THE EFFECTS OF VARIOUS CONCENTRATIONS OF PHYTASE ON BROILER GROWTH PERFORMANCE, PHOSPHORUS DIGESTIBILITY, TIBIA ASH, AND PHOSPHORUS UTILIZATION

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By: Connie T. Mou

Scientific Abstract

Two experiments were conducted to determine the effects of various phytase concentrations on broiler growth performance, carcass composition, phosphorus digestibility, tibia ash and phosphorus utilization. The first experiment contained a positive control (**PC)** diet that was sufficient in all nutrients, a diet reduced in available phosphorus, calcium, amino acids and energy utilized as a negative control (**NC)** diet and the NC diet supplemented with two different phytase products at three inclusions (500, 1000, and 2000 FTU/kg) all fed to broilers over a 42-day period. The NC fed birds resulted in reduced growth performance by 42 days of age and phytase at 500 and 1000 FTU/kg had increased growth performance compared to NC ($P \leq 0.01$), resulting in a similar response to the PC fed birds ($P > 0.05$) indicating phosphorus and other nutrient release from the NC diet with phytase supplementation. Birds fed a diet supplemented with phytase A at 2000 FTU/kg outperformed the PC fed birds in body weight gain, feed efficiency, cold carcass weight, breast weight, breast yield, breast + tender weight and yield ($P \leq 0.01$), but 2000 FTU/kg of phytase B resulted in poor responses often not improved in comparison to the NC fed birds ($P > 0.05$). The second experiment utilized a standard curve to evaluate the use of phytase at various concentrations over a 14 day feeding assay. There were no differences between the two phytase treatments (500 and 2000 FTU/kg) in body weight gain, feed efficiency, feed intake or tibia ash weight ($P > 0.05$). Standard curve analysis of tibia ash weight resulted in an estimate of 0.15 phytate phosphorus release from both phytase treatments. At 14 days, birds fed a treatment supplemented with phytase at 2000 FTU/kg showed an increase in apparent ileal phosphorus digestibility in comparison to 500 FTU/kg fed birds. The data may

suggest that birds are digesting more phosphorus at an inclusion of 2000 FTU/kg phytase than 500 FTU/kg phytase but are not able to effectively utilize or store the nutrient as tibia ash showed similar mineral deposition between the two treatments. The concentration of non-phytate (**nPP**) in the Experiment 2 was 0.20% nPP (0.30% nPP in Experiment 1), which might have precluded the growth performance effects noted in Experiment 1. These two experiments indicate that phytase can act as a viable method in supplementing phosphorus and has the potential to increase broiler growth performance but results may vary depending on the phosphorus deficiency status of the diets before phytase supplementation.

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Unless otherwise noted, all images were created by the author.

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Nomenclature

AvP	Available Phosphorus
BW	Body Weight
BWG	Body Weight Gain
BY	Breast Yield
BYW	Breast Yield Weight
CY	Carcass Yield
CCW	Cold Carcass Weight
DCP	Dicalcium Phosphate
EU	Experimental Unit
FE	Feed Efficiency
FI	Feed Intake
FTU	Phytase Unit of Measurement
IP	Polyphosphorylated Inositol
LTY	Leg Thigh Yield
LTYW	Leg Thigh Yield Weight
$NaHCO_3$	Sodium Bicarbonate
NC	Negative Control
nPP	Non-Phytate Phosphorus
P	Phosphorus
PC	Positive Control
S	Sulfur
TA	Tibia Ash Determination
Ti	Titanium

CHAPTER 1

GENERAL INTRODUCTION

Phosphorus (P) is a critical nutrient in the poultry industry that influences efficient growth performance and skeletal health. The mineral is a key molecule in the formation of phospholipid bilayers, generates the high-energy bonds for adenosine triphosphate, DNA, and RNA, and along with Ca, provides structure for skeletal integrity (Qian *et al*., 1996). Grain and oilseeds naturally contain P, however, the nutrient has low-availability due to the form of P i.e., phytate. The P bound to the phytate molecule in the seed is used for post-germination growth and is stored as the water insoluble phytate molecule until needed. This water insoluble storage form also acts as an energy reservoir and prevents nutrient loss for plants since it can only be accessed with the action of the enzyme phytase or specific acids (Wodzinski and Ullah 1996). The phytate of corn may make up 66% of the total P in the grain with only 14% of the P being bioavailable to the bird. Similar to corn, phytate makes up an estimated 61% of the total P in soybean meal (48% crude protein) with a slightly higher P bioavailability at 23% (NRC 1994). Historically, this reduced availability of the P in grains and oil seeds has been rectified by the supplementation of inorganic P to meet the birds P requirements. This has worked but is not without consequence. The addition of inorganic P has led to over application of P to the diet increasing dietary costs. The excess P can negatively impact the environment by increasing loss of P through water shed that can run off to large bodies of water and thereby leading to an increase in the potential onset of water eutrophication (McGrath *et al*., 2005). An overgrowth of plant life can occur from the excess nutrients that may cause an imbalance in the marine life (Toor *et al*., 2005). In addition to the environmental risk associated with excess P, it is costly and has resulted in a four-fold increase in price in the past decade (Augspurger *et al*., 2007). This increase in price has been

attributed to the transition from fossil fuels to ethanol-based fuels made from major grains such as corn and soybean increasing the demand for corn and soy resulting in increased demand for P as a fertilizer.

Producers have utilized phytase, an exogenously produced feed enzyme, to liberate P bound to the phytate molecules found in the grains and oil seeds in the diet. The enzyme hydrolyzes the bound P releasing it for the bird. The industry has used phytase for over 20 years once cost of enzyme production decreased and inorganic P prices increased (Wodzinski and Ullah 1996). Nelson (1967) determined that phytase is an efficient method to maximize phytate utilization for birds, so in the past few decades, producers have begun formulating diets to use phytase as a means to provide P as a substitute for inorganic phosphorus. In addition to direct effects on phosphorus utilization, phytate is a polyanionic molecule causing it to interact with cations forming complexes. Those complexes may be comprised of dietary nutrients such as proteins, lipids and carbohydrates resulting in an anti-nutritive effect as these nutrients become unavailable to the birds (Ravindran *et al*., 2000). Phytase has the ability to break the bonds among the phosphate and inositol ring reducing the ability of phytate to form unavailable complex with other nutrients. The use of phytase has resulted in an alternative to inorganic P helping to increase P available directly from the diet and subsequently reducing dietary cost (Cowieson *et al*., 2006). More recently, birds provided diets with higher inclusion rates of phytase have resulted in greater growth performance (Pirgozliez *et al*., 2011). These effects are known as an extra-phosphoric effect. With higher concentrations, the better the ability of the enzyme is to hydrolyze phosphates from the phytate molecule thereby quickly minimizing the ability of phytate-nutrient complexes to form and reduce digestibility of the diet.

Therefore, the hypothesis for this thesis is:

- At an inclusion of 500 FTU/kg, birds will perform similar to birds fed a diet with equal nutritional value but with P supplemented though inorganic P such as dicalcium phosphate.
- At a higher phytase inclusion of 1000 or 2000 FTU/kg, broilers will result in greater growth performance, P digestibility, body composition (tibia ash and carcass) and P utilization than at a lower phytase inclusion rate such as 500 FTU/kg as more enzyme will be present to act on phytate molecules reducing the anti-nutritive effects of phytate on broiler performance.

The first objective of the thesis was to evaluate the effects of high inclusion phytase with two phytase products that were supplemented to a nutrient deficient diet at three inclusion rates; commercial inclusion of 500 FTU/kg and two higher inclusion rates of 1000 and 2000 FTU/kg. The diets were fed to broilers from day of hatch to 42 days of age and effects were determined through growth performance, carcass composition and tibia ash. The second objective of this thesis was to use a standard curve to evaluate effects of two phytase concentrations (500 and 2000 FTU/kg) when supplemented to a P-deficient diet on broiler growth performance, phosphorus digestibility, and phosphorus utilization when fed from day of hatch to 14 days.

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CHAPTER 2

REVIEW OF LITERATURE

Physiological Role and Storage of Phosphorus

Phosphorus (P) is a major biological factor in bird growth and performance. It has a critical role in both plants and birds as a storage strategy and structural component. Up to about 80% of the P in animals is stored in the skeleton as hydroxyapatite. Hydroxyapatite is classified as a group of phosphate minerals with a high concentration of hydroxyl, fluoride, and chloride ions. Phosphorus plays numerous biologically important roles such as bone development, mineralization, skeletal integrity, energy metabolism, and cell signaling (Adedokun and Adeola 2013). Birds obtain P through the diet from two main sources; inorganic P or P bound within cereal grains. Ravindran *et al*. (1995) found that 50 to 85% of the P found in feed ingredients like cereal grains are bound to molecules known as either phytic acid or phytate and the salt constituents to which they are attached. The primary method in release of the phytate-bound P is by enzymatic action by such enzymes like phytase.

Phytate

Plants utilize the phytate molecule as a second form of storage to bind P in their seeds as a future P source for growth during post germination. Phytate P has the ability to bind to minerals (Ca, Mg, K) to create an insoluble salt. The compound prevents loss of P when exposed to water. However, phytate within plants is found bound as a phytic-acid protein complex in seeds. This form of phytate accumulates in the protein-rich outer aleurone layers of monocotyledonous and in dicotyledonous seeds. The complexity of this compound reduces the ease of P release, similar to the phytate-salt compounds unless the appropriate factors are present

for break down and release. When conditions are ideal for germination, plant seeds initiate growth and release the stored P via specific enzymes such as phytase or exposure to certain acids. Phytate is broken down and the stored P is shuttled towards ATP generation and aiding in the transportation of materials in the cells of plants (Wodzinski and Ullah 1996). Some plant seeds such as wheat, rye and barley possess a higher concentration of enzymatic activity. The increase in activity has been proven to make the P in those grains more readily available in diets compared to grains with reduced enzyme presence (Anderson 1985). A few of the phytate salts such as sodium phytate provided an equal response if not greater phytate P release in comparison to phytate bound in protein complexes (Nelson 1967). With the confirmation of phytate providing already available P in diets in substitution of inorganic P, directions turned towards defining a clearer understanding of how and when to incorporate the molecule into diets to maximize the bioavailability of P. The digestibility of the molecule increases with age and maturity. Older birds are able to utilize the P more efficiently (Common, 1939; McGinnis *et al*., 1944; Singsen *et al*.,1950). Birds younger than four weeks of age have a lower ability in hydrolyzing the molecule. By eight weeks of age, the ability of birds to utilize phytate has a noticeable increase through tibia ash analysis. The deposition of P in the bone had increased

levels in comparison to the levels at a younger age (McGinnis *et al*., 1944). The difficulty in utilizing phytate can be associated with the structure of the molecule leading to antinutritive effects.

Naturally, phytate is chemically polyanionic, which enables it to act as a storage strategy for plant seeds. This chemical nature causes the molecule to easily chelate with di- and trivalent cations and trap nutrients. With the attraction to bind with cations, phytate has increased interactions with the minerals, proteins and carbohydrates found within the diets of birds. Interactions of those molecules lead to formation of large molecules that lengthen the process to break down and digest (**Figure 2-1)**. Those compounds are known as binary (phytate-protein) or ternary (phytate-mineral-protein) (Selle *et al*., 2000). Depending on the state of the binary or ternary compound, they may go through the gastrointestinal (GI) tract significantly undigested wasting nutrients that could have been absorbed and utilized towards growth (Cowieson *et al*., 2006). Nutrients are exposed to a variety of pH levels as they travel down the GI tract of a bird. The solubility of phytate changes at different pH levels. At a low pH such as the environment in the stomach, phytate will form electrostatic linkages with amino acids such as arginine, lysine and histidine resulting in insoluble complexes. These complexes have been attributed to reduced

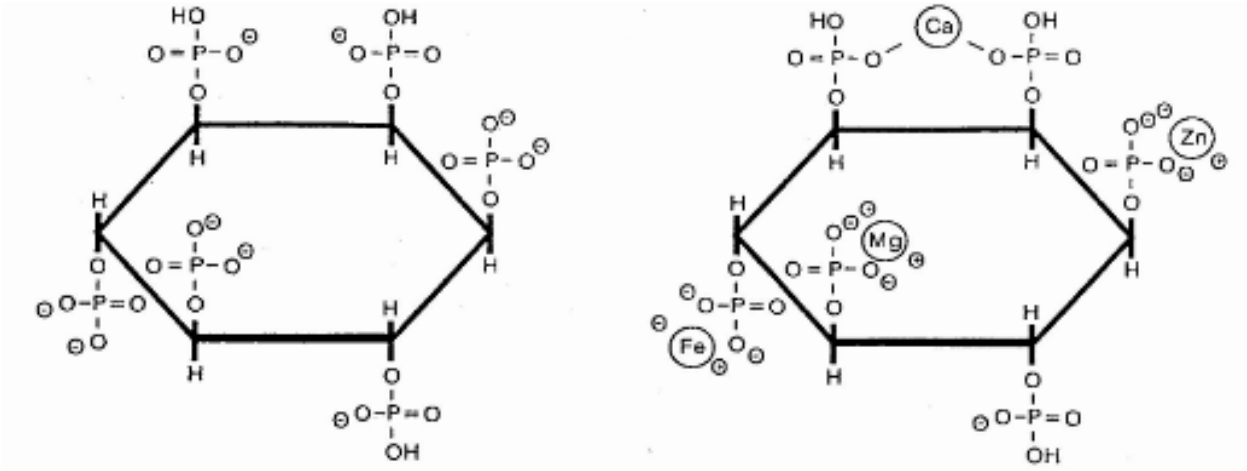

Figure 2-1. Structure of phytate molecule and phytate molecule chelated with various cations.

interaction of proteolytic enzymes with proteins since the proteins are bound to phytate (Barre *et al*., 1954). Phytate also has the ability to bind to endogenous proteases such as trypsin and chymotrypsin. With those proteolytic enzymes bound, they have reduced interaction with nutrients lowering protein digestion in comparison to diets where phytate is hydrolyzed by enzymatic action freeing up the bonds with those proteolytic enzymes (Anderson 1985). Phytate have also been attributed to decreasing other proteolytic enzymes such as pepsin, trypsin and a few other amino peptidases located within the small intestine (Liu *et al*., 2009). With the proteins bound to phytate, it can potentially decrease amino acid digestibility by 3-20% (Cowieson *et al*., 2006). Essential amino acids such as histidine, lysine and arginine have been found to have a higher affinity to bind to phytate (Cosgrove, 1966; Reddy *et al*., 1982). These collectively reduce protein digestibility requiring the bird to put more of the nutrients obtained from the diet into maintenance instead of growth (Namkung and Leeson 1999). When phytate protein complexes reach an isoelectric pH such as the intestinal tract, the charges in the linkages neutralize releasing the bound protein. The protein is now free and able to be digested by proteolytic enzymes to obtain the amino acids for absorption. The rate of breakdown of the complex is dependent if the bound protein contains divalent cations. Divalent cations will remain attached to phytate at or past isoelectric pH due to chemical attraction (Ravindran *et al*., 1999). When phytate reaches higher pH levels, the molecule can chemically bind to minerals posing an issue since minerals are most soluble at high pH levels. When minerals form complexes with phytate, the molecule becomes insoluble and a reduction in digestibility may occur (Sebastian *et al*., 1997).

Calcium (Ca) has been well studied in relation to phytate due to its dependent relationship with P. Nelson (1967) found that phytate might be inhibiting the absorption of calcium since absorption of calcium is influenced by the amount of P absorbed. Depending on

the pH of the gut and molar ratios, phytate has the capacity to bind up to 5 Ca atoms. With the phytate bound P and Ca, it negatively affects the ability of the bird to absorb proper amounts of both nutrients for growth and development thereby reducing efficient production (Liu *et al*., 2014). Findings have led to awareness that the hydrolysis of phytate is linked to the amount of Ca in the diet. There is a linear reduction in ileal phytate P degradation and Ca levels when Ca supplementation is increased from 4.7 to 11.6g/kg in broiler diets (Plumstead *et al*., 2008). Nelson *et al*. (1968) tested the limiting effects of phytate on Ca absorption in white leghorns. The experiment resulted with diets high in phytate could limit Ca absorption as the phytate complexes could bind up to 0.45% of the Ca within the diet. This complex stays insoluble as it traverses the digestive tract reducing interaction with digestive enzymes. The phytate-Ca complex may also bind to fatty acids due to the divalent nature of Ca increasing the occurrence of lipid soaps and reduce available energy as lipids are major source of energy (Ravindran *et al*., 2001). Phytate has also been found to impact the digestibility of zinc (Zn). Birds fed an inorganic feed-grade Zn ($ZnSO_4 \cdot H_2O$) resulted in reduced digestibility due to formation of complexes with phytate. In comparison, birds fed an organic Zn-methionine complex were not affected by phytate as the Zn was already bound and not available to join the phytate-Ca complex (Wetekind and Hortin 1992).

Phytate can decrease carbohydrate digestion due to the binding of starch enzymes such as amylase through phosphate links. Increased glycemic index measurements supported the significant improvement in bird performance when phytase enzyme is added compared to the low index of birds fed a diet without phytase inclusion (Thompson and Yoon 1984). A closer look into the reduced carbohydrate digestion lead to findings that phytate may be blocking certain enzymes from breaking down sugars by non-competitively blocking attachment sites.

Phytate was found to be a possible competitor to the enzyme alpha-amylase and its active binding site to its corresponding sugar (Ravindran *et al*., 2001).

The attachment of nutrients such as proteins and carbohydrates to phytate increases the occurrence of endogenous losses. The protein-phytate complexes formed in the stomach may be causing additional gastric secretions of pepsin and hydrochloric acid (**HCl**) as a compensatory response to intact protein in the small intestine (Augspurger and Baker 2004). This increase can lead to lower pH chyme entering the small intestine and damage to the intestinal lining, causing the birds' body to react with increased production of protective mucin. Some of the primary amino acids that compose mucin contain sulfur (**S**). Mucin production can be estimated through excreta by measuring S levels since mucin will shed periodically. Raised levels of S found in excreta analysis may indicate an increased production of mucin. Phytate can cause a disruption in the GI tract leading to an increase in mucin production resulting in increased energy and amino acid loss due to the poor digestibility of phytate, increasing the birds' endogenous losses. Along with mucin, the body also raises sodium bicarbonate (**$NaHCO_3$**) production to buffer the acidity of the chyme caused by the extra HCl. The bird will need to increase intake of sodium to support this body reaction. There is speculation that the depletion of Na used for $NaHCO_3$ production is causing a disruption in Na^+ -K^+- ATPase activity and in turn Na^+- dependent transport systems, which affect the absorption of nutrients in the intestine (Selle and Ravindran, 2007). These adjustments inhibit the ability of birds to efficiently breakdown nutrients found within the diet which may inhibit bird growth performance (Liu *et al*., 2014).

Lowe *et al*. (1939), Singsen and Mitchell (1945), and Singsen *et al*. (1947) found that if phytate is provided in the diet with increased amounts of vitamin D_3, there was a noticeable difference in the amount of phytate P absorbed. Phosphorus absorption is affected by the amount

of calcium absorbed and calcium absorption is regulated and promoted by vitamin D_3. Researchers have then looked into using vitamin D_3 as a way to increase phytate P absorption in birds. Chicks provided higher levels of vitamin D_3 will result in greater performance by having higher weight gain, increased retention of P in the bones of birds, and better utilization of certain phytates such as calcium phytate, which at first was poorly utilized by younger birds. These differences were demonstrated in birds given diets with imbalanced levels of Ca and P, diets with adequate Ca:P ratio resulted in little to no response from vitamin D_3.

Providing phytate with different levels of other supplements has shown to increase phytate P absorption. Li *et al*. (2000) found feeding lower recommended levels of phytate led to better and more efficient performance and growth of broilers. One of the more effective methods in releasing phytate bound P is the supplementation of phytase in the diet. Phytase is one of the main enzymes that have the ability to hydrolyze phytate. The importance of the enzyme is its ability to break down phytate since it is the highest order of the existing polyphosphorylated inositol's (**IP**). Birds possess the necessary phosphatases to hydrolyze lower order IP's such as tetra- (IP4) and triphosphate (IP3). These contribute towards calcium release into the cytoplasm, signal transduction, and lipid signaling when combined with diaglycerol. With the complex phytic acid form, birds lack enough phytase to release the bound P to absorb and use towards growth and development.

Supplementation of Phosphorus

The inclusion rate of P within the industry has presented issues as to how much to supplement in the diet, whether the bird can efficiently utilize the P within the diet, where the possible excess P ends up, and how it may affect the environment.

Efficient absorption of P is dependent on the form the nutrient is supplemented in the birds' diet and the ability of the gastrointestinal (**GI**) tract to digest and absorb the mineral. Several factors play into how well a bird's GI tract can carry out the mechanisms to take up P. It begins with the uptake and metabolism of cholecalciferol also commonly known as the inactive form of vitamin D_3. Cholecalciferol comes from animal sources which have a greater effect in broilers than plant derived vitamin D_3. Cholecalciferol goes through a 2-step hydroxylation process to become active then hydroxylated by 25-hydroxylase in the liver to its pre-active form, 25-hydroxycholecalciferol also known as calcidiol. The molecule then migrates to the kidney where 1-alpha hydroxylase catalyzes the reaction to hydroxylate the molecule to its active form, 1,25-dihydroxycholecalciferol whose name can be interchangeable with calcitriol. Levels of circulating active vitamin D within the bird are partially influenced by parathyroid hormone levels (PTH) (Shanmugasundaram and Selvaraj, 2012). The hormone promotes expression of 1-alpha hydroxylase in the kidney, which in turn will increase levels of active vitamin D, which will upregulate the amount of absorbed P (Huber *et al*., 2015). The transporters that have been found to be involved with P absorption in poultry GI tracts is sodium-dependent phosphate transport protein 2B (NaPi type IIb) and sodium-dependent phosphate transport protein 3 (NaPi type III). The activities of the transporters are influenced by a combined effect of levels of P, vitamin D3, and PTH within the bird. Only a few experiments have been conducted to see how the proteins respond when the bird is exposed to nutritional stresses such as a diet low in P (Huber *et al*., 2015). Current knowledge around the mechanisms of the transporters is that the expression is correlated with the amount of P circulating within the lumen of the GI tract. The mechanisms of the sodium transporters needs to be further evaluated to thoroughly understand P

digestibility in poultry. To maximize the birds' ability to absorb P, proper supplementation of P in the diet is critical.

It has been proposed that the industry tends to over supplement inorganic P in the diets of broiler chicks (Waldroup *et al*., 1974). An experiment was conducted demonstrating that an increase of inorganic P does not improve the performance of the birds raised from four to eight weeks when supplemented 0.12% over the recommended National Research Council (**NRC**) amount. Following this led to reconsidering the overall 1994 NRC requirements and whether they were in general too high when it came to broilers. An experiment conducted by Waldroup *et al*. (2000) found that the non-phytate P (**nPP**) starter amount could be lowered to be between 0.37% to 0.39% nPP. Angel *et al*. (2000) found for the grower that it could be lowered to 0.28% to 0.32% as well as the finisher could be lowered to 0.19% to 0.24%. McGrath *et al*. (2005) found reducing nPP diets or diets containing phytase significantly reduced total litter P. With less nPP incorporated in the diet, there was lowered P loss in the litter when exposed to water. This aspect plays a crucial role for the litter used as fertilizer in crop fields. Depicted in **Figure 2-2** is an adaptation from Busman *et* al., 1997 of the P cycle in reference to broilers. Without the supplementation of the enzyme phytase, an enzyme that aids in releasing the bound phosphates from the molecule phytate, P-containing litter from broilers spread across crop fields have the potential to contribute P to nearby waterways leading to larger bodies of water. The P in the runoff comes from the additional supplementation of inorganic P to compensate for the low P digestibility in corn when birds do not have outsides factors such as phytase to release the bound phytate-P.

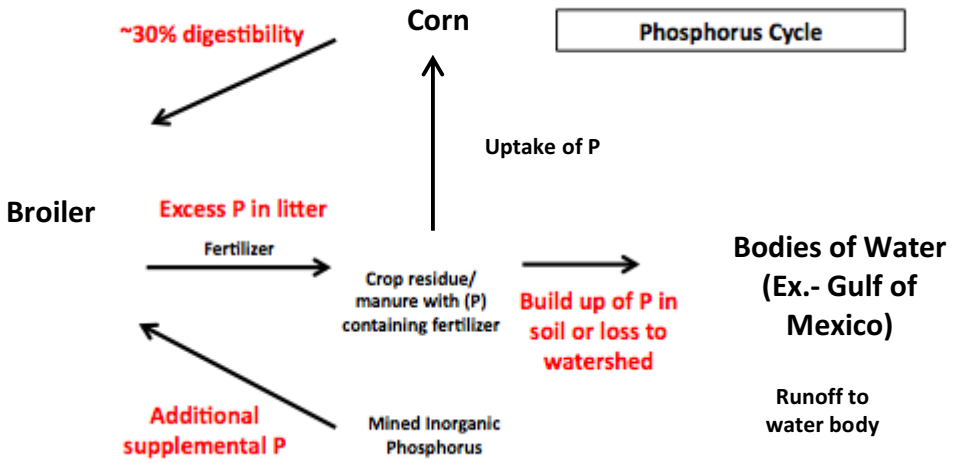

Figure 2-2. Phosphorus cycle in relation to broilers.

According to Powell *et al*. (2008), there is a large debate over the amount of P being released into the environment from the poultry litter that may be used as fertilizer for crops. The excess richness of nutrients such as P in large bodies of water may lead to plant overgrowth which may decrease oxygen availability for the marine life, which may then cause to an imbalance in marine life. In culmination with the negative impact on marine life, Withers *et al*. (2001) indicated it is a form of pollution that is inhibiting the use of those waters. McGrath *et al*. (2005) found that the P leaking into the surrounding areas near the croplands are polluting the surface and ground waters creating issues for surrounding towns needing the water for use.

In addition to environmental issues, P is a costly ingredient. Augspurger (2004) indicated that within the past several years, the price for feed-grade P for animals has risen four-fold. Out of the P supplements being used in the livestock industry, there are three dominant types: dicalcium phosphate (22% Ca, 18.5% P), defluorinated rock phosphate (33% Ca, 18% P), and mono-dicalcium phosphate (16% Ca, 21% P). Of the three, the first two are the main supplements used within the poultry industry for birds such as layers, broilers, and turkeys.

According to Augspurger (2004), poultry account for 50% of the P supplement usage. The prices of P supplements have risen the past years due to change in the direction of agriculture and how crops are utilized. An estimate of how much P supplements will cost is to observe the cost of fertilizer grade phosphates since the two relatively mirror each other. The rapid transition to using ethanol-based fuel has caused a demand in the production of corn and soybeans in which those crops require P as well. Corn demands about twice as much P than soybean per acre. With a rise in demand for crops to be used towards fuel production, a rise in crop fertilizer will occur. A higher usage of fertilizer increases the risk of over application of P. If P levels are lowered in the diet, it could aid in decreasing the amount of P in the poultry litter. With less excess P in the litter, it could allow better water quality and decreased pollution. In addition to reducing the amount of P in the diets, another solution is adding different feed additives to potentially release the already available P that is bound to the ingredients in the diet. In **Figure 2-3,** the cycle characterizes how phytase has been one such solution in alleviating the reliance on inorganic P thereby reducing feed costs and reducing the impact broiler litter may have on the environment.

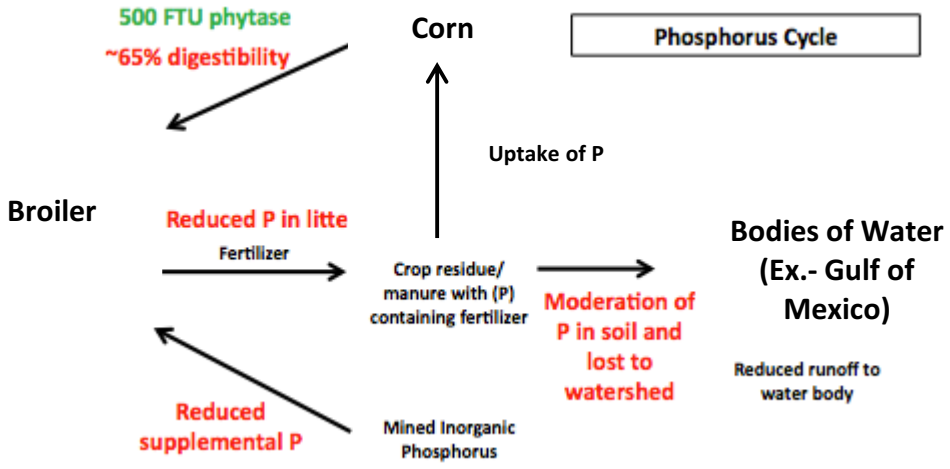

Figure 2-3. Phosphorus cycle in relation to broilers with the addition of phytase.

The addition of a commercial inclusion of phytase can potentially double P digestibility in diets reducing the amount of inorganic P to diets. The reduction in inorganic P can thereby reduce the excess P in broiler litter used as fertilizer which may help moderate the P in runoff lost to watershed and reduce the occurrence of water eutrophication. Since the early 1970s, research has demonstrated that phytase greatly aids in more efficient utilization of the P in the diet.

Phytase Inclusion in Poultry Diets

Phytase was first discovered and extracted by Suzuki in 1907 from rice bran. The first forms of phytase used as a possible feed additive were developed from the fungi *Aspergillus ficum* in 1911. The fungus derived enzyme can increase the availability of phytate bound P in diets providing an alternative to inorganic phosphorus supplementation (Angel *et al*., 2005). Through the next few decades, the enzyme was extracted, studied and purified in hopes to create a commercial product that could be used as an additive for P supplementation purposes. Phytase could be found in not only fungi but also bacteria such as *Aerobacter aerogenes, Bacillus subtilis, Escherichia coli, Klebsiella aerogenes, and Pseudomonas* sp. but outputs were low from these microorganisms and species of *Aspergillus* were found to produce the highest yields of phytase. In 1962, the International Minerals and Chemicals were of the first groups to attempt to create a commercial form of phytase (Wodzinski and Ullah 1996). After a refined phytase product was developed, researchers needed to determine whether the enzyme would retain its activity through the GI tract of animals such as birds and how it would maintain function after feed processing. Nelson *et al*., (1968) ran several trials determining that phytase can maintain activity level through the GI tract and can provide a sufficient output of phytate P.

Phytase has aided the poultry producers by saving close to two billion dollars annually since some phytase additives only cost around $0.60USD to supplement one ton of feed with a

phytase concentration of 500 FTU/kg, which at the moment is the commercial standard (Cowieson *et al*., 2012). When first generation phytase products were being incorporated into commercial diets in the early 1990s, it was found that the enzyme was able to hydrolyze between 35-40% of the phytate P (Cowieson *et al*., 2012). As technology advanced, improvements were made to the enzyme, third and fourth generation phytases improved phytate P utilization by hydrolyzing 60- 70% dietary phytate. At an inclusion rate of 500 FTU/kg in P deficient diets, phytase is able to effectively increase BWG, increase feed intake, and increase P retention in bone ($P<0.05$) without negatively affecting feed conversion (Karimi *et al*., 2013). Phytase may increase the digestibility of other nutrients such as amino acids, energy nutrients and a few minerals, reducing the antinutritive effects of phytate (Selle & Ravindran 2007). This has been termed as an extra phosphoric effect, which researchers are currently focusing on and how it could improve growth and performance.

Impact of Phytase on Growth and Performance

Phytases are classified by which phosphate they remove first from the phytate molecule. There are two main types; those that remove from the third or sixth carbon on the inositol ring (Onyango *et al*., 2004). Without the addition of phytase, diets utilizing inorganic P to supplement the birds nutritional requirement of P needed an average 0.33% increase in nPP compared to diets that had phytase added. With the increase, parameters such as BWG, feed conversion ratio, mortality, and tibia ash were observed to have comparable performance with birds fed diets treated with phytase (Yan *et al*., 2001). At an inclusion rate above 200 FTU/kg to P deficient diets, broilers raised from day-of-hatch to 21 days of age fed diets treated with phytase had an improvement in BWG as well as feed intake ($P<0.01$) (Denbow *et al*., 1995). With the addition of phytase, mortality rates can be reduced. Denbow *et al*. (1995) observed broilers that were

provided diets from day of hatch to 21 days of age with 0.20 to 0.27% nPP with no phytase supplementation, mortality rates were as high as 45%. Mortality rates were lowered by 20% when birds were fed diets with 0.20 and 0.27% nPP diets that were supplemented with phytase concentrations of 200-400 FTU/kg. At phytase concentrations higher than 600 FTU/kg, mortalities were on average 5%, closer to normal expectations at 21 days of age. Broilers raised from day 7 to 27 days fed a maize based diet with phytase responded with greater weight gain, feed conversion efficiency, feed intake, as well as nutrient utilization (AME, N retention, AMEn) ($P<0.01$) (Liu *et al*., 2014). Huff *et al*. (1998) concluded that P deficient diets with an inclusion of phytase fed to broilers could reduce the needed total P between 11 -25% with no significant decrease in bird performance or health. Karimi *et al*. (2013) observed broiler chicks raised from day one to almost three weeks of age had no significant decrease in bird performance or health when fed diets supplemented with 500 FTU/kg of phytase. In addition, phytase inclusions higher than commercial levels responded with greater improvements in broiler performance such as toe and tibia ash P retention ($P<0.05$). This could be due to phytase liberating a majority of the available phytate bound P within the diet. Bougouin *et al*. (2014) found with an addition of over 1000 FTU/kg, phytate P retention could be improved by 8.6%. With improved bird growth and performance, the supplementation can lead to better litter quality. Phytate can cause the bird to intake higher levels of sodium, shifting the mineral balance. Aggravating the osmotic balance in the body leads to increased water consumption and excretion. Litter becomes moist, reducing the quality, which increases the probability for litter quality issues. These can lead to flock health issues due to bacterial/fungal growth from the moist litter (Selle *et al*., 2009). Phytase can reduce these phytate-nutrient interactions and effectively decrease the occurrence of these issues.

Impact of Phytase on Amino Acid Digestibility

The negative charge on the phosphate groups bound to phytate may cause it to bind to the amino acids of proteins thereby blocking proper digestion and absorption of amino acids. With the supplementation of phytase, there has been significant increase in amino acid digestibility (Cowieson *et al*., 2006). Phytase cleaves the individual phosphate groups off the inositol ring reducing the active sites for forming complexes with other molecules. When differing levels of phytase were applied to individual diets made of various plant protein sources, cereals, and cereal by-products, results indicated significant improvements in most essential amino acids. Wheat and sorghum had greater improvements in the digestibility of leucine, alanine, and glutamic acid compared to other feedstuffs ($P<0.05$). Phytase increased the amino acid digestibility of the two feedstuffs by 5.3% to 10.4% (wheat) and 2.7% to 5.5% (sorghum). Digestion in oil seeds such as soybean meal, canola meal, cottonseed meal, and sunflower meal also saw significant increases in amino acid digestibility ($P<0.05$). The type of phytate-protein binding may determine the effectiveness of phytase action on amino acid digestibility in different feedstuffs (Ravindran *et al*., 1999). Amongst those amino acids, threonine was found to have consistent and significantly higher digestibility rates across all feedstuffs. This amino acid aids in the formation of glycine that in turn influence neurotransmitters in the central nervous system. Broilers fed low P diets supplemented with phytase resulted in higher amino acid digestibility in comparison to non-phytase supplemented low-P diets. This increase could be attributed to those phytase diets also demonstrating a significant degradation of phytate in the terminal ileum. This reduces the occurrence of phytate-protein complexes freeing up proteins for digestion by proteases to obtain amino acids for absorption ($P<0.05$) (Rutherford *et al*., 2004). Phytase can also affect the protein efficiency ratio (PER), which is defined as the grams of weight gain

divided by the grams of protein consumed and can help determine the ability of the bird in utilizing protein from a diet (Boling-Frankenbach *et al*., 2001). In birds fed diets comprised of canola meal, an energy source high in protein, there was a significant improvement in PER ($P<0.05$) (Kong and Adeola 2011). Ravindran *et al*. (2001) found with broilers raised from hatch to day 28, phytase was able to linearly improve the digestibility of nitrogen and all amino acids in P-adequate, lysine-deficient diets. Highest digestibility responses for nitrogen and amino acids were at a phytase inclusion of 1000 FTU/kg ($P<0.01$). These increases in amino acid digestibility and responses in energy result in the improved performance from when phytase was included in the diet. In addition to phytase hydrolyzing phytate, it could be enhancing the interaction between digestive enzymes and nutrients by disrupting cell walls of diet ingredients thereby improving digestion (Ravindran *et al*., 2001).

Impact of High Concentration Phytase (Extra-Phosphoric Effect) on Broiler Growth Performance

Phytase has been utilized within the poultry industry for decades. Recently the effects of high concentration phytase feeding have resulted in greater bird growth and performance (Walk *et al*., 2014). With the near-complete destruction of the phytate molecule, phytase reduces the ability of phytate to create complexes with other nutrients that could inhibit digestibility. Action of phytase can be affected by temperature, the fluctuating pH levels in the gastrointestinal tract, rate at which the enzyme passes through the bird, and substrate availability. Administering a dose of 24,000 FTU/kg of phytase to P deficient diets resulted with improvement in bird performance compared to lower dose phytase diets (<1,000 FTU/kg). Birds had greater toe ash percentage ($P<0.05$) as well as greater nutrient utilization in amino acids and minerals when diets had high phytase inclusion (Cowieson *et al*., 2006). Significant increases were noticed in histidine, arginine, threonine, valine, leucine, and lysine digestibility compared to birds fed diets

with no phytase supplementation (Cowieson *et al*., 2006). Olukosi *et al*. (2013) conducted an experiment testing the effectiveness of high inclusion bacterial derived phytase in marginally P deficient diets. Phytase treatments produced a significant increase in phytic acid disappearance, and P retention ($P<0.05$). The improved P retention was supported by the improved percentage tibia ash ($P<0.05$). In addition, broilers fed a diet with a phytase inclusion 2000 FTU/kg demonstrated an improvement in ileal digestibility by 36 kcal/kg in comparison with broilers fed a control diet lacking in phytase supplementation. These results indicate the bacterial phytase was effective in improving bird performance and P utilization particularly at high inclusions. Walk *et al*. (2013) found that phytase at levels exceeding 1500 FTU/kg to broiler chicks raised from day one to day 49, improved their growth and performance and feed conversion ratio compared to birds fed P-adequate or low-P diets.

The breakdown of phytate can release the inositol ring that binds the phosphate groups to the phytate molecule. Inositols aid as a structural component for secondary messengers, lipid phospholipids as well as other phosphate molecules in eukaryotic cells. Walk *et al*. (2014) demonstrated with an inclusion of phytase at 1000 FTU/kg or 1500 FTU/kg resulted in higher concentrations of inositol in the birds' gizzards, which correlated with greater performance ($P\leq0.05$). Shirley *et al*. (2003) found supplementing phytase at 12,000 FTU/kg improved phytate destruction to almost 95%, which in turn increased total P retention to 80%. Nitrogen corrected apparent metabolizable energy rose from 3216 to 3415 kcal/kg, P within tibia ash increased by 20%, plasma P increased by almost 5mg/100mL, and P rickets rates were reduced from 80 to 3%. Overall body weight gain was improved in chicks compared to ones fed a low-P, high inclusion phytase diet. Zeller *et al*. (2015) conducted a similar experiment in relation with the amount of phytase supplemented in the broiler diet at 12,500 FTU/kg but the experiment was

focused on the mechanism behind inositol release from the destruction of phytate. At a dose of 12,500 FTU/kg, the birds resulted with a greater response in the release of inositol phosphate isomers specifically myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate) (InsP6). A higher release and degradation of these inositol isomers led to an increased P absorption and retention in the broiler chicks raised to 24 days of age in the experiment. When young male chicks were fed a phytate-containing, amino acid-deficient diet supplemented with a higher dose of phytase (1200 FTU/kg), birds resulted with improvements in gain to feed ratios ($P<0.05$). The birds also demonstrated an improvement in true amino acid digestibility. This supports the theory that phytase is liberating the phosphate groups on the inositol ring. Phytate bound proteins are liberated leaving those proteins available to interact with digestive enzymes (Biehl and Baker 1997). These responses demonstrate the improvement of growth and performance in birds by the action of high phytate inclusion indicating the presence of the extra-phosphoric effect in broiler diets.

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CHAPTER 3

COMPARISON OF PHYTASE SUPPLEMENTATION AT VARIOUS INCLUSION RATES IN NUTRIENT DEFICIENT DIETS FED TO BROILERS THROUGH EVALUATION OF GROWTH PERFORMANCE, CARCASS COMPOSITION, TIBIA ASH, PHOSPHORUS UTILIZATION, AND PHOSPHORUS DIGESTIBILITY

Abstract

The objective was to evaluate the effects of phytase at various inclusions on broiler growth performance, carcass composition, tibia ash, and phosphorus digestibility. In Experiment 1, two phytase products were supplemented at 500, 1000 and 2000 FTU/kg to determine their effects on broiler growth performance, carcass composition and tibia ash. A total of 512 Cobb 500 broilers were raised in floor pens (64 pens of 8 chickens resulting in 8 replicate groups for each of the 8 treatments) from day of hatch to 42 days. Treatments included a positive control diet (**PC:** 0.45% AvP), a negative control (**NC:** 0.30% AvP), and three phytase inclusions for each of the two phytases (500, 1000, and 2000 FTU/kg) supplemented to the NC diet. Experiment 2 utilized a standard curve generated from 0.20, 0.24, 0.28, 0.32 and 0.36% non-phytate phosphorus (**nPP**) with the phosphorus (**P**) added as dicalcium phosphate (**DCP**) to evaluate the effects of 500 or 2000 FTU/kg fungal phytase. In total, 504 mixed sex Cobb 500 broilers (8 cages of 9 chicks for each of the 7 treatments) were raised in battery cages from hatch to 14 days of age. Linear regression analysis of tibia ash was performed based on dietary DCP to determine P release due to phytase. Experiment 1 demonstrated that birds fed diets with phytase at 500 and 1000 FTU/kg resulted in increased growth performance compared to NC ($P \leq 0.01$) resulting in similar responses as PC fed birds. The inclusion of 2000 FTU/kg of phytase A, resulted with increased growth performance and carcass composition in comparison to both the

NC and PC fed birds ($P \leq 0.01$) supporting the theory of extra-phosphoric effects occurring with high phytase treatment. Experiment 2 resulted in no differences in growth performance or tibia ash between the two phytase inclusions. However, a phytase inclusion of 2000 FTU/kg demonstrated higher apparent ileal phosphorus digestibility than 500 FTU/kg. The data indicate that phytase is a viable method of supplementing P and that a higher inclusion of phytase has the capability to increase growth performance but is dependent on dietary P status before phytase supplementation.

Key words: phytase, broiler, growth performance, tibia ash

Introduction

Phosphorus is a critical nutrient in the poultry industry that influences efficient growth performance. The mineral provides structure for skeletal integrity, is a key molecule in the formation of phospholipid bilayers, and generates the high-energy bonds for adenosine triphosphate (**ATP**) (Qian *et al.*, 1996). Phosphorus is naturally contained in the molecule phytate located in the grain and oilseed component of the diets provided to the birds but has low availability (NRC 1994). In the plant reproductive cycle, phytate acts as a P reservoir and prevents P loss until germination; therefore the molecule is stable and requires the action of the enzyme phytase or specific acids to release phosphate (Wodzinski and Ullah 1996). Historically, this reduced digestibility of the P in grains and oil seeds has been rectified by the supplementation of inorganic P to meet the P requirements of growing birds. However, the addition of inorganic P has led to over application of P to the diet elevating dietary costs and the amount of P in litter leading to increased management required to utilize litter to ensure litter does not become an environmental issue (McGrath *et al.*, 2005).

Exogenous phytases have been produced for nearly 100 years, but within the last 20 years, commercial production of phytase has allowed poultry producers to cost effectively utilize the enzyme to liberate and increase the absorption of the P bound to phytate in the grains and oil seeds commonly fed to poultry to support bird growth (Cowieson *et al*., 2012).

Phytate is a polyanionic molecule which can cause the molecule to interact with cations that can form complexes with minerals, proteins (amino acids), carbohydrates and lipids in the digestive process. These complexes may reduce the utilization of nutrients reducing efficient poultry production (Ravindran *et al.*, 2000). Phytase can alleviate the anti-nutritive effects of phytate by breaking the bonds attaching the phosphate groups to the inositol ring. The inositol ring has been reported as a structural component for secondary messengers, lipid phospholipids as well as other phosphate molecules in eukaryotic cells (Knuckles and Betschart, 1987). Thus, the release of inositol rings may also benefit bird growth performance. In addition to biological benefits, the use of the enzyme as an alternative to inorganic P may help reduce production costs and environmental issues (Cowieson *et al.*, 2006).

A higher concentration of phytase has the ability to quickly hydrolyze phytate minimizing the formation of phytate-nutrient complexes. In an experiment conducted by Olukosi *et al.,* (2013), birds were provided marginally P deficient diets supplemented with bacterial phytase inclusions ranging from 500 to 2000 FTU/kg. At inclusions of 1000 and 2000 FTU/kg, birds resulted with an increase in body weight gain (**BWG**), ileal digestibility of phytate, and tibia ash percent ($P<0.05$) (Olukosi *et al.*, 2013). With a phytase dose of 12,500 FTU/kg supplemented to P-deficient diets, birds resulted with increased utilization in energy yet lower feed intake in comparison to birds fed a diet supplemented with 500 FTU/kg. The additional

growth and efficiency has been termed as an extra-phosphoric effect as the response is not associated with the liberation of additional phosphorus in the diet (Walk *et al.*, 2014).

The objective of these experiments was to evaluate the effects of phytase on growth performance, bone ash, carcass composition, phosphorus bioavailability, and apparent ileal phosphorus digestibility in broilers when fed diets supplemented with different phytase concentrations.

Materials and Methods

All animal procedures were approved by the Virginia Tech Institutional Animal Care and Use of Committee. In both experiments, Cobb 500 broilers chicks were obtained from local commercial hatcheries and transported to Virginia Tech facilities on day-of-hatch. Birds were provided *ad libitum* access to experimental diets and water for the duration of both experiments.

Experimental Design and Diets

In Experiment 1, 512 male chicks were utilized over a 42 day grow out experiment. Immediately upon arrival at Virginia Tech, chicks were wing banded, individually weighed then assigned to one of eight treatments. Treatments were randomly assigned to pens. Birds were placed into floor pens on clean pine shavings at a stocking density of 567cm^2/bird. Each pen of 8 chicks was an experimental unit (**EU**) resulting in 8 EU for each treatment or 64 total birds/treatment. Temperatures were set at 33C for brooding for the first seven days. After which, temperature was decreased by 3C every seven days until day 28 where temperature was maintained at 24C for the remainder of the experiment. Birds were kept at 24 hours of light for the first day then set to 23 hours of light at day 2 though brooding lights were kept to 7 days of age to assist birds in regulating body temperature, and finding feed and water. Once birds

reached 7 days of age, lighting was adjusted to 18 hours of light until day 37 where hours of dark were reduced by an hour each proceeding day until day 42 (Cobb-Vantress 2015). Birds were fed a starter diet from hatch to day 21 before being switched to a grower diet for the remainder of the experiment. The diets were mixed as a mash, and then transported to a local feed mill where the starter diet was pelleted and crumbled and the grower diet was pelleted. The PC diet was formulated to meet Cobb management nutrient recommendations (Cobb-Vantress 2015: **Table 3-1**). The NC diet was formulated with the following nutrient reductions; 0.15% Available Phosphorus (**AvP**), 0.165% Calcium, 52.58 kcal/kg, 0.42% Crude Protein, 0.017% Lysine, 0.004% Methionine, 0.035% Cystine, 0.033% Threonine, 0.019% Tryptophan, 0.026 Isoleucine 0.023% Valine, 0.013% Arginine and 0.035% Sodium. A basal diet was generated from the common nutrients of the eight treatments to reduce error and variation among treatment diets. Experimental diets were then produced with the addition of dicalcium phosphate, limestone, DDGS, corn, and phytase to the basal diet to obtain the desired treatments; PC (0.45 AvP, 0.90 Ca), NC (0.30 AvP, 0.74 Ca), and NC with three phytase concentrations of two phytases (500, 1000, and 2000 FTU/kg). An FTU is the unit of measurement for phytase activity and is defined as the amount of enzyme required to release 1 umol of inorganic phosphate from 5.0 m*M* sodium phytate per minute at a pH of 5.5 and 37 C (Yueming *et al.*, 2014). Two phytases evaluated in this experiment were both derived from the bacteria *Buttiauxella* and expressed in fungal production systems. Phytases produced from this species of bacteria have demonstrated a greater thermostability, resistance to pepsin and higher activity towards phytic acid-protein complexes at lower pH in comparison to *Asperigillus* derived phytases (Zeng *et al.*, 2015). However, the distinction between phytases were kept blind for inclusion in this experiment and were identified as A or B.

In Experiment 2, 504 Cobb 500 broiler chicks (mixed male and female chicks) were obtained from a local hatchery for the 14-day experiment. Immediately upon arrival at Virginia Tech, birds were wing banded, weighed then assigned to one of seven treatments. Treatments were randomly assigned to pens. Nine broiler chicks were assigned per cage resulting in a total of 8 EU for each of the seven dietary treatments. Housing consisted of raised-wire cages at a stocking density of 490cm^2/bird. Temperature was set at 34C for brooding, decreasing to 31C at day 3 then to 29C for the remainder of the experiment. Chicks received continuous lighting over the entire experimental period. On day seven of the experiment, a single replicate cage of birds from treatment 0.28 DCP and 500 FTU/kg were removed due to mechanical heating issues. All seven diets were formulated to Cobb 500 nutrient recommendations with the exception of total P and Ca. In Experiment 2, five of the diets were formulated to contain 0.20, 0.24, 0.28, 0.32 or 0.36% nPP with the P supplemented by DCP (**Table 3-2**). The five DCP diets were utilized to generate a standard curve to assess P bioavailability by phytase liberation. Two phytase diets were supplemented with a fungal derived phytase (Ronozyme HiPhos, DSM Nutritional Products, Inc., Parsippany, NJ) of either 500 or 2000 FTU/kg. Titanium (**Ti**) dioxide, an inert dietary marker was added in Experiment 2 at the rate of 0.25% of the diet to determine analyze P digestibility.

Performance Data

Birds were individually weighed on 0, 21, 28, 35, and 42 days for Experiment 1 and 0, 7 and 14 days for Experiment 2. Bird weights were averaged by pen or cage for statistical analysis and final data were reported as g per bird. Feed offered was measured on 0, 21, 28, 35, and 42 days for Experiment 1 while in Experiment 2 feed offered was measured on 0, 7, and 14 days. Feed refusal was measured on 21, 28, 35, and 42 days for Experiment 1 and 7 and 14 days for

Experiment 2. Feed offered and feed refusal were then used to determine feed intake (**FI**) between 0-21, 0-28, 0-35, and 0-42 days in Experiment 1 and 0-7 and 0-14 days in Experiment 2. Feed intake was calculated for each pen or cage by (initial feed pan and feed weight + feed added over duration of experiment-feed pan weight and remaining feed). Total pen BWG and pen FI were used to calculate feed efficiency (**FE**). Mortality weight gain was collected to correct FE to account for mortality and sampled birds. Feed efficiency is expressed as g gain per kg feed intake. Mortality was monitored and recorded on a daily basis.

Tibia Ash

At the conclusion of each experiment, 42 days for Experiment 1 and 14 days for Experiment 2, birds were euthanized via cervical dislocation and the right legs of 4 birds per EU of Experiment 1 and the right legs of 7 birds per EU of Experiment 2 were collected to determine tibia ash. Collected legs were autoclaved at 121 C for 8 minutes under 6.82kg of pressure and all adhering tissue was removed including both tibia cartilage caps. Bones were then dried in a convection oven at 100 C for 24 hours for dry weight determination (Hall *et al.*, 2003). Tibias collected from 42 day old birds were fat extracted. Fat extraction was completed by EU as all bones within an EU were wrapped in gauze and tied with string to ensure sample continuity. Samples were then fat extracted using a soxhlet apparatus with hexane as the solvent. Heat was applied to the stock hexane allowing it to vaporize, rise and condense at the top of the soxhlet to fat extract the bones. Once hexane in the bell reached the release mechanism, the hexane and extracted fat were returned to the stock hexane chamber. This cycle was repeated for 48 hours to complete fat extraction. Samples were removed and air dried for 24 hours to evaporate hexane (AOAC, 2000). Air dried fat-extracted bones were placed in pre-weighed labeled crucibles, weighed and dried in a convection oven at 100 C for 24 hours. Pooled tibia bones were removed

and immediately placed in desiccators to cool for approximately 30 minutes. Crucible and dried tibias were weighed then ashed at 600 C for 24 hours. After 24 hours, the ash oven was turned off and bones were allowed to cool to approximately 150 C. Crucible and ashed tibias were then removed and placed in desiccators to cool for 1.5 hours before being weighed for fat-free dry tibia ash. Tibias from 14 day-old birds were not subjected to fat extraction but were ashed as outlined above. Tibia ash was expressed as both total grams of ash and percent of dry bone weight using the following equations:

Total tibia weight = (crucible weight + tibia ash weight -crucible weight)/# of tibias in group

Tibia ash percent = (crucible weight + tibia ash weight-crucible weight)/(crucible weight+ tibia dry weight -crucible weight) * 100

Carcass Weight and Yield

In Experiment 1, carcass yields were obtained from 4 birds per pen at 42 days of age. Feed was withdrawn 12 hours before processing to empty the digestive tract and prevent ingested feed and fecal material from contaminating carcasses during evisceration process. Each group of birds were selected and marked with spray paint to allow for quick determination of treatment before being placed into coops (8 birds per coop) for transport to the processing plant. The processing plant was within 20 minutes of the farm and birds were moved quickly from farm to coop to processing. In addition to spray painted color, all birds still maintained individual wing bands for specific identification. Birds were chosen based on the average weight of each EU. Selected birds were within 500g of the EU average weight. Birds were hung on shackles before being rendered insensible with an electric knife (VS200 Electric Stun Knife, Midwest Processing

Systems, Eden Prairie, MN) and euthanized via exsanguination. Broiler carcasses were then scalded in 60 C water for 90 seconds using a batch scalder (AM 48, Brower Equipment, Houghton, IA) before defeathering using a batch picker (Ashley SP23 Picker, Greensburg, IN) for 60 seconds. After defeathering, feet were removed before the carcasses were rehung. Once rehung, removal of the head, neck and viscera were completed by hand. Carcasses were cleaned and visually inspected before being chilled in a mixture of cold water and ice overnight. The next day, cold carcass weight was measured followed by further processing of the carcass. Breast weight (total weight of both pectoralis major and minor from both the right and left side of the carcass) and leg quarter weight (both thigh and drumstick from both sides) were determined. Carcass and parts were expressed as both a total weight and a percentage of live bird weight for carcass yield or cold carcass weight for parts (Buhr *et al.*, 2014). After leg quarter weight determination, the right tibias from all processed birds were excised for determination of tibia ash.

Apparent Ileal Phosphorus Digestibility

At the conclusion of Experiment 2 (day 14), all remaining birds were euthanized via cervical dislocation and digesta contents were collected from the ileum (Meckel's diverticulum to the ileo-cecal junction) via gentle mechanical manipulation. Ileal contents were pooled by replicate cage to generate sample and placed into plastic bags before being frozen (-20 C) until processed. Both ileal contents and feed samples were dried at 55 C for 72 hours and then ground using a cyclone mill with a 2mm screen. Once ground, both feed and ileal samples were analyzed to determine P and Ti concentrations. Feed samples were analyzed in quadruplet while ileal samples were analyzed in duplicate. One g of feed or 0.5g of dried ileal digesta was weighed into a 125mL Erlenmeyer flask. Twenty ml of sulfuric acid and 2mL of nitric acid were then added to

each flask. Samples were boiled for 5 mins then allowed to cool completely. Another 2.5mL of nitric acid were added to each flask and boiled for an additional 20 mins before being cooled completely to a clear colored solution. The acid solution sample was then standardized using a 100ml volumetric flask that had approximately 70ml of deionized water pre-added before the sample and finally additional deionized water was added. The standardized samples were then poured through circular 90mm filter paper and 50mL samples were retained in 50mL conical tubes for final analysis (Boguhn *et al.*, 2009). Samples were sent to the Virginia Tech Soil Testing Laboratory for an inductively coupled plasma atomic emission spectroscopy analysis (**ICP**) to determine P and Ti concentration. The ICP measures characteristic atomic emission spectra. Samples were sent into a nebulizer to create an aerosol, which in turn were introduced into argon plasma. In the plasma, at approximately 8000 K, molecules were almost completely dissociated and the resulting ions emit absorbed energy as light. Like other elements, P and Ti have a unique atomic structure so the wavelength of the light specific. Once the light energy passes through the diffraction grating, it is separated into its component wavelengths. The light intensity is measured in the ICP using a photomultiplier tube. The light intensity emitted at each wavelength is proportional to the amount of the element that is excited. A calibration curve is created so the unknown samples can be measured against known standards. Determined values were represented as milligrams per liter (Martin *et al.*, 1994). Total digesta and feed P and Ti values were determined by multiplying the weight of the original sample by the dilution factor and the final P and Ti concentrations in solution.

The equation below was used to determine apparent ileal phosphorus digestibility (AIPD):

$$AIPD = (P_{diet} - (P_{ileum} * (Ti_{diet}/Ti_{ileum}))/ P_{diet} * 100$$

Where (P_{diet}) is the phosphorus determined concentration in the diet; (P_{ileum}) is the concentration of phosphorus in the ileal digesta; (Ti_{diet}) is the determined concentration of titanium in the diet; (Ti_{ileum}) is the determined concentration of titanium in the ileal digesta.

Statistical Analysis

Performance data (BWG, FI, FE, carcass yield, breast yield, leg and thigh yield, tibia ash) were collected over the six week period and subjected to ANOVA. If significant differences were noted ($P \leq 0.05$), Fishers LSD test was utilized to separate means. In Experiment 2, a standard curve was created with DCP treatments to determine P bioavailability from the diet due to phytase release. The DCP treatments were formulated with dietary levels of nPP ranging from 0.20 to 0.36% in increments of 0.04%. The responses were then measured in the birds' performance and bone characteristics and plotted. The equations developed from the linear regression were used to determine the amount of P from the diet liberated by phytase. The birds BWG, FI, FE, and apparent ileal P digestibility were determined and if significant differences were noted ($P \leq 0.05$), Fishers LSD test was utilized to separate means.

Results and Discussion

Experiment 1

Performance data

From day of hatch to 21 days, birds fed the PC diets resulted in weight gain approximately 16% lower than commercial expectations. In addition, mortality was 2.54%, roughly 1.5% above expected mortality rate for birds at this age. Both the reduced BWG and high mortality rate suggest an additional factor (outside of diet) might have reduced performance in this experiment. From 21 to 42 days of age, the birds performed to expectation and by the end

of the experiment, the birds were approximately a day behind body weight expectations. From day 35 to day 42 the mortality rate was again higher than expected at 3.13%. The high mortality rate observed around day 42 was believed to be primarily due to ascites and heart failure, both signs of fast growth, without a direct treatment effect as mortality was not localized to any one specific treatment. In total, 39 of the 512 broilers were removed as mortality or culls resulting in an overall mortality rate of 7.62%, higher than expected (Tabler *et al.*, 2004). Although performance was not ideal, especially early in the growth cycle, it was not thought to affect the interpretation of the experiment.

At 21 days, birds fed the NC diet (741.9 g/bird), resulted in numerically, but not significantly, lower BWG when compared to birds fed the PC diet (775.8 g/bird: **Table 3-3**). This result was unexpected as the diet was reduced by 0.15% nPP, which should have limited FI and BWG. This lack of significance is most likely due to increased BWG variation (Pooled SEM = 25.7) over the first 21 days. This response is not unexpected due to the high mortality and low performance of these birds in general over the 0-21 day period. In addition to the poor performance and increased variation, there was a smaller than expected difference in analyzed total P reported for the PC and NC starter diets that might have also minimized differences in BWG. After 21 days, all birds were switched from starter to grower feed where analyzed dietary P for both PC and NC were closer to formulated values. Body weight gain and feed efficiency after 21 days resulted in expected results as the NC fed birds were reduced in comparison to the PC fed birds *($P \leq 0.01$)*. At day 28, 35 and 42, BWG of the NC fed birds were 153, 212 and 273 g/bird less than the PC fed birds, respectively, a consistent 10% reduction (*$P \leq 0.01$*). Overall, this response is consistent with previous reports where diets deficient in P and calcium resulted in reduced BWG (Kiarie *et al.*, 2015; Li *et al.*, 2015; Taheri *et al.*, 2015; Taheri *et al.*, 2015).

However NC fed birds resulted in numerically increased FI in comparison to PC birds which contradicts previous reports where birds fed a nutrient reduced diet will have a reduced feed intake (**Table 3-4:** Ferket *et al.,*2006). However, these data support the poor FE demonstrated by NC birds where they were not as efficient as PC fed birds in converting feed into growth even with the increased feed intake. Over the 42-day period, the reduced BWG in NC birds validate the sensitivity of the experiment.

Traditionally, phytase has been provided at an inclusion of approximately 500 FTU/kg (Selle 2008), although recently inclusions have begun to increase. As expected, over the 42 day grow out period, both phytases supplemented at 500 FTU/kg performed similar to the PC fed birds for BWG and FE ($P>0.05$). No significant differences were found in BWG among the two phytases at 500FTU/kg and NC fed birds by day 21. After 21 days, birds that received the 500 FTU/kg diets gained an average of 121, 188, 267g/bird more than NC fed birds by day 28, 35, and 42, respectively (**Table 3-3**). Similar results were observed in FE where the two phytases at 500 FTU/kg were more efficient than the NC fed birds by day 28, 35, and 42 ($P\leq0.01$: **Table 3-4**). At an inclusion of 1000 FTU/kg, both phytases resulted in BWG, (day 0-21, 0-28, 0-35, and 0-42) and FE, (0-21, 0-28, and 0-35) similar to 500 FTU/kg phytase treatments suggesting there was no indication of an extra-phosphoric effect occurring at 1000 FTU/kg diets ($P>0.05$). Experiments conducted by Cabahug *et al.* (1999) and Ravindran *et al.* (2000) observed little to no differences in broiler performance when offered P-deficient diets supplemented with phytase inclusions at 400 or 800 FTU/kg. This suggests that for an extra-phosphoric effect to occur, phytase inclusion may need to be past a particular rate when supplemented to a P-deficient diet. Phosphorus-deficient diets with phytase inclusions greater than 1000 FTU/kg have demonstrated

increased bird growth performance in comparison to diets supplemented with 500 FTU/kg of phytase (Yi *et al.*, 1996; Campasino *et al.*, 2014).

Over the 0-28, 0-35 and 0-42 day periods, birds fed a high inclusion of 2000 FTU/kg of phytase A resulted in increased BWG in comparison to all other treatments (*P≤0.01:* **Table 3-3**). The birds demonstrated higher FE than PC and other phytase treatments (**Table 3-4**). The impact that 2000 FTU/kg of phytase A had on growth and performance could be attributed to an extra-phosphoric effect (Butani and Parnerkar 2015). The improved BWG and FE demonstrated by 2000 FTU/kg of phytase A maybe a result of phytase hydrolyzing phytate molecules and reducing the occurrence of phytate-nutrient complexes (Ravindran *et al.*, 2006; Cowieson *et al.*, 2011; Manobhaven *et al.*, 2016).

Phytase B at 2000 FTU/kg did not demonstrate the growth performance observed in birds offered phytase A at 2000 FTU/kg. From 0-42 days, the birds' FE was consistently lower than PC fed birds by at least 6% while birds fed diets supplemented with 2000 FTU/kg of phytase A had FE that was consistently greater than PC fed birds by roughly 5% (*P≤0.*05: **Table 3-4**). In addition, throughout the 42-day experiment, the birds demonstrated similar growth performance as birds fed the NC diet. By day 21, 28, 35, and 42 the differences in BWG between the birds fed 2000 FTU/kg phytase B and the NC fed birds only differed by 29, 11, 35, and 47g/bird. The data would suggest the 2000 FTU/kg phytase B treatment had no enzyme activity present. However, all diets were sent in for analysis to determine enzyme activity level and results indicated presence of enzyme in the treatment 2000 FTU/kg of phytase B so it is uncertain as to the cause of the birds' poor performance.

Carcass and Bone Responses

As expected, PC fed birds had a greater response in carcass performance than NC fed birds. In contrast to the nutrient-adequate PC fed birds, NC birds resulted in approximately 200g decreased carcass weight, increased breast, breast + tender, and leg + thigh weight (*P≤0.01*: **Table 3-5**). There were no significant differences between PC and NC fed birds for percentage yield of carcass, breast, breast + tender or leg + thigh. The differences demonstrated in parts weight was due to larger bird body weights not an alteration in yield. Pillai *et al.* (2006) reported similar results where birds at 42 days fed either a P-adequate diet or P-deficient diet had a percentage yield of carcass that were not significantly different yet the birds live weight had a significant difference of 120g/bird. Birds fed the NC diet resulted in reduced tibia ash weight in comparison to the PC fed birds (*P≤0.01*), but this response was again influenced by growth rate as there were no significant differences when tibia ash was expressed on a percent of total tibia bone basis (**Table 3-6**). Tibia ash on a total g basis revealed birds displaying no differences between treatments (dos Santos *et al.*, 2012; Li *et al.*, 2015).

As expected, supplementation of NC diets with 500 and 1000 FTU/kg diet resulted in the release of P from the diet resulting with birds having performance, carcass weights and tibia ash weights similar to PC fed birds (*P>0.05*). With the exception of leg + thigh weight, birds fed a diet supplemented with either phytase product at 500 and 1000 FTU/kg alleviated the lower carcass weights associated with birds fed the NC diet. Both 500 and 1000 FTU/kg of phytase, regardless of source, resulted in increased cold carcass, breast, and breast + tender weight in comparison to the NC fed birds (*P≤0.01:* **Table 3-6**), resulting in values that were similar to those of the PC fed birds (*P>0.05*). Both phytase treatments at 500 FTU/kg, resulted in birds with an increased breast yield percentage in comparison to birds fed the PC diets. There were no

significant differences in tibia ash percent, but when bird growth was considered when looking at total tibia ash weight, the 500 and 1000 FTU/kg fed birds had increased tibia ash weight in comparison to the NC fed birds ($P \leq 0.01$), similar to the PC fed birds ($P > 0.05$: **Table 3-6**).These data are in agreement with previous reports where broiler chickens provided P through the supplementation of a commercial phytase at 500 and 1000 FTU/kg resulted in similar if not greater effects in BWG, cold carcass weight, and tibia ash weight (Qian *et al.*, 1996; Onyango *et al.*, 2005; Jendza *et al.*, 2006; Campasino *et al.*, 2014).

The 2000 FTU/kg phytase A treatment resulted in birds having increased cold carcass weight of 200g and breast weight of approximately 100 g in comparison to PC fed birds ($P \leq 0.01$: **Table 3-5**). When fed 2000 FTU/kg of phytase A, broiler breast + tender (%) and leg + thigh (%) did not result in any differences in comparison to the other phytase inclusion rates with the exception of phytase A at 500 FTU/kg and phytase B at 2000 FTU/kg. Broilers that received the NC + 2000 FTU/kg diets resulted in similar tibia ash weight in comparison to lower phytase inclusions and PC diets, suggesting the birds were able to ensure that P was not limiting for either growth or tibia ash ($P > 0.05$: **Table 3-6**). The lack of difference in tibia ash weight may indicate that the increased BWG, cold carcass weight, and breast weight with phytase A at 2000 FTU/kg was associated with extra phosphoric effects, rather than directly related to dietary P status. However, phytase B at 2000 FTU/kg did not demonstrate the results seen with phytase A at 2000 FTU/kg where birds had increased cold carcass, breast, and breast + tender weight in comparison to PC fed birds. Similar to BWG, 2000 FTU/kg of phytase B resulted in carcass weights and yield similar to the NC fed birds. In carcass parts, NC and 2000 FTU/kg phytase B fed birds resulted in a difference of 54g in carcass weight, 15g in breast weight, 0.1% difference in breast yield, 14g difference in breast + tender weight, 0.1% difference in breast + tender yield,

and a difference of 5g in leg + thigh weight. Contrary to phytase B, phytase A at 2000FTU/kg significantly outperformed NC in carcass parts weight (*P≤0.01*: **Table 3-5**). The birds fed the 2000 FTU of phytase A had an increase of 429g in carcass weight; 163g more in breast weight; a difference of 1.5% in breast yield; 199g more in breast + tender weight, a difference of 2.2% in breast + tender yield, and were 99g heavier in leg + thigh parts than NC fed birds. The small differences that resulted between NC and 2000 FTU/kg phytase B further support the speculation that other factors existed that resulted in low performance.

In conclusion, phytase supplementation of 500 and 1000 FTU/kg to a P deficient diet, resulted in birds with similar growth performance, carcass characteristics and tibia ash as to those birds provided a nutritionally adequate diet (PC diet). At 2000 FTU/kg of phytase A, broilers resulted in increased BWG and FE suggesting an extra-phosphoric effect may have contributed to the enhanced performance. As expected with the increased BWG, broilers fed 2000 FTU/kg of phytase A also had increased carcass and parts weights. Tibia ash percentage of the birds fed the 2000 FTU/kg phytase A were not significantly higher than other P adequate diets suggesting that the growth performance results were not a direct effect of P status, but were most likely associated with an extra-phosphoric effects.

Experiment 2

Experiment 2 was conducted to further quantify and define P absorption and P utilization in broilers when fed diets of various levels of available phosphorus provided through dicalcium phosphate or phytase supplementation of either 500 FTU/kg or 2000 FTU/kg. Analyzed values of total P for all diets were similar to formulated values (**Table 3-2**). In addition to the analyzed P values, phytase activity was determined in the two diets that had supplemental phytase, resulting in 750 and 3200 FTU/kg relative to the formulated activity levels of 500 and 2000

FTU/kg, respectively. Phytase additions to diets were based on company recommendations from the product label. These recommendations often are based on a minimum activity in the bag of enzyme, although it is common for the determined value to be 25 to 40% higher due to shelf-life and guaranteed minimum activity requirements. Therefore the higher than expected enzyme recovery are within expected limits. By the end of Experiment 2, there were 14 mortalities resulting in a 2.4% mortality rate. Mortalities were distributed across dietary treatments and no discernable pattern was apparent suggesting these were not a result of a treatment effect.

Birds offered DCP treatments resulted general increase in BWG as nPP increased until 0.32, 0.36 DCP and both phytase treatments where BWG plateaued (**Table 3-7**). The plateau may suggest that P was not the first limiting growth factor in this experiment. Birds fed DCP treatments resulted in increased feed intake from 0-14 days as AvP levels increase though feed efficiency did not rise as AvP levels increased (**Table 3-2**). Birds in all DCP treatments resulted in similar FE with the exception at 0.36DCP where birds had a significantly lower FE than the preceding DCP treatment (0.32). Birds fed the phytase treatments responded with FI and FE that were inversely related as both 500 and 2000 FTU/kg had the highest feed intakes and lowest FE. The high FI demonstrated by these birds fed diets supplemented with phytase corresponds to other experiments where birds were found to have higher intake when fed phytase supplemented diets with dietary deficiencies in P that ranged from 0.32 to 0.40% (Yi *et al*., 1996; Walk *et al*., 2012; Gehring *et al*., 2013). The literature is varied as others also found that phytase supplementation reduced FI by an average of 10g per bird (Silversides *et al*., 2004; Walk *et al*., 2012). In Experiment 2, birds may have increased feed consumption in attempts to meet their nutritional needs as nutrient value is a primary driving factor behind feed intake (Ferket *et al*. 2006). The inconsistency demonstrated across the different phytase inclusions through the

mentioned experiments may then be largely in part due to the dietary availability of P in the diets as they ranged from 0.20% to 0.40%. The phytase diets in Experiment 2 were formulated with a lower nPP level (0.20%) than the 0.30% of Experiment 1, with the greater deficiency of P having a larger negative effect on FI and FE rate.

Tibia ash on a percent basis resulted with a poor linear response indicated by the low r^2 value of 0.264 (*y = 52.612x + 25.61*, (*P<0.01:* **Figure 3-1**)). Tibia ash on a percentage basis is a less sensitive method than measuring on a total gram basis in detecting differences as it does not account for the size of bone. Tibia ash on a percent basis indicated both phytase treatments had the same phytate phosphorus release of 0.15. Shirley and Edwards (2003) and Walk *et al*. (2014) evaluated bird growth and performance with diets containing increasing levels of phytase past 500 FTU/kg. Similar to Experiment 2, birds in these experiments resulted in no significant difference in tibia ash weight or percent when phytase inclusions went past 500 FTU/kg. Even phytase levels at 3000 FTU/kg resulted in no significant difference in tibia ash in comparison to 500 FTU/kg. The lack in significance could be due to limitations in substrate, not enzyme or tibia ash could also be maximized and P is being excreted.

Tibia ash on a total g basis produced a better linear response with a higher r^2 value of 0.644 indicating the DCP treatments had a better fit (y = 797.31x + 63.13 (*P<0.01*: **Figure 3-2**)). On a total g basis, both phytase treatments had a phytate phosphorus release of 0.16. Although not regressed, apparent ileal phosphorus digestibility was also determined (**Figure 3-3**). At 0.20nPP, birds had the highest P digestibility which correlates to the expression of P transporters in the GI tract of a bird as the expression of transporters has been shown to increase when nutrients are low (Selle and Ravindran 2007). As P levels are increased, and the deficiency is not as severe, P digestibility plateaus at about 50% for the 0.28 and 0.32% nPP fed birds.

Interestingly, the 500 FTU/kg phytase treatment that had an available P content of 0.36% (phosphorus release of 0.16 and 0.20% nPP in the diet) resulted in apparent P digestibility similar to that of the 0.36% nPP fed birds suggesting that P provide by phytase has a similar digestibility to that provided by inorganic P. Interestingly, digestibility in the 2000 FTU/kg phytase treated birds resulted in a significant 15.6% higher P digestibility than the birds supplemented with 500 FTU/kg phytase. These results do not agree with the tibia ash determination where both phytase treatments resulted in the same amount of mineral bioavailability, suggesting that digestibility may not always correlate with bioavailability.

The 500 FTU/kg and 2000 FTU/kg phytase treatments resulted in similar BWG, FI, FE, tibia ash weight and percent *(P>0.05*). At day 14, the birds fed a diet supplemented with 2000 FTU/kg resulted in a significant increase in apparent ileal P digestion, suggesting that despite no differences seen in growth or bone composition, a high inclusion of phytase resulted in higher disappearance of P from the diet in the GI tract than the 500 FTU/kg phytase fed birds. The data also suggest that an extra-phosphoric effect may be hidden when phytase diets are formulated to lower nPP concentrations.

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Table 3-1. Formulation and nutrient composition of starter and grower basal diets for broilers fed various nutrient concentrations and three concentrations of two phytases over a 42 day grow out period, Experiment 1.

	Starter		Grower	
Ingredients	Positive	Negative[2]	Positive	Negative[2]
	%	%	%	%
Corn	60.62	63.28	69.61	72.98
Soybean meal (48% CP)	27.68	26.80	21.38	20.60
DDGS	3.21	3.83	1.00	1.00
Poultry byproduct meal	3.00	3.00	2.50	2.50
Soy oil	1.67	0.20	2.21	0.57
Salt	0.31	0.28	0.30	0.23
DL-Methionine	0.28	0.24	0.22	0.17
L-Lysine•HCl	0.27	0.27	0.20	0.20
L-Threonine	0.03	0.00	0.04	0.02
Limestone	0.89	0.99	0.71	0.79
Dicalcium Phosphate	1.74	0.82	1.28	0.38
Choline chloride (60%)	0.10	0.10	0.10	0.10
Vitamin premix[3]	0.10	0.10	0.10	0.10
Mineral premix[4]	0.10	0.10	0.10	0.10
TiO2	0.00	0.00	0.25	0.25
Chemical Composition (calculated)	%	%	%	%
ME (kcal/kg)	3060	3007	3180	3127
Crude Protein	21.42	21.31	18.16	18.00
Calcium	0.90	0.74	0.70	0.53
Phosphorus	0.74	0.58	0.62	0.45
Non-Phytate Phosphorus	0.45	0.30	0.35	0.20
Crude Fat	4.90	3.62	5.52	4.09
Digestible Met + Cys	0.88	0.84	0.74	0.71
Digestible Lysine	1.18	1.16	0.95	0.93
Chemical Composition (analyzed)				
Crude Protein	20.4	21.1	18.3	17.6
Crude Fat	4.31	3.19	4.35	3.09
Crude Ash	5.10	5.23	4.69	5.23
Phosphorus	0.58	0.53	0.62	0.43

[1] Eight treatments were mixed from a common basal including three phytase concentrations (500, 1000, 2000 FTU/kg) of the two phytases and the positive control and negative control diets without phytase.
[2] Reduced by 0.15% Available P, 0.165% Ca, 52.58 kcal/kg, 0.42% CP, 0.017% Lys, 0.004% Methionine, 0.035% Cysteine, 0.033% Threonine, 0.019% Tryptophan, 0.026% Isoleucine, 0.023% Valine, 0.013% Arginine, and 0.035% Sodium.
[3] Provided per kg of diet: vitamin A, 8818342 IU; vitamin D3, 2645503 ICU; vitamin E, 22046 IU; vitamin B12, 26 mg; biotin, 220mg; niacin, 88183 mg; menadione, 2646 mg; thiamine, 3732 mg, riboflavin, 8818 mg; d-pantothenic, 22046 mg; vitamin B6, 4339 mg; folic acid, 2205 mg.
[4] Mineral premix guaranteed analysis per kg of diet: Ca min, 7.0%; Ca max, 8.40%; Copper min, 2.0%; Iron min, 4.0%; Manganese min, 12.0%; Zinc min, 21.0%; Cobalt min, 50 ppm; Iodine min, 3000 ppm

Table 3-2. Formulation and nutrient composition of diets for broilers fed various levels of available phosphorus via dicalcium phosphate and phosphorus released from phytase over a 14 day period, Experiment 2.

Phosphorus Source	——— Dicalcium Phosphate ———					Phytase[1]
Available Phosphorus	0.20	0.24	0.28	0.32	0.36	0.32
Ingredients	%	%	%	%	%	%
Corn	63.36	63.20	63.03	62.86	62.69	63.93
Soybean Meal 48	28.08	28.11	28.14	28.17	28.20	28.32
Poultry Byproduct Meal	4.00	4.00	4.00	4.00	4.00	4.03
Soy oil	0.71	0.77	0.82	0.88	0.93	0.71
Salt	0.42	0.42	0.42	0.42	0.42	0.42
DL-Methionine	0.28	0.28	0.28	0.28	0.28	0.28
L-Lysine•HCl	0.23	0.23	0.23	0.23	0.23	0.24
L-Threonine	0.04	0.04	0.04	0.04	0.04	0.04
Limestone	1.71	1.57	1.44	1.30	1.16	1.05
Dicalcium Phosphate	0.19	0.41	0.63	0.85	1.07	---
Choline Chloride 60	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin Mineral Premix[2]	0.88	0.88	0.88	0.88	0.88	0.88
TiO2	0.25	0.25	0.25	0.25	0.25	0.25
Nutrient composition	%	%	%	%	%	%
ME (kcal/kg)	3035	3035	3035	3035	3035	3061
Crude Protein	21.51	21.51	21.51	21.51	21.51	21.70
Calcium	0.90	0.90	0.90	0.90	0.90	0.79
Phosphorus	0.46	0.50	0.55	0.59	0.64	0.46
Non-Phytate P	0.20	0.24	0.28	0.32	0.36	0.20
Fat	4.07	4.12	4.16	4.21	4.26	4.10
Crude Fiber	2.54	2.53	2.53	2.53	2.52	2.56
Digestible Met + Cys	0.88	0.88	0.88	0.88	0.88	0.89
Digestible Lysine	1.18	1.18	1.18	1.18	1.18	1.19
Digestible Threonine	0.77	0.77	0.77	0.77	0.77	0.78
Chemical Composition (analyzed)						
Phosphorus	0.48	0.50	0.57	0.57	0.63	0.48
Determined Non-Phytate P	0.22	0.24	0.30	0.30	0.35	0.22
Titanium	0.14	0.14	0.14	0.14	0.14	0.14

[1]Phytase provided at 500 and 2000 FTU/kg. Analyzed values 750 and 3200 FTU/kg diet.

[2]Provided per kilogram of diet: cobalt (min), 30ppm; copper (min), 4.75ppm; iodine (min), 1.18ppm; iron (min), 59.40ppm; manganese (min), 75.50ppm; zinc (min), 57.20ppm; vitamin A (min), 7749.28IU; vitamin D3 (min), 2596.01ICU; vitamin E (min), 1.94IU; vitamin B12 (min), 0.01mg; menadione (min), 1.36mg; riboflavin (min), 4.84mg; D-pantothenic acid (min), 7.13mg; niacin (min), 23.25mg; choline (min), 448.43mg.

Table 3-3. Influence of two phytases at three different concentrations on body weight gain (g/bird) of broilers over a 42 day grow out period, Experiment 1.[1]

Diet	Body Weight Gain			
Bird age (d)	0-21	0-28	0-35	0-42
	(g/bird)			
PC[2]	775.8[bc]	1439[b]	2194[b]	2856[b]
NC[3]	741.9[bc]	1286[c]	1982[c]	2583[c]
NC+500A	750.4[bc]	1394[b]	2155[b]	2827[b]
NC+1000A	797.8[b]	1448[b]	2265[b]	2905[ab]
NC+2000A	887.8[a]	1595[a]	2388[a]	3066[a]
NC+500B	786.0[b]	1421[b]	2168[b]	2874[b]
NC+1000B	766.3[bc]	1448[b]	2229[b]	2808[b]
NC+2000B	712.3[c]	1297[c]	2017[c]	2630[c]
A				
SEM	25.7	32	41	58
P-value	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01

[1] Body weights are the response of 8 replicate pens (64 total chicks/treatment) with an average initial weight of 40.0 g/chick.

[2] PC = Positive Control

[3] NC = Negative Control

[a-c] Within a column, least squares means without a common superscript differ (*P≤0.05*).

Table 3-4. Influence of two phytases at three different inclusions on feed intake (kg/pen) and mortality corrected feed efficiency (g weight gain/kg feed consumed) of broilers over a 42 day grow out period, Experiment 1[1].

Diet	Feed Intake				Feed Efficiency			
Bird age (d)	0-21	0-28	0-35	0-42	0-21	0-28	0-35	0-42
	(kg/pen)				(g/kg)			
PC[2]	9.78	18.39	27.79	38.98	626^{ab}	617^{bc}	621^{bc}	575^{bc}
NC[3]	11.21	20.15	29.85	40.80	529^{c}	504^{e}	520^{d}	491^{d}
NC+500A	10.33	18.00	26.66	36.86	569^{bc}	588^{bcd}	606^{bc}	570^{b}
NC+1000A	9.81	17.56	27.35	37.21	624^{ab}	636^{ab}	636^{ab}	593^{ab}
NC+2000A	10.60	18.88	27.48	37.68	662^{a}	669^{a}	671^{a}	617^{a}
NC+500B	10.48	18.11	26.34	36.55	559^{bc}	577^{cd}	597^{bc}	566^{bc}
NC+1000B	11.34	19.74	28.35	38.70	530^{c}	571^{cd}	607^{bc}	555^{c}
NC+2000B	10.34	18.48	27.25	37.74	550^{c}	559^{d}	585^{c}	545^{c}
SEM	0.41	0.66	0.86	1.01	24.6	18.0	18.0	13.0
P-value	0.08	0.11	0.16	0.16	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01

[1] Feed intake and feed efficiency are the response of 8 replicate pens (64 total chicks/treatment).
[2] PC = Positive Control
[3] NC = Negative Control
[a-e] In the same column, least squares means not sharing a common superscript differ (*P≤0.05*).

Table 3-5. Influence of two phytases at three different inclusions on carcass weight, breast weight, breast + tender weight and leg + thigh weight all expressed as either g or % of weight from broilers over a 42 day grow out period, Experiment 1[1].

Diet	Cold Carcass		Breast		Breast + Tender		Leg + Thigh	
	(g)	(%)	(g)	(%)	(g)	(%)	(g)	(%)
PC[2]	2180[b]	76.7	667[b]	23.6[b]	811[b]	28.3[cd]	819[ab]	28.6
NC[3]	1949[c]	75.4	606[c]	23.6[b]	720[c]	27.9[d]	760[c]	29.5
NC+500A	2178[b]	77.1	697[b]	24.6[a]	824[b]	29.0[bc]	805[bc]	28.6
NC+1000A	2239[ab]	77.1	715[b]	24.6[a]	853[b]	29.3[ab]	834[ab]	28.8
NC+2000A	2370[a]	77.3	769[a]	25.1[a]	919[a]	30.1[a]	859[a]	28.0
NC+500B	2224[b]	77.4	721[b]	25.1[a]	847[b]	29.5[ab]	817[ab]	28.5
NC+1000B	2180[b]	77.6	704[b]	25.1[a]	824[b]	29.3[ab]	803[bc]	28.6
NC+2000B	2003[c]	76.0	621[c]	23.5[b]	734[c]	28.0[d]	765[c]	29.0
SEM	46.8	0.01	16.8	0.003	20.4	0.003	17.5	0.003
P-value	≤ 0.01	0.07	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	0.10

[1] Means represent the response of 4 birds for each of the 8 replicate pens (32 total birds/treatment).
[2] PC = Positive Control
[3] NC = Negative Control
[a-d] In the same column, least squares means not sharing a common superscript differ (*P≤0.05*).

Table 3-6. Influence of two phytases at three different inclusions on tibia ash percent (%) and weight (g/bird) of broilers over a 42 day grow out period, Experiment 1[1].

Diet	Tibia Ash	
	(%)	(g/bird)
PC[2]	49.2	3.27[ab]
NC[3]	48.4	2.90[c]
NC+500 Phytase A	48.3	3.25[ab]
NC+1000 Phytase A	49.9	3.22[ab]
NC+2000 Phytase A	50.3	3.40[a]
NC+500 Phytase B	51.1	3.19[ab]
NC+1000 Phytase B	50.6	3.16[b]
NC+2000 Phytase B	49.4	3.11[b]
SEM	0.69	0.07
P-value	0.06	≤ 0.01

[1] Tibia ash are the response of 4 birds for each of the 8 replicate pens (32 total birds/treatment).
[2] PC = Positive Control
[3] NC = Negative Control
[a-c] In the same column, least squares means not sharing a common superscript differ (*P≤0.05*).

Table 3-7. Influence of available phosphorus and phytase on body weight gain (g/bird), feed intake (g/pen), and mortality corrected feed efficiency (g weight gain/kg feed consumed) of broilers from 0-14 days, Experiment 2[1].

Diet	Body Weight Gain	Feed Intake	Feed Efficiency
Bird age (d)		0-14	
	(g/bird)	(g/pen)	(g/kg)
0.20DCP	268.1[b]	2708[d]	656[bc]
0.24DCP	325.4[a]	3193[c]	727[b]
0.28DCP	337.5[a]	3180[c]	701[ab]
0.32DCP	331.2[a]	3252[c]	727[a]
0.36DCP	334.9[a]	3823[b]	605[c]
500 FTU	326.8[a]	4400[a]	500[d]
2000 FTU	320.1[a]	4283[a]	526[d]
SEM	7.04	78.64	14.34
P-value	≤0.05	≤0.05	≤0.05

[1] Body weight gain, feed intake and feed efficiency are the response of 8 replicate pens (72 total birds/treatment). Average initial weight, 39.6 g/chick.

[a-d] In the same column, least squares means not sharing a common superscript differ (*P≤0.05*).

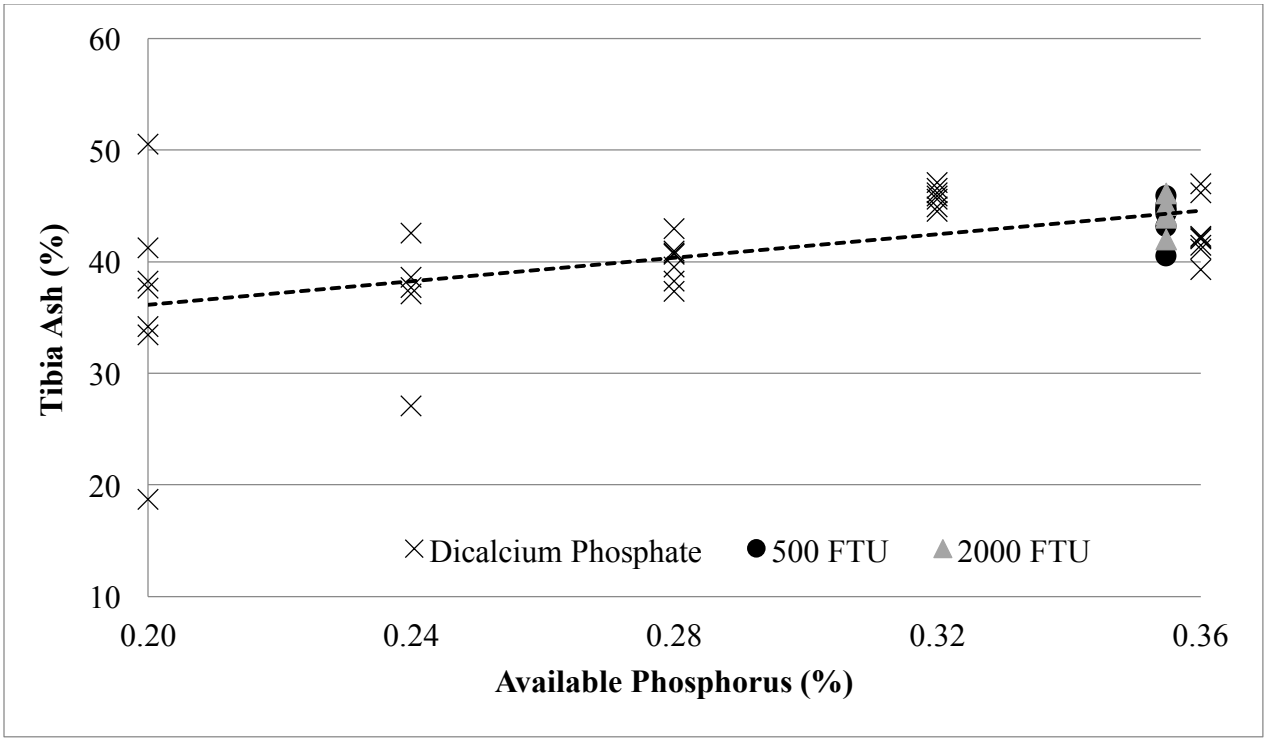

Figure 3-1. Standard curve analysis of dicalcium phosphate on tibia ash percent (%) for broilers at day 14, Experiment 2.

Linear equation analysis resulted in the equation *y = 52.612x + 25.61 (P<0.01)* with an r^2 = 0.264. Phytase inclusion (500 FTU and 2000 FTU) are also graphed. Phytase plotted to determine phytase efficacy (amount of phosphorus from the source as a result of phytase action i.e., phytate-phosphorus **PP)**. Both phytase treatments resulted in an equal amount of phosphorus availability and a release of 0.15PP.

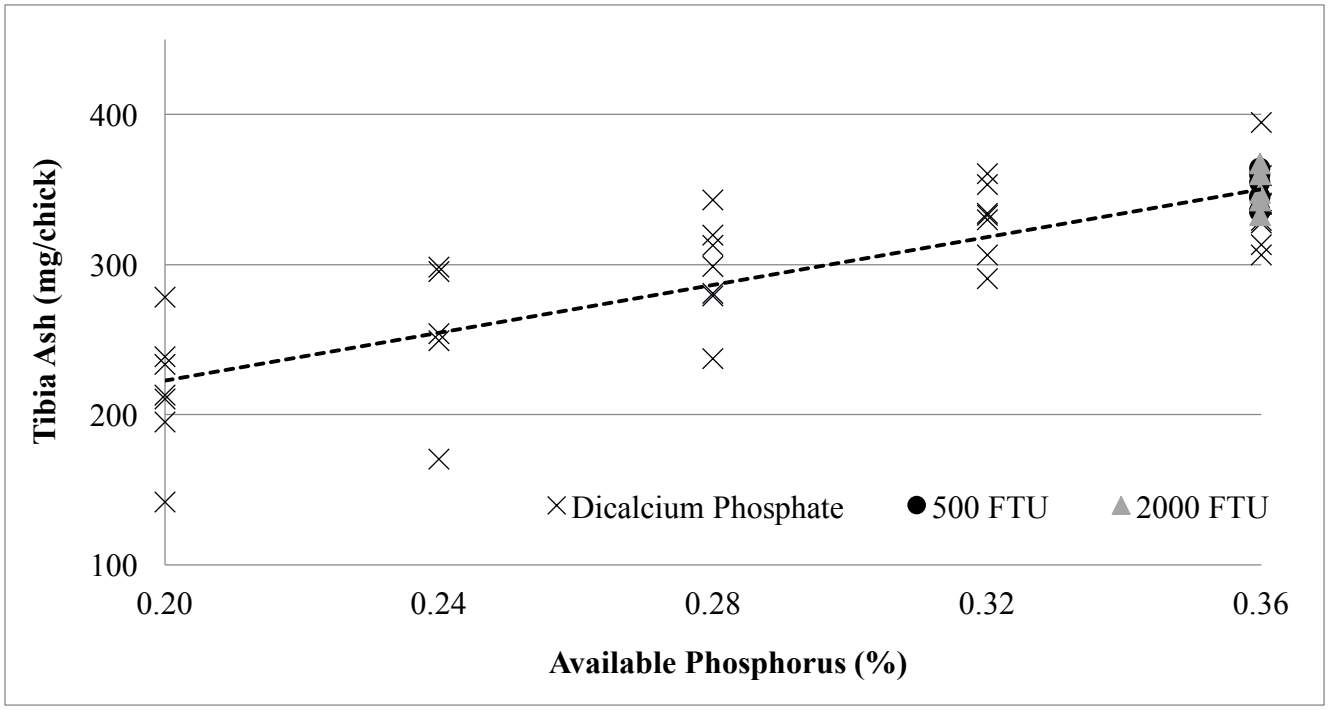

Figure 3-2. Standard curve analysis of dicalcium phosphate on tibia ash (mg/chick) for broilers at day 14, Experiment 2.

Linear equation analysis resulted in the equation y = 797.31x + 63.13 *(P<0.01)* with an r^2 = 0.*644*. Phytase inclusion (500 FTU and 2000 FTU) are also graphed. Phytase plotted to determine phytase efficacy (amount of phosphorus from the source as a result of phytase action i.e., PP). Both phytase treatments resulted with a PP release of 0.16.

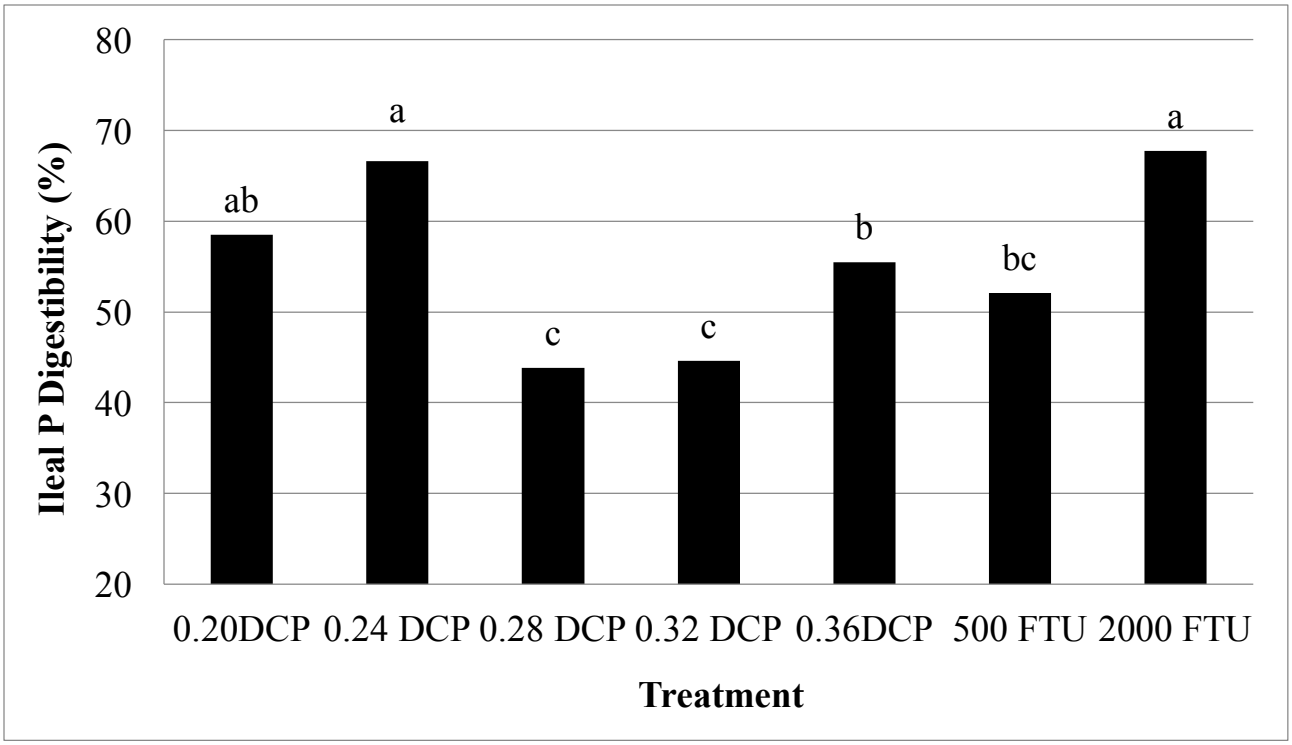

Figure 3-3. Influence of available phosphorus and phytase on apparent ileal digestibility of broilers at day 14, Experiment 2.

[a-c] Between columns, least squares means without a common letter differ (*P≤0.05*).
Columns are mean values of 8 pens per treatment with 8 birds per pen and bars are defined as overall SEM (1.73).

CHAPTER 4

GENERAL CONCLUSIONS

The objective of this thesis was to determine the effects of phytase concentration on broiler growth performance, carcass composition, P utilization, and P digestibility. Experiment 1 determined the effects of feeding a nutrient deficient (P was formulated to be limiting) diet supplemented with two phytases at three concentrations (500, 1000, and 2000 FTU/kg) on Cobb 500 broilers raised from day of hatch to 42 days. Birds fed the NC diets with the 500 and 1000 FTU/kg of phytase resulted in performance as expected with the release of P from the diet allowing those birds to grow and perform similar to the PC fed birds and better than the NC fed birds. Although the phytase B did not respond at 2000 FTU/kg (this response was unexpected and the explanation for the response is unknown), Phytase A at 2000 FTU/kg resulted in both BWG and FE significantly increased in comparison to the PC fed birds. Experiment 2 was designed to further quantify and define the P absorption and P utilization when birds were provided diets supplemented with phytase at 500 and 2000 FTU/kg. To this end, standard curve analysis using tibia ash weight demonstrated that P utilization of both 500 and 2000 FTU/kg phytase were similar despite a higher P ileal digestibility noted with 2000 FTU/kg phytase inclusion. These data might seem contrary at first observation, but taken with the extra-phosphoric growth effects noted in Experiment 1 might suggest that the major response of high phytase inclusion is not related to P absorption or P utilization, but an independent mode of action.

A possible mode of action that may explain the response in Experiment 1 is that higher concentrations of phytase have the ability to quickly hydrolyze phytate molecules reducing the formation of complexes among phytate and other dietary nutrients such as proteins (amino

acids), carbohydrates, and minerals found in the diet (Selle 2008). Phytate nutrient complexes have been observed to cause anti-nutritive effects and increase endogenous losses of the bird. These complexes have the potential to increase the production of sodium bicarbonate within the bird (**$NaHCO_3$**). The increased production of $NaHCO_3$ is a buffering response to increased acidity of digesta, a result of intact protein reaching the intestine due to indigestible phytate-protein complexes (Selle 2008). The production of $NaHCO_3$ leads to the reduction of available Na for Na-dependent transporters, which may affect the ability of the bird to actively absorb nutrients like amino acids. Gehring *et al*. (2012) observed broilers fed diets supplemented with phytase at 1000 FTU/kg and 2000 FTU/kg demonstrated increased apparent ileal digestibility of amino acids compared to 500 FTU/kg showing evidence of an extra-phosphoric effect. At 2000 FTU/kg, birds resulted in an increase in ileal digestible energy in comparison to the control diet which had no enzyme supplementation. The increased nutrient and energy digestibility has the potential to positively impact a birds' performance. Tibia ash in Experiment 1 resulted in birds demonstrating similar tibia ash percent and weight indicating similar P utilization despite being offered diets with differing phytase inclusions (phytase A at 500, 1000, and 2000 FTU/kg). This would suggest the increased growth performance resulting from the 2000 FTU/kg of phytase A was likely due to factors outside of dietary P. This was demonstrated in carcass weights from Experiment 1 where phytase A at 2000 FTU/kg resulted in increased breast weight in comparison to other phytase inclusions without differences in leg + thigh weights. In general a result of an increase in leg + thigh weights and not breast weights (bone out) would indicate increased P deposition in the carcass (Tizziani *et al*., 2016). No differences were observed in leg + thigh bone confirming tibia ash results where similar P utilization occurred between the phytase A treatments (500, 1000, and 2000 FTU/kg). The increased breast meat was a caused by

the release of nutrients other than P as the cuts measured contained no bone tissue suggesting the presence of an extra-phosphoric effect. The bird was able to utilize more of the nutrients within the diet and directed those increased nutrients towards growth performance.

The performance results of Experiment 1 and 2 were contrary as 2000 FTU/kg phytase outperformed the PC and 500 FTU/kg phytase fed birds in Experiment 1, but had similar performance in Experiment 2. In conjunction with the outcome of Experiment 1, previous experiments (Silversides *et al*., 2004; dos Santos *et al*., 2012; Walk *et al*., 2012; Walk *et al*., 2013; Walk *et al*., 2014) have demonstrated that higher inclusions of phytase above 1000 FTU/kg typically result in greater BWG and improved FE in comparison to lower inclusion rates. However, in Experiment 2, both 500 and 2000 FTU/kg phytase resulted in similar growth performance despite 2000 FTU/kg phytase fed birds displaying a higher P digestibility. In Experiment 2, phytase supplementation occurred in diets that contained 0.20% nPP which was lower than Experiment 1 where phytase treatments were supplemented to a diet that contained 0.30% nPP. This key difference could explain the contrasting results as an extra-phosphoric effect might be masked if the diet is P-limiting negating any benefits from the phytase- freed nutrients. As both the 500 and 2000 FTU/kg of phytase were added to the same diet, the concentration of phytate or substrate might have limited the phytase reaction limiting the potential growth performance of the 2000 FTU/kg phytase fed birds. In conjunction with the similar growth response observed by the 500 and 2000 FTU/kg phytase treatments, tibia ash resulted in similar mineral deposition, further suggesting that the effects of the enzyme may reach a plateau. Shirley and Edwards (2003) found that 16 day old broilers fed diets containing phytase concentrations ranging from 93.75 to 12,000 FTU/kg had no significant differences in tibia ash or P retention suggesting that the enzyme is liberating a majority of the phytate

molecules present in the diet at lower concentrations. Interestingly, the birds in this experiment demonstrated similar results as in Experiment 1 where tibia ash results revealed similar P utilization among all phytase treatments yet birds had significantly greater BWG and FE at with the 2000 FTU/kg phytase A.

The first experiment demonstrated the presence of what is interpreted as an extra-phosphoric effect when diets were supplemented with 2000 FTU/kg of phytase A. The data in Experiment 2 demonstrate that birds may not always display an increased growth performance to high inclusion phytase if total dietary nPP or available P is still a limiting factor in the growth response. Based on these experimental observations, total dietary phosphorus and the availability of this P to the bird can alter growth performance from high inclusion of phytase. As the phytase diets in Experiment 1 were formulated to have an nPP level of 0.30% and with the addition of phytase, birds either met or exceeded their P requirement (0.45%) thereby P was not a limiting. However, in Experiment 2, diets were formulated at 0.20% and even with the 2000 FTU/kg inclusion; birds were exposed to up to 0.36% AvP, roughly 0.10% lower than the recommendation.

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