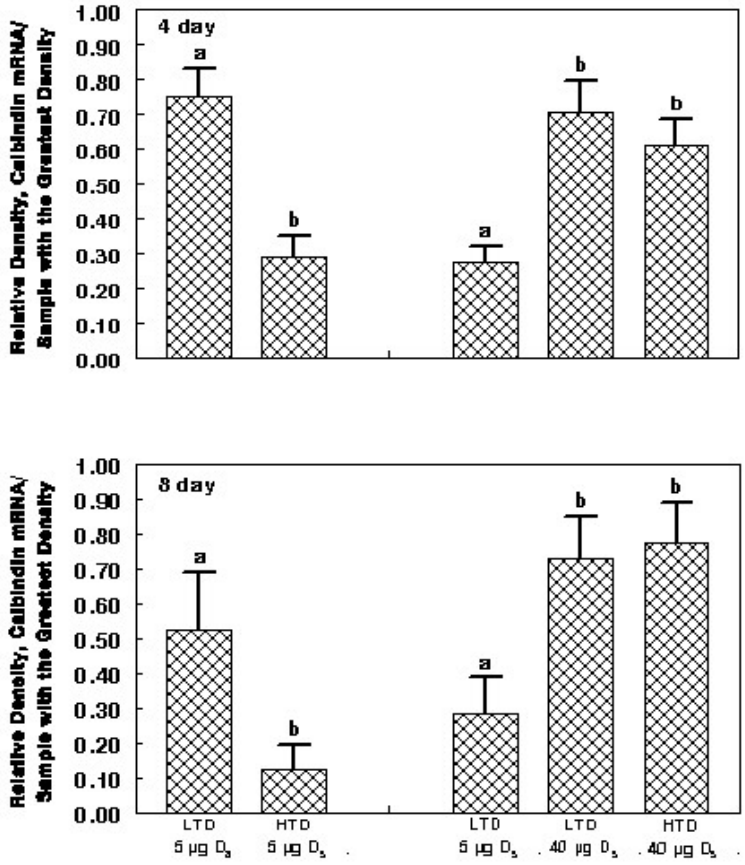
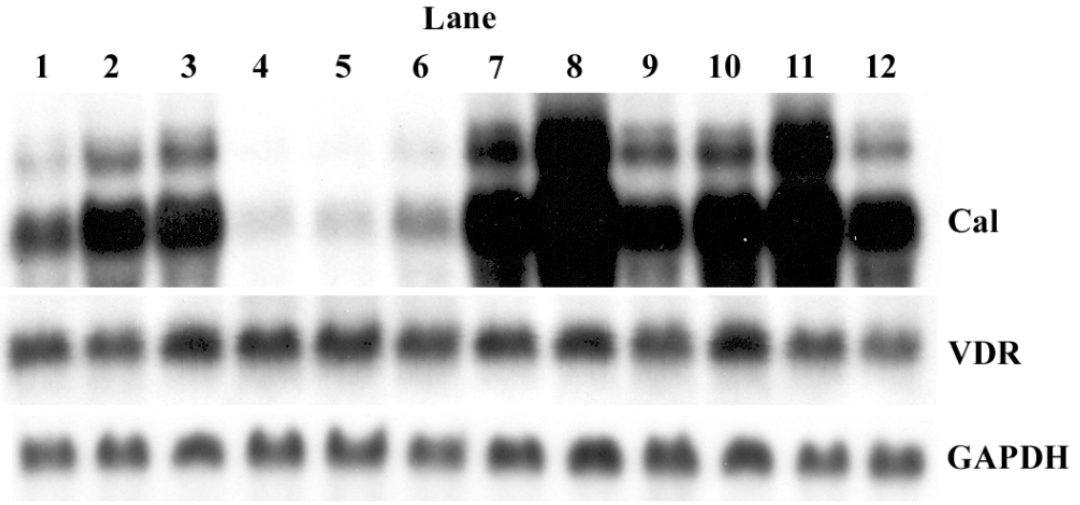
Tables and Figures

FIGURE 5.1.



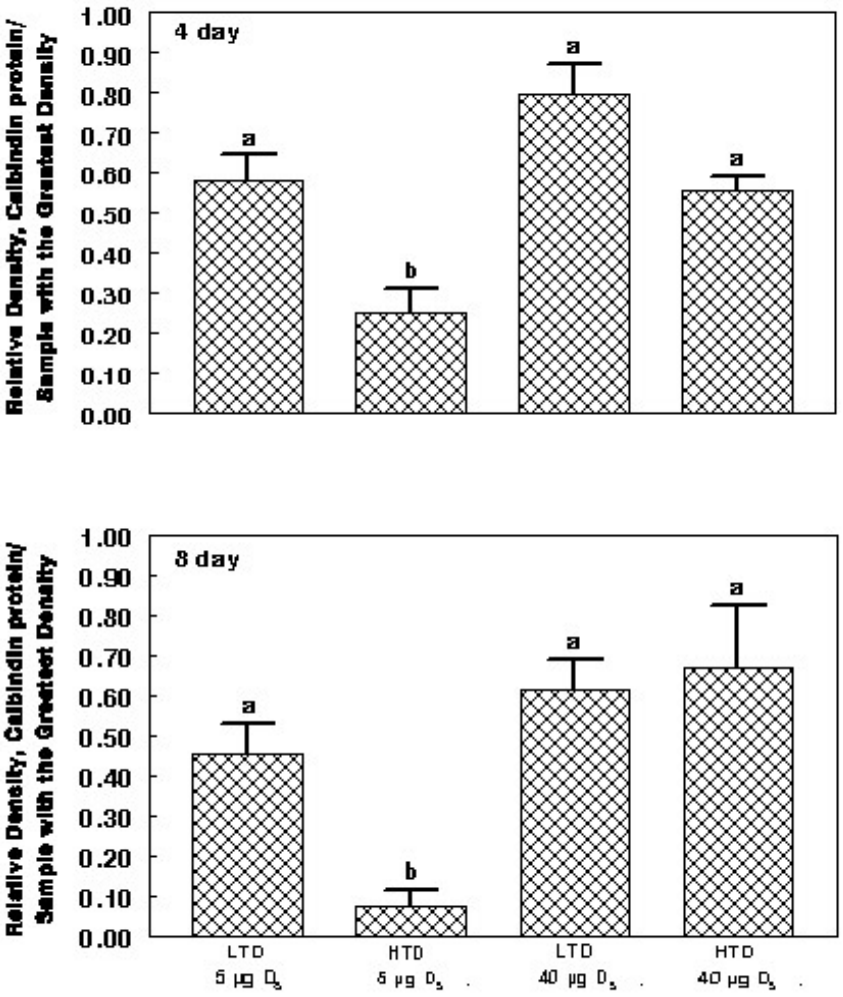
The relative density of intestinal calbindin-28 kd mRNA in LTD and HTD genetic lines of broilers fed either 5 or 40 µg D_3/kg diet for the 4 and 8 day experimental durations (Experiment 1). Values are means ± SEM, n = 6 replicate pens. In order to accurately quantitate the density of the samples from the chicks fed the 40µg D_3/kg diet, film exposure had to be 24 h or less, however for the samples from the HTD chicks fed 5µg D_3/kg diet to be visible the film exposure had to be 48 hours or greater. Therefore, the mRNA expression of calbindin-28 kd in the HTD chicks fed the 5µg D_3/kg diet could only be calculated relative to the LTD chicks fed the same level of D_3. Means with different letters in section of the 4 and 8 d figures differ, $P < 0.05$.

FIGURE 5.2.



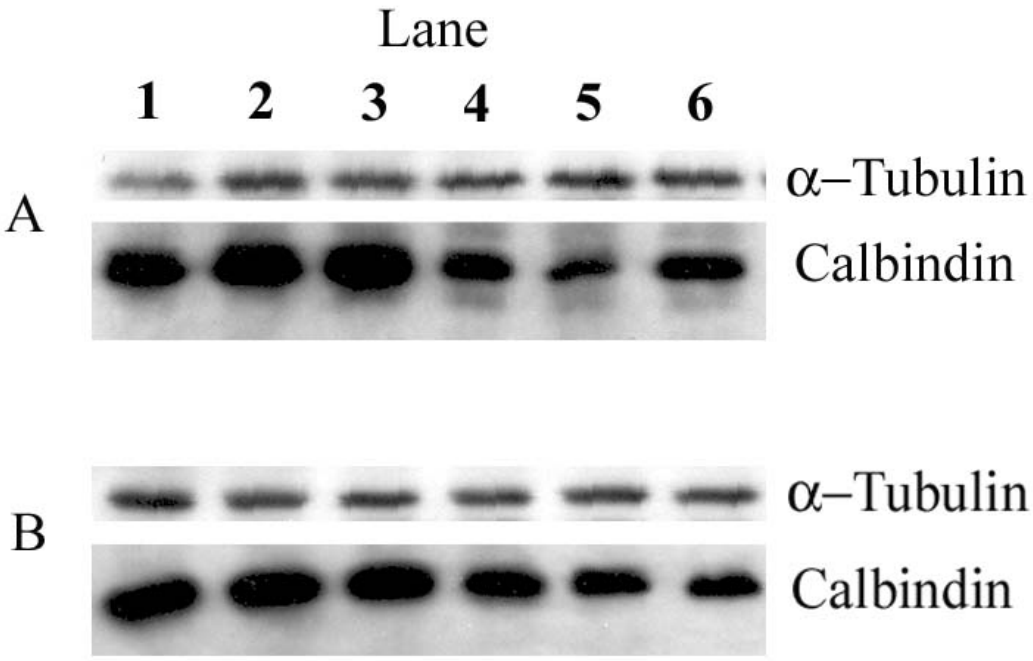
Autoradiograms from the Nothern analysis of calbindin-28 kd and vitamin D receptor, showing 3 of the 6 replicate samples from each genetic line of broilers given both dietary treatments for the 4 d experimental duration (Experiment 1). Total RNA (40 µg) was loaded for each sample. Samples obtained from the LTD and HTD genetic lines of broilers fed 5µg D_3/kg diet are in lanes 1-3 and 4-6, respectively while samples obtained from the LTD and HTD broilers fed 40 µg D_3/kg diet are in lanes 7-9 and 10-12, respectively. The film exposure times were 50, 22, and 3 h for the calbindin-28 kd, vitamin D receptor and GAPDH Northern blots, respectively. Abbreviations: cal, calbindin-28 kd; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VDR, vitamin D receptor.

FIGURE 5.3.



The relative density of intestinal calbindin-28 kd protein in LTD and HTD genetic lines of broilers fed either 5 or 40 µg D_3/kg diet for the 4 and 8 day experimental durations (Experiment 1). Values are means ± SEM, n = 6 replicate pens. Means with different letters in section of the 4 and 8 d figures differ, $P < 0.05$.

FIGURE 5.4.



Autoradiograms from Western analysis of calbindin-28 kd, showing 3 of the 6 replicate samples from each genetic line of broilers and dietary treatment for the 4 d experimental duration (Experiment 1). For each sample 40 µg of protein was loaded. Samples obtained from the LTD and HTD genetic lines of broilers fed 5µg D_3/kg diet are in lanes 1-3 and 4-6 of panel A, respectively while samples obtained from the LTD and HTD broilers fed 40 µg D_3/kg diet are in lanes 1-3 and 4-6 of panel B, respectively.

TABLE 5.1. Composition of the Basal Diet for Experiments 2 and 3[1]

Ingredients	Amount
	(%)
Corn (9.05% CP)	56.200
Soybean meal (47.99% CP)	36.817
Soybean oil (8.8 kcal/g)	3.420
Limestone	1.093
Dicalcium phosphate	1.700
Salt (NaCl)	0.450
DL-Methionine	0.186
Vitamin mix[2]	0.0548
Trace mineral mix[3]	0.0800
Total	100.00

[1]All diets contained a calculated 13.08 MJ/kg diet, 22.74% CP, 0.90% calcium, and 0.45 available phosphorus (0.70% total phosphorus). Cholecalciferol was added as a top-dressing to the basal diet at either 5 µg/kg diet (200 IU/kg diet) or 40 µg/kg diet (1,600 IU/kg diet).

[2]Vitamin mix provided the following (per kilogram of diet): thiamin•mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 8.8 mg (source concentration, 50%); D-calcium pantothenate, 12 mg; vitamin B_{12} (*cobalamin*) (source concentration, 0.1%), 12.1 ug; pyridoxine•HCl, 2.7 mg; D-biotin, 0.11 mg (source concentration, 2%); folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.3 mg; choline chloride, 308.6 mg (source concentration, 60%); trans-retinyl acetate, 18.4 mg (source concentration, 300 IU/mg); all-rac-tocopherol acetate, 22.0 mg (source concentration, 0.5 IU/mg); ethoxyquin, 125 mg.

[3]Trace mineral mix provides the following (per kilogram of diet): manganese ($MnSO_4•H_2O$), 60 mg; iron ($FeSO_4•7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4•5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg.

TABLE 5.2. Performance Data of Commercial, LTD, and HTD Broiler Chicks Given Two Levels of Vitamin D_3 (Cholecalciferol), Experiment 3[1,2]

Broiler Strain	Vitamin D_3	BWG	FI	G:F	Plasma Ca
	(µg/kg diet)	----- g/chick ----			mg/100 mL
Control	5	446[a]	621[a]	0.716[a]	10.7[ab]
Low TD		334[bc]	477[b]	0.700[ab]	10.5[b]
High TD		311[bc]	477[b]	0.652[c]	10.5[b]
Control	40	405[ab]	591[a]	0.683[abc]	11.1[a]
Low TD		330[c]	495[b]	0.667[bc]	11.0[a]
High TD		354[c]	548[ab]	0.647[c]	10.9[ab]
	PSEM[3]	13.4	12.1	0.0069	0.09
Main effect means Vitamin D_3 (µg/kg diet)					
5		364	525	0.689[a]	10.6[b]
40		363	545	0.666[b]	11.0[a]
	PSEM	16.5	20.9	0.0085	0.11
Broiler Strain					
Control		426[a]	607[a]	0.700[a]	10.9
Low TD		332[b]	486[b]	0.684[a]	10.8
High TD		333[b]	512[b]	0.650[b]	10.7
	PSEM	23.3	29.6	0.0120	0.154
ANOVA					
R^2		0.58	0.55	0.59	0.46
Source of variation	df	---------- Probability > F ----------			
Treatment	3	0.0052	0.0087	0.0039	0.0338
Broiler Strain	1	0.0009	0.0018	0.0018	0.5413
Vitamin D_3	1	0.9886	0.4259	0.0247	0.0015

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Broiler Strain x Vitamin D_3	1	0.2241	0.2576	0.4236	0.8568

[1]Average initial chick weights (g/chick): Control (45.7 ± 2.1), LTD (35.2 ± 1.4), and HTD (34.4 ± 1.4).
[2]All diets contained a calculated 13.08 MJ/kg diet, 22.74% CP, 0.90% calcium, and 0.45 available phosphorus (0.70% total phosphorus). Vitamin D_3 was added as a top-dressing to the basal diet at either 5 µg/kg diet (200 IU/kg diet) or 40 µg/kg diet (1,600 IU/kg diet).
[3]PSEM = the calculated pooled standard error of the mean.

TABLE 5.3. Tibia Data for Commercial, LTD, and HTD Broiler Chicks Given Two Levels of Vitamin D_3 (Cholecalciferol), Experiment 3[1]

Broiler Strain	Vitamin D_3 (µg/kg diet)	Score		Incidence		Ash
		TD	D_3 Rickets	TD	D_3 Rickets	
				(%)		
Control	5	0.282[c]	0.558[ab]	23.3[bc]	35.0[ab]	39.2[bc]
Low TD		1.000[b]	0.300[abc]	45.0[ab]	15.0[bc]	38.4[cd]
High TD		2.082[a]	0.750[a]	73.3[a]	44.2[a]	37.3[d]
Control	40	0.000[c]	0.050[c]	0.0[c]	5.0[c]	41.6[a]
Low TD		0.082[c]	0.000[c]	8.3[c]	0.0[c]	40.9[ab]
High TD		1.200[b]	0.200[bc]	50.0[ab]	10.0[c]	40.9[ab]
	PSEM[2]	0.1157	0.0840	5.97	4.00	0.58
Main effect means						
Vitamin D_3 (µg/kg diet)						
5		1.122[a]	0.536[a]	47.2[a]	31.4[a]	38.3[b]
40		0.428[b]	0.083[b]	19.4[b]	5.0[b]	41.1[a]
	PSEM	0.1417	0.1029	7.31	4.90	0.34
Broiler Strain						

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Control			0.141[b]	0.304[ab]	11.7[b]	20.0[ab]	40.4[a]
Low TD			0.541[b]	0.150[a]	26.7[b]	7.5[b]	39.6[ab]
High TD			1.641[a]	0.475[b]	61.7[a]	27.1[a]	39.1[b]
	PSEM		0.2000	0.1455	10.33	6.94	0.41
ANOVA							
R^2			0.82	0.53	0.67	0.64	0.70
Source of variation		df	---------- Probability > F ----------				
Treatment		3	0.0001	0.0120	0.0007	0.0014	0.0003
Broiler Strain		1	0.0001	0.1103	0.0004	0.0344	0.1060
Vitamin D_3		1	0.0005	0.0013	0.0041	0.0002	0.0001
Broiler Strain x Vitamin D_3		1	0.2319	0.6615	0.7608	0.3683	0.5813

[1]All diets contained a calculated 13.08 MJ/kg diet, 22.74% CP, 0.90% calcium, and 0.45 available phosphorus (0.70% total phosphorus). Cholecalciferol was added as a top-dressing to the basal diet at either 5 µg/kg diet (200 IU/kg diet) or 40 µg/kg diet (1,600 IU/kg diet).

[2]PSEM = the calculated pooled standard error of the mean for phytase supplemented diets.

TABLE 5.4. Thyroid Hormone Data for 16-d Ross 308, LTD, and HTD Broiler Chicks Given Two Levels of Vitamin D_3 (Cholecalciferol), Experiment 3[1]

Broiler Strain	Vitamin D_3 (µg/kg diet)		Thyroid Hormones		
			T4	T3	Free T3
			ug/dL	ng/dL	pg/mL
Control	5		2.22[a]	172.8[b]	8.03[c]
Low TD			2.07[a]	265.3[a]	10.73[a]
High TD			2.01[ab]	173.9[b]	8.12[c]
Control	40		2.13[a]	168.7[b]	7.61[c]
Low TD			1.80[b]	226.9[a]	9.42[b]
High TD			1.82[b]	176.1[b]	8.10[c]
	PSEM[2]		0.069	13.76	0.405
Main effect means Cholecalciferol (µg/kg diet)					
5			2.10[a]	204.0	8.96
40			1.92[b]	190.6	8.38
	PSEM		0.040	7.94	0.234
Broiler Strain					
Control			2.18[a]	170.7[b]	7.82[b]
Low TD			1.94[b]	246.1[a]	10.08[a]
High TD			1.91[b]	175.0[b]	8.11[b]
	PSEM		0.049	9.73	0.286
ANOVA					
R^2			0.62	0.70	0.70
Source of variation		df	---------- Probability > F ----------		
Treatment		3	0.0023	0.0003	0.0004
Broiler Strain		1	0.0023	0.0001	0.0001

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Vitamin D_3	1	0.0050	0.0930	0.2475
Broiler Strain x Vitamin D_3	1	0.4954	0.2932	0.3063

[1]All diets contained a calculated 13.08 MJ/kg diet, 22.74% CP, 0.90% calcium, and 0.45 available phosphorus (0.70% total phosphorus). Cholecalciferol was added as a top-dressing to the basal diet at either 5 µg/kg diet (200 IU/kg diet) or 40 µg/kg diet (1,600 IU/kg diet).
[2]PSEM = the calculated pooled standard error of the mean for phytase supplemented diets.

APPENDIX TABLES

APPENDIX 1

APPAPPENDIX TABLE 1.1. Composition of the Basal Diet (B8-01-TD)[1,2]

Ingredient	Amount
	(%)
Corn (8.8% protein)	52.729
Soybean meal (48.5% protein)	36.564
Poultry fat (8.2 kCal/kg)	5.333
Salt (NaCl)	0.450
Trace mineral mix[3]	0.080
Vitamin mix[4]	0.250
DL-Methionine	0.191
Cr_2O_3	0.100
Limestone	2.103
Dicalcium phosphate	0.411
Various[5]	2.240
Total	100.00

[1]Dietary analyses were [in percent]: 91.03 ± 0.003 (DM); 24.01 ± 0.471 (CP); 0.934 ± 0.077 (Ca); 0.413 ± 0.017 (P); 0.340 ± 0.0121 (phytate P), and a calculated ME of 3,123 kcal/kg diet.

[2]Sand was used as an inert ingredient, removed from the respective diets as either Natuphos™ phytase or Natugrain ™ (1,650 U/g xylanase and 1,200 U/g $-glucanase) was added.

[3]Trace mineral mix provides the following (per kilogram of diet): manganese ($MnSO_4$•H_2O), 60 mg; iron ($FeSO_4$•$7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4$•$5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg.

[4]Vitamin mix provided the following (per kilogram of diet):
thiamin•mononitrate (B_1), 1.1 mg; nicotinic acid, 44 mg; riboflavin (B_2), 4.5 mg; D-Ca pantothenate, 12 mg; vitamin B_{12} (*cobalamin*), 6.6 ug; pyridoxine•HCl (B_6), 2.2 mg; D-biotin, 0.11 mg; folic acid, .55 mg; menadione sodium bisulfate complex, 1.1 mg; ascorbic acid, 250 mg, choline cholride, 220 mg; cholecalciferol, 1,100 IU; trans-retinyl acetate, 5,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 125 mg.

[5]The ingredients listed under the heading “Various” represent the sand that was used as an inert ingredient, and subsequently removed from the respective diets as dicalcium phosphate, calcium, or phytase was added.

APPENDIX TABLE 1.2. The Effect of Phytase and Natugrain on 16-d Broiler Performance (B8-01-TD)[1, 2]

Phytase	Natugrain	BWT	FI	Gain:feed	Mortality
(U/kg diet)	(%)	(g)	(g)	(g:g)	---- % ----
0	0.00	210[c]	294[c]	0.714[c]	33[a]
0	0.10	191[c]	257[c]	0.748[c]	30[a]
0	2.00	196[c]	240[c]	0.817[ab]	41[a]
1,500	0.00	342[b]	431[b]	0.793[b]	0[b]
1,500	0.10	353[b]	441[ab]	0.801[b]	0[b]
1,500	2.00	363[b]	450[ab]	0.807[b]	0[b]
12,000	0.00	406[a]	495[a]	0.821[ab]	0[b]
12,000	0.10	414[a]	500[a]	0.828[ab]	0[b]
12,000	2.00	413[a]	489[ab]	0.845[a]	0[b]
	PSEM[3]	13.9	19.2	0.0115	8.2
Main effect means					
Phytase (U/kg diet)					
0		199[c]	264[c]	0.760[c]	34[a]
1,500		353[b]	441[b]	0.800[b]	0[b]
12,000		411[a]	495[a]	0.831[a]	0[b]
Natugrain (%)					
0		319	407	0.776[b]	11
1		319	399	0.792[b]	10
2		324	393	0.823[a]	14
	PSEM[3]	8.0	11.1	0.0066	4.7
ANOVA					
Source of variation	df	---------- Probability ≥ F ----------			
Treatment	8	0.0001	0.0001	0.0001	0.0035
Phytase Level	2	0.0001	0.0001	0.0001	0.0001
Naytugrain level	2	0.8941	0.6823	0.0003	0.8541
Phytase*Naytugrain	4	0.7126	0.4349	0.0095	0.9563

[1]Dietary analyses were [in percent]: 91.03 ± 0.003 (DM); 24.01 ± 0.471 (CP); 0.934 ± 0.077 (Ca); 0.413 ± 0.017 (P); 0.340 ± 0.0121 (phytate P), and a calculated ME of 3,123 kcal/kg diet.
[2]Sand was used as an inert ingredient, removed from the respective diets as either Natuphos™ phytase or Natugrain ™ (1,650 U/g xylanase and 1,200 U/g $-glucanase) was added.
[3]PSEM = the calculated pooled standard error of the mean (o(MSE ÷ N)).
[4]Means that differ within a column significantly differ (P # 0.05).

APPENDIX TABLE 1.3. The Effect of Phytase and Natugrain on 16-d Plasma P, Tibia and Acites Data (B8-01-TD)[1, 2]

			Score			Incidence				
Phytase	Natugrain	Plasma P	P Rickets	TD	Water belly	P Rickets	TD	Water belly	Tibia Ash	Tibia Ash Weight
(U/kg diet)	(%)	(%)				----------	%	----------		(g/tibia)
0	0.00	1.78^{cd}	2.7^{ab}	0.29	0.64^{b}	91^{a}	9.5	21.4^{bc}	24^{d}	0.164^{c}
0	0.10	2.09^{cd}	1.9^{bc}	0.20	0.57^{b}	88^{a}	6.7	35.5^{b}	26^{c}	0.175^{c}
0	2.00	1.61^{d}	3.0^{a}	0.25	1.97^{a}	100^{a}	8.3	65.5^{a}	27^{c}	0.172^{c}
1,500	0.00	2.62^{c}	1.5^{c}	0.19	0.11^{b}	63^{b}	7.5	3.7^{c}	33^{b}	0.337^{b}
1,500	0.10	2.56^{cd}	1.2^{c}	0.15	0.00^{b}	56^{b}	7.5	0.0^{c}	32^{b}	0.340^{b}
1,500	2.00	2.11^{cd}	1.1^{c}	0.08	0.00^{b}	51^{b}	7.9	0.0^{c}	33^{b}	0.374^{b}
12,000	0.00	4.50^{b}	0.0^{c}	0.37	0.00^{b}	4^{c}	14.8	0.0^{c}	39^{a}	0.520^{a}
12,000	0.10	4.08^{b}	0.0^{c}	0.11	0.00^{b}	0^{c}	3.7	0.0^{c}	39^{a}	0.525^{a}
12,000	2.00	5.38^{a}	0.0^{c}	0.33	0.00^{b}	4^{c}	11.1	0.0^{c}	40^{a}	0.546^{a}
	PSEM[3]	0.292	0.288	0.169	0.207	8.30	5.93	6.61	0.7	0.0141
Main effect means										
Phytase (U/kg diet)										
0		1.83^{c}	2.5^{a}	0.25	1.06	93^{a}	8.2	40.8	26^{c}	0.170^{c}
1,500		2.43^{b}	1.2^{b}	0.14	0.04	57^{b}	7.6	1.2	33^{b}	0.350^{b}
12,000		4.65^{a}	0.0^{c}	0.27	0.00	3^{c}	9.9	0.0	40^{a}	0.531^{a}
Natugrain (%)										
0		2.97	1.4	0.28	0.251^{b}	52	10.6	8.4^{b}	32	0.340
1		2.91	1.0	0.15	0.189^{b}	52	5.9	11.9^{ab}	32	0.346
2		3.03	1.4	0.22	0.655^{a}	48	9.1	21.8^{a}	33	0.364
	PSEM[3]	0.168	0.17	0.097	0.1198	4.8	3.42	3.82	0.4	0.0141

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ANOVA

Source of variation[4]	df	Probability ≥ F								
Treatment	8	0.0001	0.0001	0.9342	0.0001	0.0001	0.9666	0.0001	0.0001	0.0001
Phytase Level	2	0.0001	0.0001	0.5949	0.0001	0.0001	0.8852	0.0001	0.0001	0.0001
Naytugrain level	2	0.8790	0.2090	0.6578	0.0267	0.7673	0.6245	0.0582	0.0523	0.1302
Phytase*Naytugrain	4	0.0335	0.2976	0.9263	0.0069	0.7726	0.9095	0.0146	0.2095	0.7394

[1]Dietary analyses were [in percent]: 91.03 ± 0.003 (DM); 24.01 ± 0.471 (CP); 0.934 ± 0.077 (Ca); 0.413 ± 0.017 (P); 0.340 ± 0.0121 (phytate P), and a calculated ME of 3,123 kcal/kg diet.

[2]Sand was used as an inert ingredient, removed from the respective diets as either Natuphos™ phytase or Natugrain ™ (1,650 U/g xylanase and 1,200 U/g $-glucanase) was added.

[3]PSEM = the calculated pooled standard error of the mean (o(MSE ÷ N)).

[4]Means that differ within a column significantly differ (P # 0.05).

APPENDIX TABLE 1.4. The Effect of Phytase and Natugrain on 16-d Nutrient Utilization Data (B8-01-TD)[1, 2]

Phytase	Natugrain	Retentions Ca	Retentions P	Ca	P	Phytate Disappearance	Ca:P	AME
(U/kg diet)	(%)	%						kcal/kg
0	0.00	39	53^{c}	0.400	0.241^{c}	53^{c}	1.66^{ab}	3514^{ab}
0	0.10	45	54^{c}	0.466	0.246^{c}	59^{c}	1.91^{a}	3524^{ab}
0	2.00	39	58^{c}	0.399	0.261^{c}	57^{c}	1.56^{ab}	3536^{ab}
1,500	0.00	44	66^{b}	0.452	0.298^{b}	72^{b}	1.52^{ab}	3497^{b}
1,500	0.10	46	67^{b}	0.474	0.301^{b}	74^{b}	1.57^{ab}	3497^{b}
1,500	2.00	42	65^{b}	0.429	0.295^{b}	70^{b}	1.46^{b}	3572^{a}
12,000	0.00	47	82^{a}	0.477	0.370^{a}	92^{a}	1.29^{b}	3531^{ab}
12,000	0.10	46	80^{a}	0.476	0.363^{a}	91^{a}	1.31^{b}	3562^{ab}
12,000	2.00	46	84^{a}	0.471	0.379^{a}	91^{a}	1.24^{b}	3552^{ab}
	PSEM[3]	2.9	2.0	0.0302	0.0089	2.2	0.133	21.2
Main effect means								
Phytase (U/kg diet)								
0		41	55^{c}	0.422	0.250^{c}	56^{c}	1.71^{a}	3524
1,500		44	66^{b}	0.452	0.298^{b}	72^{b}	1.52^{a}	3521
12,000		46	82^{a}	0.474	0.371^{a}	91^{a}	1.28^{b}	3549
Natugrain (%)								
0		43	66	0.443	0.303	72	1.5	3514
1		46	67	0.472	0.303	75	1.6	3527
2		42	69	0.433	0.312	73	1.4	3553
	PSEM[3]	1.7	1.1	0.0175	0.0051	1.3	0.077	12.3

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ANOVA

Source of variation[4]	df	Probability ≥ F						
Treatment	8	0.3896	0.0001	0.3949	0.0001	0.0001	0.0501	0.1924
Phytase Level	2	0.1276	0.0001	0.1303	0.0001	0.0001	0.0034	0.2633
Naytugrain level	2	0.2815	0.4161	0.2823	0.4163	0.4392	0.2873	0.1007
Phytase*Naytugrain	4	0.7886	0.5963	0.7913	0.5941	0.6181	0.8234	0.3520

[1]Dietary analyses were [in percent]: 91.03 ± 0.003 (DM); 24.01 ± 0.471 (CP); 0.934 ± 0.077 (Ca); 0.413 ± 0.017 (P); 0.340 ± 0.0121 (phytate P), and a calculated ME of 3,123 kcal/kg diet.

[2]Sand was used as an inert ingredient, removed from the respective diets as either Natuphos™ phytase or Natugrain ™ (1,650 U/g xylanase and 1,200 U/g $-glucanase) was added.

[3]PSEM = the calculated pooled standard error of the mean (o(MSE ÷ N)).

[4]Means that differ within a column significantly differ (P # 0.05).

APPENDIX 2

TABLE 2.1. Composition of Basal Diet (B4-02-TD)[1]

Ingredient	Amount
	(%)
Corn (8.8% protein)	56.160
Soybean meal (48.5% CP)	36.719
Soybean oil (8.8 kcal/kg)	3.413
Limestone	0.872
Salt (NaCl)	0.450
Dicalcium phosphate	0.379
Vitamin mix[2]	0.250
DL-Methionine	0.186
Cr_2O_3	0.100
Trace mineral mix[3]	0.080
Various[4]	1.391
Total	100.000

[1]On an as-is basis, all phytase-treated diets were analyzed to contain: 90.18% ± 0.129 (DM); 23.20% ± 0.353 (CP); 0.86% ± 0.015 (Ca); 0.44% ± 0.038 (P); 0.292% ± 0.018 (phytate P), and a calculated ME of 3,123 kcal/kg diet, while the P level of the positive control diet was 0.72.
[2]Vitamin mix provided the following (per kilogram of diet):
thiamin•mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B_{12} (*cobalamin*), 12.0 ug; pyridoxine•HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-retinyl acetate, 5,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.
[3]Trace mineral mix provided the following (per kilogram of diet): manganese ($MnSO_4•H_2O$), 60 mg; iron ($FeSO_4•7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4•5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg.
[4]Various refers to the sand that was used as an inert ingredient, and removed from the respective diets as either dicalcium phosphate or Natuphos phytase was supplemented.

TABLE 2.2. The effect of breed difference and high phytase levels on 16-d broiler performance (B4-02-TD)[1,2]

Natuphos phytase		BWT	FI	Gain: feed	Mortality
U/kg diet	Breed	-------- (g/chick) -------		(g:g)	----- % -----
0	Cobb	213[d]	308[c]	0.693[c]	27[a]
0	Ross	218[d]	321[c]	0.679[c]	10[b]
600	Cobb	300[c]	400[b]	0.751[ab]	2[b]
600	Ross	310[bc]	416[ab]	0.745[ab]	2[b]
1,200	Cobb	309[bc]	418[ab]	0.740[b]	4[b]
1,200	Ross	325[ab]	429[a]	0.757[ab]	2[b]
2,400	Cobb	326[ab]	428[a]	0.760[ab]	2[b]
2,400	Ross	336[a]	438[a]	0.766[a]	2[b]
(+) Control	Cobb	340	439	0.775	0
(+) Control	Ross	328	429	0.764	0
PSEM[3]		7.3	8.7	0.0069	3.4
Main effect means					
Phytase level					
0		215[c*]	314[c*]	0.686[c*]	18[a*]
600		305[b*]	408[b*]	0.748[b*]	2[b]
1,200		317[ab]	424[ab]	0.748[b*]	3[b]
2,400		331[a]	433[a]	0.763[a]	2[b]
PSEM		5.2	5.8	0.0049	2.4
MSD[4]		20.4	22.8	0.0187	7.8
Breed of chick					
Cobb		287	388[b]	0.736	9
Ross		297	401[a]	0.737	4
PSEM		3.7	4.1	0.0035	1.7
ANOVA					
Source of variation[5]	df	---------- Probability > F ----------			
R^2		0.93	0.91	0.86	0.66
Treatment	7	0.0001	0.0001	0.0001	0.0002
Phytase level	3	0.0001	0.0001	0.0001	0.0001
Breed[6]	1	0.0676	0.0476	0.9317	0.0554
Phytase*Breed	3	0.9146	0.9844	0.1600	0.0465
Regression analysis[7]		---------- Probability > t ----------			
R^2		0.86	0.87	0.75	0.53
Phytase level	1	0.0001	0.0001	0.0001	0.0002
Breed	1	-----	0.0727	-----	0.0146
Phytase*Breed	1	-----	-----	-----	0.0692
Phytase*phytase	1	0.0001	0.0001	0.0001	0.0032

Continued on the following page.

[1]Average, initial chick weight = 41.5 ± 1.02g/chick
[2]On an as-is basis, all phytase-treated diets were analyzed to contain: 90.18% ± 0.129 (DM); 23.20% ± 0.353 (CP); 0.86% ± 0.015 (Ca); 0.44% ± 0.038 (P); 0.292% ± 0.018 (phytate P), and a calculated ME of 3,123 kcal/kg diet, while the P level of the positive control diet was 0.72; sand was used as an inert ingredient, and removed from the respective diets as either dicalcium phosphate or Natuphos phytase was supplemented.
[3]PSEM = the calculated pooled standard error of the mean for phytase supplemented diets
[4]MSD = minimal significant difference ('' = 0.05) between the mean of the positive control groups and any the mean of any one phytase group; astrics denote significance
[5]Probability values (P $\geq$ F) are only for phytase treatments 0 through 2,400 units
[6]In the prediction equations, Cobb is denoted by "1" and Ross is denoted by "2"
[7]Prediction equations were based on the phytase supplemented diets:

Body weight gain = 221.848 + 0.134 x phytase level + 0.0000374(phytase level)^2

Feed intake = 300.899 + 0.145 x phytase level + 12.780 x breed - 0.0000413 x (phytase level)^2

Gain-to-feed = 0.692 + 0.0000831 x phytase level - 0.0000000226x (phytase level)^2

Mortality = 32.929 - 0.031 x phytase level - 10.769 x breed x 0.006 phytase x breed + 0.00000674 x (phytase level)^2

APPENDIX TABLE 2.3. The effect of breed difference and high phytase levels on 16-d plasma and tibiae data (B4-02-TD)[1]

				Score		Incidence			
Natuphos phytase		Plasma Ca	Plasma P	P Rickets	TD	P Rickets	TD	Tibia Ash	Tibia Ash
U/kg diet	Breed	------ (mg/dL) ------				---------- % ----------			(g/tibia)
0	Cobb	10.4	2.4^{ab}	2.82^{a}	0.225	95.2^{a}	8.4	26.2^{a}	0.180^{e}
0	Ross	11.3	2.1^{b}	2.72^{a}	0.352	93.5^{a}	13.2	25.3^{a}	0.180^{e}
600	Cobb	11.7	1.9^{b}	1.65^{b}	0.195	64.8^{b}	11.7	30.4^{b}	0.277^{d}
600	Ross	11.9	1.8^{b}	1.46^{bc}	0.458	64.7^{b}	21.6	30.8^{b}	0.287^{d}
1,200	Cobb	11.5	2.3^{ab}	1.14^{cd}	0.320	50.0^{bc}	13.9	32.6^{c}	0.300^{cd}
1,200	Ross	11.8	1.7^{b}	0.87^{de}	0.215	41.2^{cd}	11.9	32.8^{c}	0.323^{bc}
2,400	Cobb	11.3	2.5^{ab}	0.56^{e}	0.280	25.8^{d}	13.9	34.5^{d}	0.342^{ab}
2,400	Ross	11.2	3.2^{a}	0.49^{e}	0.508	29.3^{d}	19.6	34.6^{d}	0.362^{a}
(+) Control	Cobb	10.6	5.2	0.06	0.673	5.8	28.9	37.8	0.408
(+) Control	Ross	10.2	5.1	0.02	1.00	1.9	36.5	37.5	0.407
PSEM[2]		0.59	0.310	0.124	0.1107	5.83	4.56	0.45	0.0088

Continued on the following page.

Main effect means

Phytase level								
0	10.8	2.2[ab*]	2.77[a*]	0.289[*]	94.4[a*]	10.8[*]	25.7[d*]	0.180[d*]
600	11.8	1.8[b*]	1.55[b*]	0.326[*]	64.7[b*]	16.7[*]	30.6[c*]	0.282[c*]
1,200	11.6	2.0[b*]	1.0[c*]	0.268[*]	45.6[c*]	12.9[*]	32.7[b*]	0.311[b*]
2,400	11.3	2.9[a*]	0.52[d*]	0.394[*]	27.6[d*]	16.8[*]	34.5[a*]	0.352[a*]
PSEM	0.42	0.22	0.068	0.0724	3.11	2.87	0.32	0.0062
MSE[3]	1.44	2.57	0.230	0.3567	13.62	14.62	1.10	0.0270
Breed of chick								
Cobb	11.2	2.3	1.54[a]	0.255	58.9	12.0	30.9	0.275[b]
Ross	11.5	2.2	1.38[b]	0.383	57.2	16.6	30.9	0.288[a]
PSEM	0.29	0.16	0.048	0.0512	2.20	2.03	.22	0.0044

ANOVA

Source of variation[4]	df	Probability > F						
R^2		0.15	0.42	0.94	0.24	0.86	0.21	0.95	0.95
Treatment	7	0.7443	0.0464	0.0001	0.4083	0.0001	0.5225	0.0001	0.0001
Phytase level	3	0.4069	0.0167	0.0001	0.6854	0.0001	0.4877	0.0001	0.0001
Breed[5]	1	0.4618	0.8609	0.0851	0.1148	0.6739	0.1692	0.8707	0.0434
Phytase*Breed	3	0.8793	0.2156	0.8433	0.3612	0.7566	0.6247	0.4708	0.3720
Regression analysis[6]		Probability > t							

Continued on the following page.

[1]On an as-is basis, all phytase-treated diets were analyzed to contain: 90.18% ± 0.129 (DM); 23.20% ± 0.353 (CP); 0.86% ± 0.015 (Ca); 0.44% ± 0.038 (P); 0.292% ± 0.018 (phytate P), and a calculated ME of 3,123 kcal/kg diet, while the P level of the positive control diet was 0.72; sand was used as an inert ingredient, and removed from the respective diets as either dicalcium phosphate or Natuphos™ phytase was supplemented.

[2]PSEM = the calculated pooled standard error of the mean for phytase supplemented diets

[3]MSD = minimal significant difference ('' = 0.05) between the mean of the positive control groups and any the mean of any one phytase group; astrics denote significance

[4]Probability values ($P \geq F$) are only for phytase treatments 0 through 2,400 units

[5]In the prediction equations, Cobb is denoted by "1" and Ross is denoted by "2"

[6]Prediction equations were based on the phytase supplemented:

Plasma phosphorus = 2.185 - 0.000691 x phytase level + 0.000000406 x (phytase level)^2

Phosphorus rickets score = 2.969 - 0.0021 x phytase level - 0.157 x breed x + 0.000000493 (phytase level)^2

Phosphorus rickets incidence = 94.058 - 0.054 x phytase level + 0.000011 x (phytase level)^2

Percent tibia ash = 25.885 + 0.008 x phytase level - 0.00000198 x (phytase level)^2

Tibia ash weight = 0.168 + 0.000157 x phytase level + 0.0125 x breed - 0.0000000372 x (phytase level)^2

APPENDIX TABLE 2.4. The effect of breed difference and high phytase levels on 16-d nutrient utilization (B4-02-TD)[1]

Natuphos phytase	Breed	Retentions					
		Ca	P	Phytate disappearance	Ca:P[3]	N Retemtion[4]	AME_n[5]
U/kg diet		---------- % ----------				----- % -----	kcal/kg
0	Cobb	63	63^{bcd}	59^{d}	1.95^{abc}	62.2^{c}	3307^{b}
0	Ross	64	59^{cd}	57^{d}	2.14^{ab}	63.5^{bc}	3313^{b}
600	Cobb	61	57^{d}	67^{c}	2.17^{a}	64.4^{bc}	3393^{ab}
600	Ross	61	65^{bcd}	76^{b}	1.83^{abc}	64.5^{bc}	3345^{ab}
1,200	Cobb	58	67^{bc}	73^{bc}	1.69^{c}	67.6 ^{ab}	3389^{ab}
1,200	Ross	60	67^{bc}	73^{bc}	1.74^{bc}	66.0 ^{abc}	3393^{ab}
2,400	Cobb	60	69^{b}	78^{ab}	1.70^{c}	69.4^{a}	3417^{a}
2,400	Ross	66	77^{a}	84^{a}	1.65^{c}	68.1^{ab}	3427^{a}
(+) Control	Cobb	64	61	43	1.26	68.0	3422
(+) Control	Ross	66	66	46	1.21	71.7	3466
PSEM[6]		2.5	2.8	2.2	.132	1.44	27.0

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Main effect means

Phytase level							
0		63	61[c]	58[c*]	2.04[a*]	62.9[c*]	3310[b*]
600		61[*]	61[c]	72[b*]	2.00[a*]	64.5[bc*]	3369[a*]
1,200		59	67[b]	73[b*]	1.71[b*]	66.8[ab]	3391[a]
2,400		63	73[a*]	81[a*]	1.68[b*]	68.8[a]	3422[a]
PSEM		1.8	2.0	1.5	.093	3.33	19.1
MSD[7, 8]		6.16	7.2	5.4	.316	1.02	63.8
Breed of chick							
Cobb		60	64	69	1.88	17.0	3377
Ross		63	67	72	1.84	16.9	3370
PSEM		1.2	1.4	1.1	0.066	0.72	13.5
ANOVA							
Source of variation[9]	df	---------- Probability > F ----------					
R^2		0.23	0.60	0.84	0.41	0.47	0.46
Treatment	7	0.4490	0.0011	0.0001	0.0550	0.0204	0.0243
Phytase level	3	0.2623	0.0004	0.0001	0.0195	0.0022	0.0029
Breed[10]	1	0.2409	0.1485	0.0502	0.6716	0.7380	0.7130
Phytase*Breed	3	0.7142	0.0881	0.0507	0.2494	0.7319	0.6802
Regression analysis[11]		---------- Probability > t ----------					
R^2		0.13	0.51	0.74	0.24	0.42	0.37
Phytase level	1	0.0503	0.9040	0.0001	0.0048	0.0001	0.0001
Breed	1	-----	0.6900	0.0931	-----	-----	-----
Phytase*Breed	1	-----	0.0893	-----	-----	-----	-----
Phytase*phytase	1	0.0441	-----	0.0111	-----	-----	-----

Continued on the following page.

[1]On an as-is basis, all phytase-treated diets were analyzed to contain: 90.18% ± 0.129 (DM); 23.20% ± 0.353 (CP); 0.86% ± 0.015 (Ca); 0.44% ± 0.038 (P); 0.292% ± 0.018 (phytate P), and a calculated ME of 3,123 kcal/kg diet, while the P level of the positive control diet was 0.72; sand was used as an inert ingredient, and removed from the respective diets as either dicalcium phosphate or Natuphos phytase was supplemented.

[3]Ca:P = Calcium-to-phosphorus ratio between the utilized Ca and P intakes were calculated as ((%tCa retention x % analyzed dietary Ca on a DM basis)/100)/((%tP retention x % analyzed dietary P on a DM basis)/100)

[4]Apparent N retention of each diet was calculated as (100- (100 x (% Cr_2O_3 feed/% Cr_2O_3 excreta) x (%N excreta/%N feed)))

[5]Nitrogen-corrected, apparent metabolizable energy (AMEn) of each diet was calculated as: (GE Feed [kcal/g] - ((GE Excreta [kcal/g] x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) + (g N Feed - (g N Excreta x (Cr_2O_3 Feed / Cr_2O_3 Excreta)) x 8.28))) x 1,000

[6]PSEM = the calculated pooled standard error of the mean for phytase supplemented diets

[7]MSD = minimal significant difference (" = 0.05) between the the mean of the positive control groups and the mean of any one phytase group; astrics denote significance

[8]Phytate P MSD for Cobb chicks = 6.94, and phytase treatments from 0 to 2,400 U/kg diet are significantly different than the positive control diet that was fed to Cobb chicks (P > 0.05); phytate P MSD for Ross chicks = 9.09, and phytase treatments from 0 to 2,400 U/kg diet are significantly different than the positive control diet that was fed to Ross chicks (P > 0.05)

[9]Probability values ($P \geq F$) are only for phytase treatments 0 through 2,400 units

[10]In the prediction equations, Cobb is denoted by "1" and Ross is denoted by "2"

[11]Prediction equations were based on the phytase supplemented diets:

Total calcium retention = 63.589 - 0.007 x phytase level + 0.00000278 x (phytase level)^2

Total phosphorus retention = 61.402 - 0.000436 x phytase level - 1.257 breed + 0.004 x (phytase level)^2

Phytate phosphorus disappearance = 54.315 + 0.018 x phytase level + 3.152 x breed - 0.00000386 x (phytase level)^2

Ratio of retained Ca:P = 2.028 - 0.000163 x phytase level

Apparent %N retention = 63.104 + 0.0025 x phytase level

AME_n = 3327.34 + 0.044 x phytase level

APPENDIX 3

APPENDIX TABLE 3.1. Composition of the Basal Diet (B1-01-T3)

Ingredient	Amount
	(%)
Corn (8.8% CP)[1]	53.44
Soybean meal (48.5% CP)	38.09
Poultry fat (8.2 kcal/g)	5.00
Dicalcium phosphate	1.88
Limestone	0.66
Salt (NaCl)	0.40
DL-Methionine	0.20
Trace mineral mix[2]	0.25
Vitamin mix[3]	0.075
Total	100.00

[1]Corn was replaced on an equivalent weight basis with limestone (w:w) in treatments where calcium concentrations were lower. The basal diet contained a calculated ME of 3211 kcal/kg, 23.1% CP, 0.78% Ca, 0.47% P.
[2]Trace mineral mix provides the following (per kilogram of diet): manganese ($MnSO_4 \cdot H_2O$), 60 mg; iron ($FeSO_4 \cdot 7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4 \cdot 5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg.
[3]Vitamin mix provided the following (per kilogram of diet): thiamin•mononitrate (B_1), 1.1 mg; nicotinic acid, 44 mg; riboflavin (B_2), 4.5 mg; D-Ca pantothenate, 12 mg; vitamin B_{12} (*cobalamin*), 6.6 ug; pyridoxine•HCl (B_6), 2.2 mg; D-biotin, 0.11 mg; folic acid, .55 mg; menadione sodium bisulfate complex, 1.1 mg; ascorbic acid, 250 mg, choline cholride, 220 mg; cholecalciferol, 1,100 IU; trans-retinyl acetate, 5,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 125 mg.

APPENDIX TABLE 3.2. The effect of dietary T_3 (0 and 0.25 mg/kg diet) and 0.75 and 1.0% calcium on body weight, feed intake, feed conversion ratio, and incidence of calcium rickets and tibial dyschondroplasia in LTD and HTD TD broiler lines (B1-01-T3)[1]

Breed	Dietary Ca	Dietary T3	BWT	FI	G:F	Plasma Ca	Plasma P	Ca Rickets	TD	Bone ash
	%	mg/kg diet	g/chick		g:g	----------	----------	%	----------	----------
LTD	0.75	---	307	429	0.717	7.86	5.71^{bc}	19	27^{c}	38.8^{ab}
HTD	0.75	---	269	403	0.667	8.34	5.58^{bc}	25	69^{a}	37.3^{c}
LTD	1.00	---	287	412	0.693	7.92	5.57^{bc}	8	9^{d}	39.3^{a}
HTD	1.00	---	277	416	0.664	8.20	4.80^{c}	22	52^{b}	38.2^{abc}
LTD	0.75	0.25	303	422	0.718	7.69	6.41^{ab}	27	43^{b}	38.0^{abc}
HTD	0.75	0.25	267	400	0.667	7.44	6.86^{a}	23	82^{a}	35.8^{d}
LTD	1.00	0.25	280	403	0.694	7.91	5.06^{c}	17	27^{c}	37.6^{bc}
HTD	1.00	0.25	262	402	0.650	8.11	4.97^{c}	24	72^{a}	35.8^{d}
Pooled SEM			11.0	10.7	0.0144	0 .725	.845	15.5	13.3	1.04

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Main effect means									
Breed									
LTD		294[a]	416	0.706[a]	7.85	5.69	18	27[b]	38.4[a]
HTD		269[b]	405	0.661[b]	8.02	5.55	24	69[a]	36.8[b]
Calcium level (%)									
0.75		286	413	0.692	7.83	6.14[a]	24	55[a]	37.5
1.00		276	408	0.675	8.04	5.10[b]	18	40[b]	37.7
T3 level (mg/kg diet)									
0		285	415	0.685	8.08	5.41	19	39[b]	38.4[a]
0.25		278	407	0.682	7.79	5.83	23	56[a]	36.8[b]
Pooled SEM		5.5	5.4	0.0072	0.148	0.173	24.3	2.7	0.21
ANOVA									
Source of variation	df	---------- Pobability ≥ F ----------							
Treatment	7	0.0495	0.4566	0.0096	0.4883	0.0012	0.5354	0.0001	0.0001
Calcium level	1	0.2002	0.4957	0.1086	0.3431	0.0001	0.2185	0.0003	0.3922
T3 level	1	0.3732	0.2715	0.7791	0.1712	0.0986	0.3743	0.0001	0.0001
Breed	1	0.0021	0.1520	0.0001	0.3950	0.5761	0.1872	0.0001	0.0001
Calcium x T3	1	0.6221	0.6579	0.7236	0.2601	0.0219	0.7840	0.5383	0.1388
Calcium x breed	1	0.1573	0.1052	0.5085	0.7729	0.2311	0.3175	0.6585	0.4857
T3 x breed	1	0.8450	0.9462	0.6697	0.3295	0.2064	0.3689	0.9240	0.2210
Calcium x T3 x breed	1	0.7492	0.7703	0.7236	0.4422	0.9108	0.8910	0.6583	0.9625

[a-g] Means within a column that have different superscripts are significantly different ($P \leq 0.05$).

[1]This was a 16-day experiment; and, each treatment contained six replicates of ten, day-of-hatch Ross x Arbor Acre mixed-sex chicks; average initial weight = 42.2 g; the basal diet contained a calculated ME of 3211 kcal/kg, 23.1% CP, 0.78% Ca, 0.47% P.

APPENDIX TABLE 3.3. The effect of dietary T_3 (0 and 0.25 mg/kg diet) and 0.75 and 1.0% calcium on thyroid hormone levels in LTD and HTD TD broiler lines (B1-01-T3)[1]

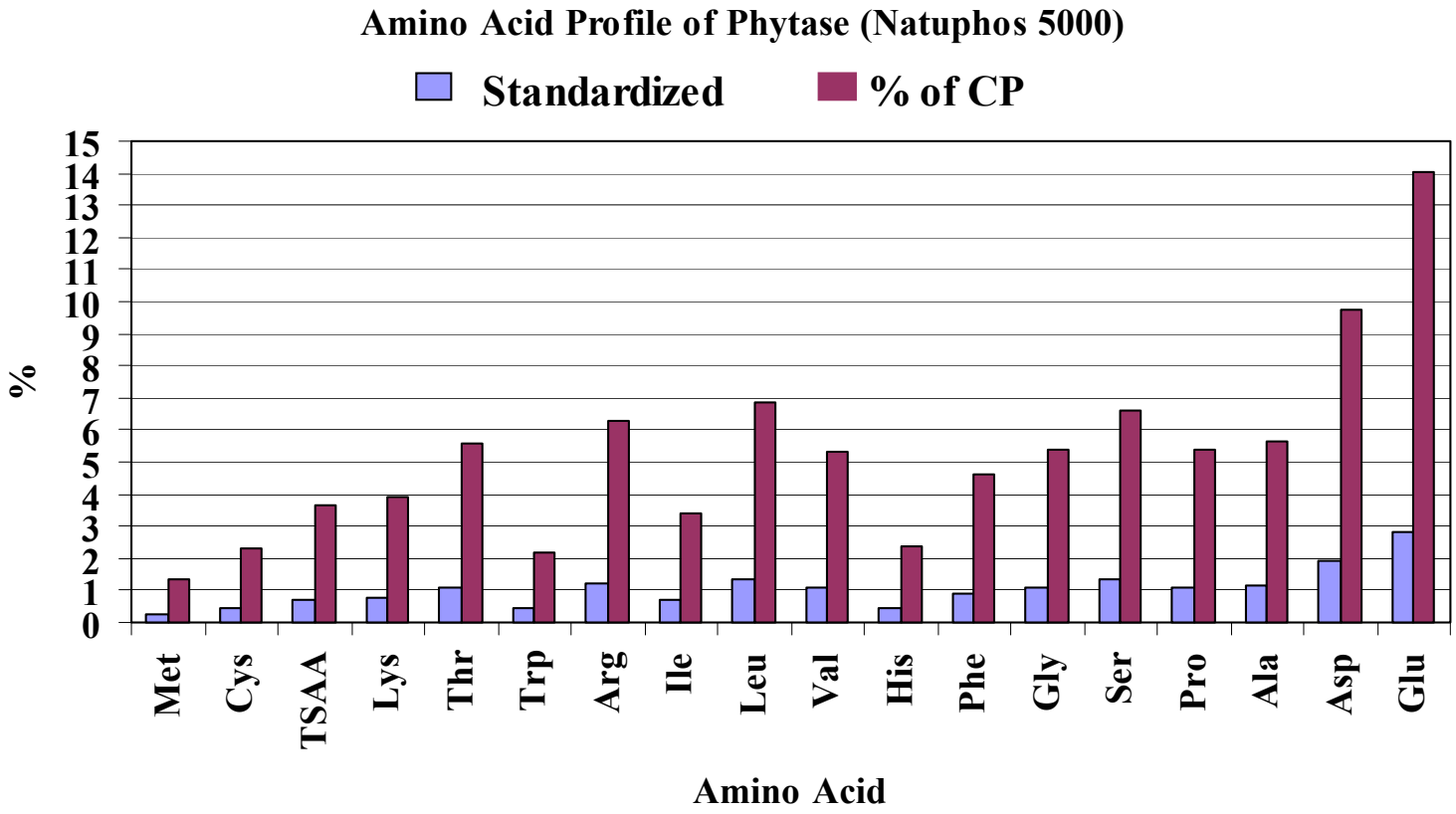
			Plasma		
Breed	Dietary Ca	Dietary T3	T4	T3	Free-T3
	%	mg/kg diet	: g/dl	ng/dl	pg/ml
LTD	0.75	---	10.9[a]	121.8[c]	5.2[d]
HTD	0.75	---	11.3[a]	121.6[c]	5.0[d]
LTD	1.00	---	10.8[a]	111.0[c]	4.6[d]
HTD	1.00	---	10.9[a]	116.5[c]	5.0[d]
LTD	0.75	0.25	8.4[b]	241.7[b]	8.3[c]
HTD	0.75	0.25	9.6[ab]	358.9[a]	9.7[b]
LTD	1.00	0.25	10.3[ab]	339.4[a]	10.8[a]
HTD	1.00	0.25	9.5[b]	260.3[b]	9.6[b]
Pooled SEM			1.60	43.84	.980
Main effect means					
Breed					
LTD			10.1	203.5	7.2
HTD			10.3	214.3	7.3
Calcium level (%)					
0.75			10.0	211.0	7.0
1.00			10.4	206.8	7.5
T3 level (mg/kg diet)					
0			11.0[a]	117.7[b]	5.0[b]
0.25			9.4[b]	300.1[a]	9.6[a]
Pooled SEM			0.33	0.95	0.20
ANOVA					
Source of variation	df		---------- Pobability $\geq$ F ----------		
Treatment	7		0.0503	0.0001	0.0001
Calcium level	1		0.4688	0.7402	0.1133
T3 level	1		0.0019	0.0001	0.0001
Breed	1		0.6838	0.3964	0.7911
Calcium x T3	1		0.2249	0.7711	0.0119
Calcium x breed	1		0.2249	0.0005	0.0768
T3 x breed	1		0.8909	0.5207	0.9103
Calcium x T3 x breed	1		0.3471	0.0003	0.0068

Continued on the following page.

[a-g] Means within a column with no common superscripts are significantly different ($P \leq 0.05$).
[1]This was a 16-day experiment; and, each treatment contained six replicates of ten, day-of-hatch Ross x Arbor Acre mixed-sex chicks; average initial weight = 42.2 g; the basal diet contained a calculated ME of 3211 kcal/kg, 23.1% CP, 0.78% Ca, 0.47% P.

APPENDIX 4 - FIGURES

Figure 4-1.



Amino acid profile of Phytase A (Natuphos® 5000). This product contains an analyzed dry matter of 92.71%, a crude protein of 22.19% and 6,054 units of phytase per gram of Natuphos® 5000.