Effect of Dietary Phosphorus Level and Phytase Enzymes on Broiler Performance and Bone Mineralization

by

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Abstract

A series of experiments evaluated dietary levels of non-phytate phosphorus (npP), with or without phytase enzyme supplementation in corn-soybean meal diets, on growth and skeletal performance in the broiler chicken. A preliminary experiment determined that muscle and flesh removal from bone was not required to adequately assess tibia breaking force. The first experiment investigated the effects of dietary npP concentration, maternal flock age, and bird sex on live performance. Body weight and bone strength were improved by higher concentrations of dietary npP, an increase in breeder flock age, and in male broilers. Experiment two evaluated the effects of three commercial phytase enzymes included into a 0.25% npP diet. Enzyme use improved body weight and bone strength, while decreasing overall mortality, in comparison to the unsupplemented diet. A third experiment investigated the effects of two phytase enzymes in a 0.35% npP diet on the performance of two commercial broiler strains. Results indicated no differences in body weight or bone strength between the two strains. Similar to Experiment 2, enzyme usage led to improvements in performance and bone strength in comparison to the unsupplemented diet. Two final experiments were implemented to assess the effects of phytase enzyme supplementation on birds undergoing an induced challenge with *Eimeria* spp. Birds were challenged or unchallenged and subjected to one of three coccidiosis control strategies: vaccination,

feed coccidiostat, or none. Data from both experiments confirm that phytase supplementation may release an equivalence of 0.20% npP when dietary Ca and npP are adjusted accordingly. Birds undergoing a coccidiosis challenge had reduced performance and decreased bone strength when not provided phytase supplementation. In addition, coccidiosis control programs provided improvements in bird performance and decreased incidence of coccidiosis. In Experiment 5 immune function was also assessed via cytokine production. Results indicated that IFN-γ and IL-17 expression was most likely 18 d following exposure to *Eimeria* spp. These experiments conclude that phytase supplementation of low and marginal npP diets may lead to improvements in growth and skeletal performance and may have an effect on incidence of infection and immune function in growing broiler chickens.

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Table of Contents

List of Tables

List of Figures

1.0 General Introduction

Poultry skeletal strength and development has been of interest in research for many years. As commercial broiler strains continue to be selected for increased meat production and early maturity, bone development fails to keep pace with overall growth, resulting in excess physical load, which may lead to bone deformity and fragility. Both calcium (Ca) and phosphorus (P) are intricately involved in the formation of inorganic matrix in bone, thus both minerals are vital to proper growth and development of the bone. The high costs associated with inorganic P sources, which are traditionally included into a broiler diet to meet the birds' P requirement, has often led to the inclusion of marginal levels of dietary P. Failure to meet the birds' dietary P requirement not only leads to the development of abnormal skeletal growth, but can also result in reduced performance and increased mortality.

Inorganic P sources are needed in the broiler diet due to the presence of phytic acid, a natural storage form of P in many plants. As with other monogastric animals, the chicken lacks the ability to break down this molecule in large quantities, resulting in much of the bound-P being underutilized and ultimately excreted. Phytase enzymes, naturally produced by plants and microbial populations, are capable of initiating the release of the phytic acid-bound P. The use of these enzymes enable inorganic P usage to be decreased in the diet without negatively impacting bird growth and skeletal development. In addition to dietary mineral supplementation, bird growth and skeletal

development can be influenced by a number of other factors including dietary mineral levels, maternal age, chick sex, genetics, environment and proper health.

Coccidiosis is one of the most commonly reported diseases in the poultry industry. This parasitic infection often leads to reduction in growth performance and decreased nutrient absorption. Control programs have been implemented to reduce the incidence of coccidiosis. Vaccination strategies may be included in these programs, which may lead to reductions in initial bird performance due to an acute parasitic infection. Dietary supplementation with a variety of enzyme combinations have been utilized to combat the negative side effects of coccidiosis, yet little research exists with regard to the effects of phytase supplementation alone.

As with many infections, infiltration of the intestine by *Eimeria* spp. leads to an immune response. Dependent on the type of infection, the immune system activates both antibody and cell-mediated immune responses. Cell-mediated immunity plays an active role in disease resistance and heavily relies on the activation and production of a number of cytokines. The assessment of immunity via cytokine gene expression is still a relatively new phenomenon since many cytokine genes in chickens were not discovered until the initiation of the chicken genome project. Therefore, little research has been conducted to assess the possibility of enzyme supplementation or dietary mineral levels on the immune response via the production of cytokines.

Given this knowledge, the research described in the following chapters represents the evaluation of phosphorus levels and/or phytase enzymes under a variety of different parameters.

2.0 Review of Literature

2.1 Calcium and Phosphorus

Extensive reviews and book chapters on calcium (Ca) and phosphorus (P) in poultry have been previously published by Larbier and Leclercq (1994), Klasing (1998), and Leeson and Summers (2001).

Calcium

Ca is the most abundant mineral in the body, constituting more than one third of the total mineral content in the adult bird. It is an essential constituent of all living cells, is required for numerous enzyme activities and has a fundamental role in blood coagulation. Ca is also one of the most metabolically active minerals, requiring its metabolism to be regulated within ± 5% of the normal range (Cunningham, 1992; Klasing, 1998). Despite it's many functions, the Ca demand is highest for the mineralization of the skeleton and the eggshell.

The majority of Ca is concentrated in the skeleton as hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, along with non-crystalline phosphates and Ca carbonate. The hydroxyapatite form provides the bone with structural rigidity, but can be solubilized to meet tissue demands for its minerals when needed (Klasing, 1998). Low concentrations of Ca are also present in the intra- and extracellular fluids. This supply allows Ca to play its role in cellular functions such as the trasmission of nerve impulses and muscle contractions (Leeson and Summers, 2001).

Dietary Ca is absorbed in the duodenum and jejunum, via passive diffusion and active transport (Norman and Hurwitz, 1993; Hurwitz et al., 1995). Ca absorption is dependent on the plasma protein calbindin, whose synthesis is controlled by the hormones 1,25-dihydroxycholecalciferol ($1,25\text{-}(OH)_2D_3$; 1,25-dihydroxy D_3) and parathyroid hormone (PTH). When ionized Ca (Ca^{2+}) levels in the blood become low, PTH is secreted from the parathyroid to stimulate the mobilization of bone Ca and increase kidney resorption (Leeson and Summers, 2001). This leads to an increase in plasma Ca, resulting in the activation of 1,25-dihydroxy D_3 synthesis in the kidneys. Synthesis of 1,25-dihydroxy D_3 in turn stimulates the synthesis of calbindin, which binds Ca for transport across the epithelial cells of the intestine.

Many factors influence the rate of intestinal absorption. Low dietary concentrations of Ca will increase intestinal absorption, while higher levels will cause a reduction. Dietary source can affect absorption, depending on the size and solubility of the ingredient. Dietary constituents may also negatively impact absorption via the formation of insoluble salts. A deficiency of vitamin D_3 can also dramatically reduce the absorption of intestinal Ca (Larbier and Leclercq, 1994). Physiological status of the bird also has a great impact on the amount of Ca absorbed.

About 25% of blood Ca can be found as the ionized form, while the remainder is bound to proteins or complexed with citrate, phosphate or sulfate (Klasing, 1998). Several hormonal control mechanisms are in place to maintain proper plasma concentrations of Ca for intracellular and metabolic activities. When plasma Ca levels are low (hypocalcemia), the hormone mechanism previously described for Ca absorption occurs. In the event of hypercalcemia (excess plasma Ca), an inverse reaction is induced.

High blood Ca stimulates the secretion of calcitonin (CT) from the thyroid, causing the inhibition of osteolysis and thus the liberation of Ca (Larbier and Leclercq, 1994). This results in increased mitochondrial uptake of Ca^{2+} and renal excretion of Ca and other minerals such as P, Na, K, and Mg.

Ca requirements may be separated into two components, those associated with maintenance and those with production. The Ca required for maintenance is based on the daily amount of endogenous loss via the excreta. Loss through these routes is dependent on other dietary factors such as P level and dietary acidity (Klasing, 1998). Since chickens are rarely maintained when out of growth or egg production, the maintenance requirement for Ca is not well established, but is generally less than 0.2% of the diet for an adult chicken (Klasing, 1998).

Ca requirements for the growing chick have been established by determining the dietary level that maximizes bone ash and bone strength. At hatch, the requirement is close to 1.0% of the diet, assuming available P levels are 0.45-0.50% (NRC, 1994). As the growth rate declines, the Ca requirement also declines. Until sexual maturity, Ca requirements can be met with dietary levels of 0.8 to 1.0% (Leeson and Summers, 2001). Ca requirements in laying hens are greater than any other stage of production due to the high Ca draw during eggshell formation. Typically an egg-producing hen requires Ca to be included at a rate of 3.0-4.0% of the diet (NRC, 1994). These levels allow for maintenance of eggshell quality without over-depleting the hen's Ca stores.

Phosphorus

Phosphorus is an essential nutrient for the development and maintenance of the skeletal system. This mineral also plays an important role in the metabolism of amino

acids, carbohydrates and fats, muscle coordination, the blood buffering system, and lipid transport. As an integral part of the phosphate molecule, P is important for DNA and RNA, is a component of many coenzymes, and is involved in the storage and transfer of energy in phosphorylated compounds (Leeson and Summers, 2001).

An abundant portion of P is bound in the bone as hydroxyapatite and non-crystalline phosphates. The remaining P may be found in phospholipids, nucleic acids, and several other metabolic molecules. High-energy phosphate bonds are involved in short-term storage of energy as adenosine triphosphate (ATP) and in energy transfer during metabolism (Klasing, 1998).

Phosphorus absorption, in the form of phosphate, occurs in the jejunum either via either passive diffusion or active transport (Larbier and Leclercq, 1994). The active transport of P is coupled with the co-transport of sodium and the rate of transport is stimulated by 1,25-dihydroxy D_3. Dietary P can be absorbed from inorganic sources such as mineral or inorganic phosphate, or from organic sources such as phosphoproteins or phospholipids (Larbier and Leclercq, 1994; Ravindran et al., 1995a; Soares, 1995).

Much like Ca, there are many factors influencing the intestinal absorption of P. Dietary sources and available P concentrations can impact absorption. For example, plants sources that are high in phytic acid may reduce the utilization of P. High levels of Ca can also decrease P absorption by forming precipitates in the intestine (Klasing, 1998). Levels of vitamin D_3 can also affect intestinal absorption.

Phosphorus concentrations are controlled by the same systems controling Ca (Cunningham, 1992). As described previously for Ca, P is mobilized from the bone via the stimulation of PTH. The kidney also maintains P balance. By augmenting the

synthesis of 1,25-dihydroxy D_3, PTH stimulates P resorption from the proximal kidney tubules, while simultaneously promoting excretion from the distal kidney tubules. This results in an increased phosphate secretion in the urine.

P requirements coincide with the bird's requirements for Ca, which typically equates to about half of the required amount of Ca (NRC, 1994). Adult birds at maintenance can meet their P requirements via bone reserves and output from the kidney. On the other hand, young growing birds and laying hens must be supplied with dietary levels of P sufficient to meet requirements for synthetic processes (Larbier and Leclercq, 1994). The requirement for both Ca and P decreases with increasing age in growing broilers. Young pullets require similar P levels until about 18-20 weeks of age, when the bird is preparing to go into egg production (NRC, 1994). At that time, P levels may continue to decline as low as 0.10%, while the requirement for Ca is significantly increased.

Dietary Calcium to Phosphorus Ratio

Dietary sources of Ca and P interact in the body during absorption, metabolism and excretion (Klasing, 1998). The ratio of Ca to P in bone is approximately 2:1. In order to maintain optimal levels of these minerals in bone, dietary inclusion rates of available Ca and P levels must also be maintained at a ratio of 2:1. Adequate concentrations of Ca and P are important for promoting bone deposition rather than bone resorption. Decreasing dietary P content results in an increase in P retention, but also leads to a decrease in Ca retention (Viveros et al., 2000). While a ratio of 2:1 is often used by the industry, Shafey (1993) indicated that ratios ranging from 1.4:1 to 4:1 may be tolerated when adequate amounts of vitamin D are supplied.

Calcium and Phosphorus in the Bone

Requirements for Ca and P are closely interrelated with bone formation. New bone formation is initiated by osteoblastic degradation of cartilage at the epiphyseal growth plate, located on either end of long bones, and is followed by the deposition of collagen (Leach and Gay, 1987; Hurwitz and Pines, 1991; Bain and Watkins, 1993). Ca and P are transported to the growth plate via invading blood vessels for precipitation into hydroxyapatite crystals, allowing the collagen matrix to calcify (Leeson and Summers, 2001). This continued calcification on the outer surface allows for the bone to elongate and increase in width.

Unlike osteoblasts, osteoclasts are responsible for the removal of inorganic bone mineral. They are typically located on the inner surface of the bone and stimulate the breakdown of hydroxyapatite crystals during growth and repair (Cunningham, 1992; Klasing, 1998; Leeson and Summers, 2001). This functions to prevent excess thickening or over calcification of the bone.

The process of osteoclastic and osteoblastic activity is referred to as bone remodeling and permits a simultaneous increase in bone length and diameter. Once bone growth has ceased, the bone remodeling continues to be hormonally regulated. PTH stimulates osteoclastic activity, while calcitonin triggers the osteoblasts. Bone deposition and resorption is a continuous process that results in a constant turnover of Ca, P, and other minerals for bodily functions.

Calcium and Phosphorus Deficiencies

Calcium deficiencies may result from low dietary levels of Ca, excess dietary levels of P, or from insufficient levels of vitamin D. Phosphorus deficiency can be

caused by low dietary intake of the mineral or by a wide Ca-to-P ratio. For both minerals, symptoms of deficiency are typically more evident in young, growing birds. Initial signs of a deficiency for either mineral in growing birds include retarded growth, decreased feed consumption, reduced activity, and poor bone mineralization. As the deficiency becomes more severe, it can lead to skeletal abnormalities such as rickets, dyschondroplasia, lameness, joint enlargement, and mis-shapen bones. If not corrected, a deficiency in either mineral can result in increased mortality. Adult birds consuming a Ca-deficient diet mobilize bone at a rate faster than it is deposited, causing the bones to become weak. If the bones become too demineralized, the bird may be unable to stand and can appear paralyzed (NRC, 1994). In addition, the sternum and rib bones will often become deformed and the chances of bone breakage throughout the body are significantly increased (NRC, 1994; Klasing, 1998).

Assessing Calcium and Phosphorus Adequacy

Bone status is commonly used as an indicator of Ca and P adequacy in poultry diets since these minerals form the majority of the inorganic bone matrix and therefore, have a direct effect on bone strength (Reichmann and Connor, 1977; Ali, 1992; Watkins, 1992; Rath et al., 1999). In the poultry industry, changes in bone integrity and quality have historically been measured using invasive techniques such as toe, foot or bone ash, mineral analysis of ash, and bone breaking force.

The tibia is the fastest growing bone in the body of the chick, making it the most susceptible to Ca and P deficiencies (Mclean and Urist, 1961; Nelson and Walker, 1964). For this reason, the tibia bone is most often chosen for analysis of bone ash to determine availability of Ca and P in broilers (Qian et al., 1996). There are multiple methods used

to estimate tibia ash, though the Association of Official Agricultural Chemists (AOAC, 1955) method has historically been the method most often used for assessing tibia ash. This method includes the extraction of fats with ethanol and anhydrous ether prior to ashing to minimize the effects of differences in growth rate. Other methods may differ in the way flesh and muscle is removed, how the fat is extracted, whether the bones are dried or not, and how the bone is ashed, including whether the cartilage caps remain or are removed. Orban et al. (1993) showed variations in methodology could alter the final results.

Toe ash is another means for evaluating bone development. This method is typically considered to be both simpler and less labor intensive since it does not require lipid extraction. Since it's initial use in the early 1940's, several authors have shown toe ash to be at least as sensitive to changes in dietary Ca and P as tibia ash (Baird and MacMillen, 1942; Evans and Carver, 1944; Fritz and Roberts, 1968; Yoshida and Hoshii, 1983; Yan et al., 2005). Ravindran et al. (1995b) actually reported toe ash, in combination with body weight, to be slightly more sensitive at detecting differences in P availability than tibia ash.

Dale and Garcia (2004) suggested the use of foot ash as an alternative to toe ash for evaluating bone mineralization. In addition to providing a larger sample size, it was theorized that ashing the foot would be more sensitive than other tests because the heads of each bone contain the growth plate, which are more sensitive to differences in mineralization (Garcia and Dale, 2006). Results from this work showed that foot ash is able to reflect differences in dietary levels of P at least as well as tibia ash.

While ashing of the foot, toes, or tibia bones is beneficial in assessing Ca and P

adequacy, these techniques can be time consuming and labor intensive. As an alternative method, bone-breaking strength via shear force has been utilized to assess bone mineralization. As with preparing bones for ashing, this method involves the complete removal of associated flesh and muscling prior to analysis. This can be conducted by either boiling the leg for five to six minutes, removing the flesh and allowing the bones to dry for 18 to 48 hours (Rowland et al., 1971; Travis et al., 1983; Ruff and Hughes, 1985) or by removing the raw flesh without cooking. Bones can then be tested, either via shear force or a three-point bending test, immediately or frozen and thawed. The latter is typically used to allow time to evaluate a large number of samples.

Several researchers have investigated the effects of preconditioning (fresh, dried, and frozen-thawed bones) on bone strength. Using the three-point bending test, Littlefield et al. (1973) assessed the humerus of five-week-old broilers and detected no difference in breaking strength between bones that were fresh, frozen-thawed, or dried and stored. Using the same testing mechanism to assess tibiae of four-week-old broilers, Lott et al. (1980) also found no differences between fresh and frozen-thawed bones. These researchers did, however, detect a 50% decrease in strength for bones that had been dried. Furthermore, they noted that the three-point bending test was more sensitive to differences in bone strength between male and female birds if the bones were fresh or frozen-thawed. Wilson et al. (1989) assessed laying hen tibia, humerus, and radius bones for strength via shear force when they were fresh or frozen thawed. Their results indicated no difference between pre-conditioning for any of the three bone types. Wilson and Mason (1992) also used shear force to evaluate preconditioning of laying hen tibia bones on bone strength. Their findings indicated no difference in breaking strength due

to the three types of preconditioning.

2.2 Phytic Acid

Historical Background

Knowledge of phytic acid was first associated with a discovery by Hartig (1855). Small particles were isolated from the seeds of various plants, which he considered to be essential for seed germination and growth of the plant. In 1872, Pfeffer compiled a group of seeds that he called 'globoids', which had no traces of N, but contained Ca, Mg and P. These globoid particles were later fully removed from the seed via a chemical method established by Palladin (1894). In 1897, Winterstein suggested the globoids be named 'inosite-phosphoric acid' since the compound yielded inosite and phosphoric acid following hydrolysis.

During the early 1900's, Posternak (1900; 1903; 1905) successfully prepared the compound in a pure form to better study its physical and chemical characteristics. He rejected the name proposed by Winterstein (1897) and proposed that the compound, which did not contain the inositol ring, be called 'phytin'. Upon further examination, Posternak determined that inosite could be synthesized from the products of hydrolysis when the phytin was heated under pressure. In 1910, Contardi demonstrated that phytin was a salt of the inosite-phosphoric acid. By 1921, many agreed and it was accepted that phytic acid was the hexaphosphate of myo-inositol (Starkenstein, 1914; Posternak, 1921).

Structure and Occurrence

Phytic acid (myo-inositol 1,2,3,4,5,6 hexakis phosphate), serves as a storage form for P in many plant tissues. This molecule is composed of six phosphate groups

extending from a central inositol ring structure (Figure 1). Phosphate groups are attached in an equatorial position at carbons 1, 3, 4, 5, and 6 while the phosphate at the 2-carbon (C_2) position is in the axial position, thus making it difficult to remove (Reddy et al., 1982).

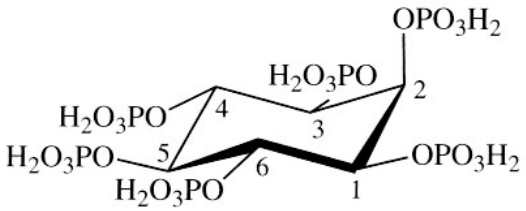

Figure 2.1 Structure of phytic acid (myo-inositol 1,2,3,4,5,6 hexakis phosphate).

The salts of phytic acid are referred to as phytates. Approximately two thirds of P may be dispersed throughout the plant as phytate, with the location differing from plant to plant. Low amounts of phytate are found in the roots and trace amounts are found in the vegetative portion of the plants (Nahm, 2007). Most of the phytate is located in the seed (Angel et al., 2001). For corn, phytate is localized within the germ, but is often found in the allerone layers and pericarp of other small grains (O'Dell and de Boland, 1976). Phytic acid is located within the protein bodies of legumes (Baker, 1991). With the exception of soybeans, which have no known specific location for phytate, phytate accumulates within the cotyledon of legumes (Kornegay, 2001).

There have been extensive reviews depicting the occurrence of phytic acid in a wide variety of plants (Reddy et al., 1982; Ravidran et al., 1995a). The amount of phytic acid varies from 0.50 to 1.89% in cereal grains, from 0.40% to 2.06% in legumes, and from 2.00 to 5.20% in oil seeds (Reddy et al., 1982). Phytic acid content varies and is

dependent on plant variety and climate, location and soil type. Average concentrations of phytate in some commonly used poultry feedstuffs are as follows: 0.24% in corn, 0.27% in wheat, 0.27% in barley, 0.70% in canola meal, and 0.39% in soybean meal (Ravindran et al., 1995a). Utilizing a combination of these plant sources in the diet could result in the inclusion of bound P at over 1%.

Chemical Characteristics

Phytic acid contains a total of twelve dissociable protons (Costello et al., 1976). Six of these are highly acidic, with a pKa value of approximately 1.8, which means when the molecule is exposed to an extremely low pH, the protons will remain attached to the molecule. If, however, the pH begins to increase, hydrogen atoms will release from the phytic acid molecule, resulting in six negative charges. Three of the remaining dissociation sites are weakly acidic, with pKa values of 5.7, 6.8, and 7.6. The remaining three hydrogens are very weakly acidic, with pKa values greater than 10.

Anti-nutritional effects

The interaction between phytic acid and proteins is thought to be of ionic type and can lead to decreased protein solubility (de Rham and Jost, 1979; Cheryan, 1980). Complexes between proteins and phytic acid can form at either an acidic or alkaline pH, though success of the complex is dependent on the protein having an electrical charge. Phytate has a strong negative charge at a pH of about 2.0, while many proteins have a strong positive charge at the same pH (Reddy et al., 1982). Under such circumstances, a phytate-protein complex can rapidly form, resulting in nonionic irreversible reactions (Hill and Tyler, 1954; Smith and Rackis, 1957; Saio et al., 1967; Okubu et al., 1975). The complex between phytate and protein works differently at an alkaline pH. At higher

pHs, multivalent cations such as Ca^{2+} mediate the interaction, resulting in a tertiary complex between the cation, phytate and protein (Saio et al., 1967; Okubo et al., 1975; Omosaiye and Cheryan, 1979). As a result of either phytate-protein complex, protein solubility can be severely reduced which may lead to decreased functional capabilities.

Due to phytate being a strong acid, it not only complexes with proteins, but can form a wide variety of insoluble salts with di- and trivalent cations at a neutral pH (Oberleas, 1973). Phytate will often complex with the following multivalent cations at different intensities, rendering them unavailable to the bird: $Zn^{2+} >> Cu^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+} > Mg^{2+}$ (Maddaiah et al., 1964; Vohara et al., 1965; Maenz et al., 1999). In general, an inverse relationship exists between the dietary phytate content and the digestibility of these minerals, resulting in an increased nutrient requirement each of the minerals. The complex between phytate and Ca or Zinc has received the most attention within the area of poultry nutrition.

In a typical broiler diet, Ca is the mineral added in the highest concentration, making it the cation with the greatest impact on mineral availability, even though it has one of the lowest affinities for binding with phytic acid (Oberleas, 1973; Nelson and Kirby, 1987; Maenz et al., 1999). Nelson and associates (1968) noted the Ca requirement of chicks fed a phytate-free diet was 0.50%, which increased to 0.95% with the addition of 1.25% phytate. From this, it was recommended that broiler Ca requirements be calculated as follows:

$$\% \text{ Dietary Calcium} = 0.6\% + (\% \text{ phytate phosphorus} + 1.1)$$

Thus, diets containing high levels of phytate should require increased amounts of Ca to offset the portion that is unavailable due to the calcium phytate complex.

Zinc (Zn) bioavailability is also affected by phytate content of the diet (Forbes et al., 1984; Saha et al., 1994). At a ph of 6.0, the approximate pH associated with the upper small intestine where most mineral absorption occurs, phytate forms a highly insoluble complex with Zn (Maddaiah et al., 1964). This complex most often leads to a growth depression, which can be overcome by the addition of supplemental Zn (O'Dell, 1962). Dietary Ca concentrations may also affect the availability of Zn. Mills (1985) suggested that excess Ca initiates a co-precipitation with zinc to the phytate molecule. The resulting Ca-Zn-phytate complex has been shown to be less soluble than the Zn-phytate complex at a pH of 6.0 (Reddy et al., 1982).

2.3 Phytase Enzymes

Suzuki et al. (1907) were the first investigators to make a preparation of phytase. They detected phytase activity in rice and wheat bran and isolated inositol as a product of the reaction. In 1937, Patwardhan first showed that via the action of phytase, the intestine could remove phosphate from the phytic acid molecule.

Characteristics

Phytase (myo-inositol hexaphosphate phosphohydrolase) is from a family of enzymes which catalyzes the stepwise removal of inorganic phosphate from phytic acid (Nayani and Markakis, 1986). The enzyme acts on inositol hexaphosphate to yield inositol and orthophosphate, resulting in inositol penta- to monophosphate as intermediary product(s) (Reddy et al., 1982). The Nomenclature Committee of

International Union of Biochemistry (IUB, 1979) lists two types of phytase. The first, 3-phytase, (enzyme classification 3.1.3.8) is typically derived from microbial and fungal populations and will initiate hydrolysis of the phosphate at the C_3 position:

myo-inositol 1,2,**3**,4,5,6 hexakisphosphate + H_2 →
D-myo-inositol 1,2,4,5,6 pentakisphosphate + orthophosphate

The second phytase classification, 6-phytase (enzyme classification 3.1.3.26), is typically isolated from plants and will first hydrolyze the molecule at the C_6 position:

myo-inositol 1,2,3,4,5,**6** hexakisphosphate + H_2O →
1-L-myo-inositol 1,2,3,4,5 pentakisphosphate + orthophosphate

The 6-phytase can completely dephosphorylate the phytic acid molecule, while the 3-phytase cannot hydrolyze the axial phosphate at the C_2 position (Wodzinski and Ullah, 1996).

Phytase enzymes may have up to two pH optima, though the number of optima each enzyme has is dependent on the original source. These differences in pH optima can result in varied effectiveness per unit of activity (Eeckhout and De Paepe, 1996). Regardless of the source or number of optima, phytase enzymes are generally active at a pH found within the crop, proventriculus and gizzard, with a tendency to lose activity within the small intestine.

Sources

Phytase is naturally produced by a variety of animals, plants and microbes (Patwardhan, 1937; Cosgrove, 1966). Some phytase activity, independent of non-specific phosphate enzymes, is located at the intestinal brush border membrane in poultry (Maenz et al., 1997). The amount of activity is dependent on the age of the bird, since the enzyme tends to be present at higher concentrations with increased age (Peeler, 1972). Axe (1998) indicated that poultry might utilize phytate up to 10% as chicks and 20% as laying hens. Since the amount of phytase activity within the intestine tends to vary, it is generally assumed to be of little significance without exogenous enzyme supplementation.

Almost all plants contain some phytase activity, although the level of activity varies greatly among plant species and stage of development. High levels of activity have been determined in cereal grains such as rye, triticale, wheat and barley while other grains and oil seeds contain little to none of the enzyme (Eeckhout and De Paepe, 1994). Phytase is inactive in dry seeds, though activity rapidly increases during germination, when phytase is synthesized to release P to meet the needs of the growing plant (Ma and Shan, 2002).

Diets formulated with cereals or cereal by-products with high phytase activity can improve P retention (Pointillart, 1991, 1993; Barrier-Gillot et al., 1996). During the 1960's, Temperton and colleagues (1965a, b) conducted research, which indicated that plant phytase was active when fed to the animal. During these studies, birds were fed a diet with 32-36% wheat and 10% barley meal that was free of animal or inorganic P sources, yet chicks were able to utilize the phytate P for proper bone mineralization.

The effectiveness of plant phytases are dependent on the pH of the digestive tract, which will be discussed in more detail later, and on how the ingredients and total feed are processed. Plant phytases are susceptible to denaturation at normal pelleting temperatures. Research has shown that plant phytases are stable at pelleting temperatures ranging from 40-60 ^{o}C, with partial or total inactivation at temperatures exceeding 70 ^{o}C (Pointillart, 1988; Jongbloed and Kemme, 1990).

Eeckhout and De Paepe (1994) previously demonstrated that microbial phytases hydrolyze phytate more efficiently than phytase derived from plants. Microbial phytase can be derived from fungi, bacteria and yeast sources. A detailed list of specific phytase producing microorganisms may be found in the paper by Vohra and Satyanarayana (2003). Nelson et al. (1971) were among the first to investigate the hydrolysis of dietary phytate in a corn-soybean meal diet via the use of an *Aspergillus* produced phytase. Because of this, phytase enzymes produced by the *Aspergillus spp.* are the most extensively studied.

Two major factors led to the development of affordable commercial microbial phytases for use in poultry and swine rations. The first was due to the advances in biotechnology that led to techniques for genetic modification of fungi. The second was the mandate for Dutch farmers to reduce P excretion (Kornegay, 2001). Other contributing factors included identification and isolation of the phytase gene, as well as improvements in fermentation technology.

Traditionally, the phytase producing microbial organism has been subjected to either submerged or solid state fermentation (Pandey et al., 2001). Once phytase has been produced as a metabolite of the microbe, it is purified and utilized as a commercial

enzyme. Progress in recombinant DNA technology has allowed for the production of phytases with improved functional properties. These phytases are commonly obtained from fungal or bacterial sources and the DNA code is spliced into yeast for mass production. Alterations are made within the genetic sequence to slightly alter the amino acid composition at specific locations. This allows for improvements in functionality such as improved heat stability. As with sources of plant phytase, the efficacy of microbial phytase supplementation is dependent on the source, pH optima, dietary mineral concentrations, diet manufacturing, and other factors (Ravindran et al., 1995a).

Site of Activity

The optimum pH for phytase activity varies between each phytase product and is highly dependent upon the source. Plant phytases generally have an optimum pH of 4.0-7.5 and optimum temperatures of 40-60 ^{o}C (Wodzinski and Ullah, 1996). Microbial phytases have an optimum pH range of 2.5-7.5 and a temperature range of 35-65 ^{o}C (Wodzinski and Ullah, 1996). In comparison with plant phytases, microbial phytases are typically more effective within the gastrointestinal environment because of a wider pH range, allowing them to retain activity in the proventriculus and gizzard where the pH is much lower (Simons et al., 1990).

Liebert et al. (1993) reported that 69-86% of phytase activity occurs in the crop and 31-38% of the activity takes place in the proventriculus. This activity coincides with the peak solubility of phytin, however a lack of phytase activity in the small intestine limits the time phytase can act to hydrolyze the substrate. This lack of activity may be due to the more basic pH (5.6-7.0) of the small intestine (Farner, 1942) that is less

favorable for phytase activity or to the presence of proteolytic enzymes that may degrade the phytase (Kornegay, 2001).

Phytase activity is measured in terms of inorganic P released from phytic acid. Within the scientific literature, the activity of a phytase enzyme may be expressed as FYT, FTU, PU, or U. All of these units have similar meaning in that one unit of phytase can be defined as the amount of enzyme that liberates 1 micromole of inorganic P per minute from an excess of sodium phytate at pH 5.5 and 37^{o}C. A pH of 5.5 was chosen because this is the activity optimum for the *A. niger* phytase (Engelen et al., 2001), while 37^{o}C was chosen based on its similarity to the temperature of the digestive tract.

Factors affecting activity

The effectiveness of each phytase enzyme is dependent on a multitude of factors that occur from the processing of the feed through to the environment of the digestive tract once the enzyme has been consumed by the bird.

Pelleting temperature. Enzymes used as animal feed supplements should be able to withstand temperatures of 60 to 90°C, which may be reached during the feed pelleting process. Many exogenous phytase enzymes are susceptible to denaturation at these temperatures, which can have an effect on the phytase activity within the intestine. Wyss et al. (1998) compared two microbial phytases to determine their heat stability. Neither phytase derived from *A. niger* or *A. fumigatus* were thermostable at pelleting temperatures between 75 to 80^{o}C. This instability led to a loss of 70-80% activity of the *A. niger* phytase due to its inability to refold properly after denaturation. On the other hand, the *A. fumigatus* phytase was able to refold to its native state following heat denaturation, resulting in full enzymatic activity. This same study went on to show that

exposure to temperatures greater than 90°C led to irreversible denaturation and a loss of 49-86% enzymatic activity of the *A. fumigatus* phytase.

pH. All phytase enzymes have the ability to hydrolyze phytate, however the range of activity can vary with enzyme source and diet composition. As previously mentioned, most phytase enzymes have optimal hydrolyzing activity within a pH range of 2.5-7.5, with an average optimal pH of 5.0 (Maenz, 2001). Phytase is therefore most active prior to reaching the small intestine. As the digesta moves toward the small intestine, its pH increases, which favors the formation of phytate-mineral complexes. Given the relatively short length of the avian digestive tract, it is important to try to increase retention time in the areas of lower pH (crop, gizzard, and proventriculus) to achieve maximum hydrolysis.

An alternative to increasing retention time is to include organic acids in the feed. The addition of organic acids, such as citric, formic, and ascorbic acids, is known to lower diet acidity (Nahm, 2003). Via their effect on the diet and intestinal pH, these acids are able inhibit or delay certain strains of bacteria. Boling-Frankenbath et al. (2001) found that the inclusion of 6% citric acid into a corn-soybean meal broiler diet spared up to 0.15% P. Nassiri-Moghaddam et al. (2007) provided data suggesting that the combination of 500 U/kg phytase and 2 to 4% citric acid in a 0.30% available P diet improved phytate hydrolysis, leading to an improvement in mineral bioavailability. In pigs, Omogbenigum and colleagues (2003) found the combination of 500 U/kg phytase plus 0.35% citric acid was able to completely replace the need for inorganic P due to the increased phytase hydrolysis at the reduced pH.

Mineral level. The effectiveness of phytase enzymes is influenced by dietary levels of Ca and P (Edwards and Veltman, 1983; Mohammed et al., 1991). Ca is generally included in higher concentrations within the diet of poultry, allowing for an easily formed precipitate with phytate in the gastrointestinal tract, making it inaccessible to the phytase enzyme. Most trials have shown that phytase activity is less beneficial when included into high Ca diets, suggesting that phytate hydrolysis is inversely related to dietary Ca levels (Singh, 2008). Mohammed et al. (1991) showed that phytate hydrolysis was increased by 15% when dietary Ca levels were reduced from 1% to 0.5%.

Phytate hydrolysis also decreases with a high inclusion of inorganic P (Ballam et al., 1984; Karim, 2006). Wise (1983) suggested that excess intake of inorganic P may inhibit the catalytic activity of microbial phytase within the avian digestive tract. The Ca:P ratio has also been shown to have an effect on efficacy of phytase. Nelson (1967) reported a dietary Ca:P ratio of 2:1 impaired the digestion of phytate. A Ca:P ratio of 2:1 causes the formation of an insoluble penta-calcium phytate complex in the intestine, which was resistant to phytase activity. Others have shown that feeding a narrower Ca:P ratio, such as 1:1 vs. 2:1, results in improved phytase hydrolysis (Vandepepopuliere et al., 1961; Qian et al, 1996).

Vitamin D availability. Diets marginal or deficient in vitamin D_3 or its metabolites (*i.e.* 1,25 $(OH)_2D_3$) decrease the efficacy of phytase (Ewing, 1963; Carlos and Edwards, 1998). This may be due to the stimulation of P transport mechanisms within the intestine (Mohammed et al., 1991; Biehl and Baker, 1997). Phytase activity was enhanced up to 68% in chickens provided low P diets supplemented with vitamin D_3 (Mohammed et al., 1991; Edwards, 1993).

Nutritional Benefits

Minerals. It is well documented that phytase increases the bioavailability of phosphorus. Different criteria have been used to determine availability of dietary phosphorus including tibia, toe or foot ash, bone breaking strength, and weight gain. Simons et al. (1990) reported that supplementation of a low phosphorus corn-soybean meal diet with a microbial phytase was able to increase the availability of phosphorus by over 60%. McKnight (1996) found that inclusion of a microbial phytase into a broiler grower diet containing 0.325% available phosphorus resulted in increased P content of the bone. The addition of microbial phytases in low P broiler diets consistently releases P from phytate, generally increasing P availability by 20-40% (Selle and Ravindran, 2007; Singh, 2008). The amount of P made available with the use of phytase enzymes is dependent on factors previously discussed such as dietary mineral and vitamin D_3 content as well as source (Simons et al., 1990) and concentration (Kornegay et al., 1996).

Previous research indicates that microbial phytase plays a role in improving calcium availability in broilers (Schoner et al., 1993, 1994; Sebastian et al., 1996b; Singh and Khatta, 2003). The inclusion of 500 U/kg of microbial phytase was found to equate to 0.46g of Ca based on body weight gain and bone ash (Schoner et al., 1994). Calcium digestibility and retention were improved with the inclusion of phytase into broiler diets (Yi et al., 1996a; Qian et al., 1997; Kornegay et al., 1998). Ahmad et al. (2000) observed an increase in apparent availability of Ca in low P diets supplemented with phytase. Other research has shown improvements in bone mineralization due to the liberation of Ca and P by the phytase enzyme (Perney et al., 1993).

Phytate is known to form a variety of complexes with several cations besides Ca, rendering them biologically unavailable to the bird (Maddaiah et al., 1964; Oberleas, 1973). Variable amounts of these bound minerals can be released when phytic acid is hydrolyzed by microbial phytases. Multiple researchers have shown that microbial phytase inclusion in a corn-soybean meal diet increases zinc availability and utilization, allowing for zinc levels to be reduced in the diet (Thiel and Weigand, 1992; Yi et al., 1996b; Mohanna and Nys, 1999). Jondreville et al. (2007) determined that 100 FTU of phytase was equivalent to 1 mg of zinc sulphate.

Inclusion of phytase into the diet has led to improvements in the utilization of copper and magnesium as well (Pallauf et al., 1992; Sebastian et al., 1996a; Mohanna and Nys, 1999). Aoyagi and Baker (1995) found that Cu utilization was improved in cottonseed meal by 34% but was decreased by 21% in soybean meal, suggesting improvements associated with phytase enzyme inclusion are dependent on dietary ingredients. Magnesium utilization has also been improved by approximately 20% in broilers fed a low P diet that included a microbial phytase (Viveros et al., 2002). Despite findings for the aforementioned minerals, there is little other research available with regard to the effects of phytase on other phytate-bound minerals.

Protein and Amino Acids. While much research has been conducted to determine the effects of phytase on mineral bioavailability, relatively little research has focused on the enzyme's effects on protein or amino acid digestibility. Selle et al. (2000) concluded that phytase supplementation can improve protein utilization in poultry by countering the anti-nutritive properties of phytic acid. Farrell et al. (1993) reported improvements in nitrogen (N) retention by more than 1% for broilers fed a sorghum-

soybean meal based diet supplemented with phytase. Namkung and Leeson (1999) reported a 2% increase in N digestibility with the inclusion of Natuphos phytase in a corn-soybean meal diet. Others have also reported similar improvements in N retention when the enzyme is included in standard corn-soybean meal diets (Kornegay, 1996a; Yi et al., 1996b).

There have also been reports of improvements in amino acid digestibilities in birds fed phytase-containing diets. Kornegay and colleagues (1996) detected improvements in the digestibility of all amino acids except methionine and proline when phytase was included into 17 and 21% crude protein diets. Yi et al. (1996b) reported similar results where, with the exception of methionine and cystine, all amino acid digestibilities were improved with the addition of 750 FTU/kg phytase. Inclusion of phytase at 1220 FTU/kg was found to positively influence utilization of methionine, as well as lysine and valine, in soybean meal protein diets (Biehl and Baker, 1997). In these and several other experiments presented in the literature, amino acid digestibility was found to improve by 1.3 to 2.3% (Yi et al., 1996b; Sebastian et al., 1997; Namkung and Leeson, 1999; Ravindran et al., 2000). In 2001, Ravindran and collegues found that the inclusion of 500 FTU phytase/kg further increased the amino acid digestibility of wheat-sorghum based diets by 3.4%. Overall, reports suggest that both amino acid digestibility and N retention are improved when phytase enzymes are used to supplement diets with decreased non-phytate phosphorus (nPP) and/or crude protein (Pourreza and Ebadi, 2006; Ravindran et al., 2000; Panda et al., 2007).

Energy Metabolism. Previous research suggests phytate is capable of complexing with starch (Thompson and Yoon, 1984). During the late 1960's to early

1970's, research indicated that apparent metabolizable energy (AME) could be improved by including phytase enzymes into cottonseed meal based diets (Rojas and Scott, 1969; Miles and Nelson, 1974). Research in Australia also indicated improvements in AME for broilers fed sorghum-soybean meal based phytase-supplemented diets (Farrell et al., 1993; Selle et al., 1999). In the U.S., improvements in the AME value of wheat and sorghum-based broiler diets were reported when supplemental phytase was included (Bryden and Ravindran, 1998; Ravindran et al., 1999). In his review, Ravindran (1999) suggested phytic acid likely affects starch digestion by either binding α-amylase or by binding starch directly through a protein linkage. He went on to mention that the hydrolysis of phytate would result in the release of either the enzyme or the bound starch.

Growth Performance. Response with regard to growth rate has been inconsistent within the literature. Some researchers have noted no difference in body weight gain between broilers fed low P diets supplemented with 300 to 1000 FTU/kg microbial phytase and those fed standard commercial diets (McKnight, 1996; Boling et al., 2001; Nassari Moghaddam et al., 2007; Timmons et al., 2008). On the contrary, numerous studies have indicated that body weight gain, feed intake, and feed efficiency can be improved in broilers when phytase is included into the diet (Simons et al., 1990; Denbow et al., 1995; Kornegay, 1996b; Sebastian et al., 1996b; Singh and Khatta, 2002). Singh (2008) compiled a summary of results indicating the influence of phytase on broiler performance parameters. Conflicting data cited within the literature could be due to an array of factors including type of phytase employed, feed ingredients utilized, and dietary mineral supply.

Environmental impact

Poultry manure, when applied properly, is the most valuable of all livestock produced manures because of its N and P content. It makes an excellent organic fertilizer source and can also be utilized as a soil enhancer. The use of poultry manure has become a major burden on the environment due to its nutrient content. Phytate-bound nutrients are passed through the bird into the manure, which is then applied to the soil. The soil microorganisms are able to degrade the phytate, releasing P, N and other nutrients. The accumulation of these nutrients may exceed crop requirements, causing excess nutrients to run off into surface and ground waters during rainfall (Saylor, 2000).

In aquatic ecosystems, over enrichment with P and N leads to eutrophication (the loss of oxygen due to increased bacterial activity), resulting in an environment favoring plant life over animal life. P is the limiting nutrient for eutrophication in fresh water systems (Schindler, 1977). In fresh water, blooms of cyanobacteria contribute to the death of aquatic animals, foul odors, and unpalatable drinking water (Singh, 2008). When this water is chemically treated, the organic detritus react with chlorine to form trihalomethanes, which are toxic to the nervous system and liver (Singh, 2008).

A reduction in nutrient content of poultry manure due to improved nutrient utilization could significantly decrease the negative impacts on the environment. The use of microbial phytase is a practical way to release the phytate-bound P and N in the bird, thus decreasing the excretion of these and other nutrients (Singh and Khatta, 2002). Coelho (1994) suggested that a reduction in P excretion by 30 to 50% could be achieved if inorganic P levels were reduced in a diet supplemented with phytase. Denbow et al. (1995) reported similar results, observing that P availability was improved and P

excretion was reduced by 50% in broilers supplemented with 1200 units/kg of a phytase enzyme. Saitoh (2001) determined that feeding low P diets in combination with phytase supplementation could reduce P excretion by up to 40% without causing a decline in productivity in poultry. Therefore, reducing dietary inorganic P sources in combination with phytase supplementation can be effective in reducing P excretion and can lead to improvements in soil and water quality (Afsharmanesh and Pourreza, 2005; McGrath et al., 2006).

Economical significance

The inclusion of P supplements into poultry diets to meet nutrient requirements is costly and results in an increase in total production costs. The utilization of phytase enzymes can lead to a decrease in the cost of poultry production by replacing a portion of the inorganic P and improving feed efficiency. Kundu et al. (2000) reported a net income increase of 9.47% by including phytase in place of dicalcium phosphate in broiler diets. By supplementing corn or wheat-based diets with phytase, the cost per unit of body weight gain was reduced by 10% and 6%, respectively (Singh and Khatta, 2004). According to the latest feed ingredient prices, feed costs can be reduced by approximately $7 USD per tonne of feed (Hruby, 2009).

2.4 Coccidiosis

Coccidiosis is one of the most common diseases affecting poultry. It is caused by intracellular protozoan parasites of the genus *Eimeria*, which multiply in the intestinal lumen and cause tissue damage. There are nine identified species of coccidia known to infect the chicken, eight of which are known to be pathogenic (Edgar and Siebold, 1964).

E. tenella inhabits the ceca and causes bloody lesions and the formation of cecal cores. *E. acervulina* is most frequently encountered in North and South America (McDougald et al., 1997). This *Eimeria* species is often found in the upper half of the small intestine, especially in the duodenal loop, and produces white lesions. *E. maxima* infects the mid-small intestine from below the duodenal loop to beyond the yolk stalk, and is found as a large oocyst, often accompanied by yellow-orange mucus. *E. mitis* affects the lower small intestine from the yolk stalk to the cecal junction. The lesions of this species are easily overlooked since the oocysts do not colonize, but will cause the area it inhabits to appear pale and flaccid (McDougald, 2003). *E. praecox* is confined to the duodenal loop and may cause watery intestinal contents and small pinpoint hemorrhages on days 4-5 of infection. *E. necatrix* causes large white or red lesions, ballooning in the small intestine below the duodenal loop, and free flowing blood in the intestinal tract and feces. *E. brunetti* is found in the lower small intestine from the yolk stalk to the cecal junction and can cause a caseous eroded surface over the entire mucosa (McDougald, 2003). *E. mivati* affects the small intestine from the duodenal loop to the cloaca. This species causes lesions that resemble those of *E. acervulina*, though they are more circular in shape (Edgar and Siebold, 1964).

The Eimeria Lifecycle

Although there is variation in the number of asexual generations and time required for each developmental stage, all of the Eimeriad coccidia exhibit a similar complex life-cycle, with stages inside and outside of the host. Depending on the species, the entire life cycle can take place in 4 to 6 days and includes three different phases: sporogony, mezogony and gametogony.

Sporogony. When excreted in the feces, oocysts contain a sporont. In the presence of oxygen, proper temperature and humidity, the sporont transforms into four sporocytes each containing two sporozoites. The bird ingests the sporulated oocyst and the mechanical action of the gizzard removes the protective covering of the oocyst, releasing the sporocysts into the digestive tract. In the duodenum, the external wall of the sporocyst is degraded by trypsin and bile salts and the sporozoites are released.

Mezogony. Each newly released sporozoite penetrates the wall of an epithelial cell, developing into a round body known as a schizont. Asexual development, known as schizogony or merogony occurs next. During this time the nucleus divides repeatedly and the newly replicated nuclei develop into merozites. The merozites break free and invade neighboring epithelial cells to repeat the process of asexual multiplication.

Gametogony. After two to four asexual generations, the merozites invade the epithelial cells and develop into either microgametocytes (male cells) or macrogametocytes (female cells) in preparation for the sexual phase. The motile microgametocytes are released into the lumen where they seek and unite with macrogametocytes to form zygotes. The newly formed zygote forms a thick wall, transforming into an oocyst. Upon maturation, the oocsyt is released from the intestinal mucosa and is excreted in the feces, completing the coccidial lifecycle.

Coccidiosis Effects on Broiler Performance

An acute coccidial infection is known to result in decreased body weight gain, feed intake and feed:gain ratios (McDonald et al., 1982; Southern and Baker, 1983; Matthews and Southern, 2000). In addition to weight loss, Kouwenhoven (1972) also reported anorexia and diarrhea during an infection with *Eimeria* spp. Others have shown

that infection can also lead to increased morbidity (Hein, 1968), lesions on the mucosal epithelium (Kouwenhoven and van der Horst, 1972), and bloody droppings and enteritis (Stephens et al., 1974).

Coccidiosis has been shown to lower the pH of the small intestine, especially within the duodenum. Under normal conditions, the pH of the duodenum is 6.0 and above, but during a coccidiosis infection the pH is reduced to 5.0 and below (Stephens et al., 1974; Fox et al., 1987; Giraldo et al., 1987).

Coccidisosis infections are also associated with decreased absorption of Ca and P (Takhar and Farrel, 1979) and improved absorption of other minerals. Multiple researchers have reported a decrease in tibia ash due to the decline in mineral absorption (Willis and Baker, 1981, Watkins et al., 1989; Ward et al., 1990). Contrary to many reports Turk (1973) found no decrease in P concentrations of the bone and Giraldo and collegues (1987) reported increases in bone P concentrations, though both reported decreased absorption of Ca. Southern and Baker (1982a, 1982b) reported an increase in the absorption of Cu, Fe, and Mn in birds infected with *Eimeria*. Co absorption and tissue mineral concentrations have also been found to improve during a coccidiosis infection (Brown and Southern, 1985).

Coccidiosis control

Anticoccidial drugs. During the 1930's, Herrick and Holmes (1936) discovered that sulfur had an anticoccidial effect. In the years to come, sulfa drugs were developed and served as the first practical anticoccidial drugs (Collins, 1949). The sulfonamides act as an alternative substrate for enzymes required by the coccidia. This blocks the synthesis of vital metabolites needed for the synthesis of DNA, inhibiting multiplication

of the schizonts. Currently, comparatively small amounts of sulfamonaides, such as sulfaquinoxaline, are used. They are efficient in the treatment of *E. acervulina* and *E. maxima*, but have little effect on other *Eimeria* strains (Fanatico, 2006). The poultry industry has been heavily dependent on the use of anticoccidial drugs for the prevention and control of coccidiosis since they were first introduced in the 1940's (Chapman, 1999).

Anticoccidial drugs can be divided into two classes: synthetic (chemical) drugs and polyether ionophorus (ionophore) compounds. Synthetic drugs commonly used today have different specific modes of action, though they all work to prevent coccidiosis by acting during the initial stages of the parasite's lifecycle. Many of the existing synthetics are similar in chemical composition, but have different trade names depending on the pharmaceutical company that produces them. Selection of specific anticoccidials for use is typically based on the drugs ability to improve performance and suppress the development of coccidial lesions (Reid, 1975). Due to their mode of action, the synthetic drugs can easily cause resistance to develop.

Ionophores are commonly used in the large-scale industry. They work by altering the function of the cell membrane and rupture the parasite. Ionophores also have antibacterial action and help prevent secondary gut diseases. These drugs are produced by fermentation and include monensin, salinomycin, narasin, and maduramycin. Like many other drugs, some ionophores are now completely ineffective against coccidia because of developed resisitance, though the resistance observed is much less than that seen with synthetic anticoccidials.

Companies will often use a shuttle program, rotational program, or combination

of the two in order to avoid drug resistance by the parasite. A shuttle program entails a rotation of different drugs or drug classes to be used across the same flock. Due to convenience, it is common to provide a particular compound for a period during which one type of feed is given. For example, a synthetic drug may be included in the starter while an ionophore is included in the grower ration. A rotational program involves switching the drugs used after a period of time, whether it is after each season or so many flocks. For example, an ionophore may be used during the summer months while switching to a synthetic drug in the cooler months of fall and winter.

Vaccines. The first live vaccine (Coccivac®; Schering-Plough, Union, NJ) was made available in the United States during the 1950's (Chapman et al., 2002). Live vaccines are generally utilized for breeder stock due to the well developed protective immunity following a primary infection with coccidiosis, though they are used for commercial broilers and replacement hens to a lesser extent (Williams, 2002). There are at least ten commercially available live vaccines. These vaccines, which contain live oocysts, differ in a variety of ways including the type of *Eimeria* species (virulent vs. attenuated), drug resistance, species composition, and method of administration (Dalloul and Lillehoj, 2004).

One of the main differences between the available live vaccines is whether the strains of *Eimeria* are live or attenuated (Williams, 2002). The virulent or nonattenuated vaccines contain unmodified field or laboratory isolated strains. These include vaccines such as Coccivac® and Immucox® (Vetech Laboratories, Guelph, Ontario). Live vaccines may not contain sufficient numbers of the more pathogenic species for inducing long-lasting protective immunity, and are dependent on the autoreinfection from recycled

parasites. Live vaccines also carry the possibility of introducing a new species or unexpected pathogen into the flock if pathogenicity prevails over immunogenicity. This can be avoided with the use of attenuated vaccines, which consist of parasites whose virulence has been artificially reduced.

Virulence reduction can be accomplished by passing the *Eimeria* through embryonated eggs or by selecting for precocious strains (Dalloul and Lillehoj, 2004). One advantage to using an attenuated vaccine is that protective immunity can be induced without suffering a decline in performance parameters, which is often the case with the more conventional live oocsyt formulations. The disadvantage, however, is the high costs associated with generating this type of vaccine.

Most live vaccines contain drug-sensitive strains, which permits medication with ionophores while allowing time for immunity to develop. In most cases, live vaccines will include two or more *Eimeria* species. This is advantageous because it is most economically feasible to vaccinate against those species most prevalent within a geographic area. The disadvantage, since protective immunity against coccidiosis is species specific, is the vaccine may be ineffective in protecting against outbreaks caused by other species.

Immune Response to Coccidiosis

Humoral immune response. Humoral immunity, which is the aspect of immunity mediated by the secretion of antibodies from B-lymphocytes, has been shown to be stimulated by *Eimeria* parasites. When infected, the parasites stimulate the release of different antibodies into circulation and the intestinal mucosa (Rose et al., 1984; Lillehoj, 1987; Nash and Speer, 1988; Hughes et al., 1989). Within circulation,

immnuglobulin (Ig) M and IgA can be found (Trees et al., 1985; Lillehoj and Ruff, 1986). IgM is most effective in eliminating protozoal pathogens. IgA is the predominant antibody located in the gastrointestinal tract of infected animals (Rose et al., 1984; Trees et al., 1985; Lillehoj and Trout, 1996). IgA has a multitude of functions including the prevention of the entrance of environmental antigen into the gastrointestinal and respiratory tracts, neutralization of viruses and microbial toxins, and prevention of pathogens adhering and colonizing mucosal surfaces (Lillehoj and Trout, 1996; Kindt et al., 2007). Despite the stimulation of humoral immunity, it is still unclear how these responses participate in protection against *Eimeria* parasites.

Cell-mediated immunity. Cell-mediated immune responses include both antigen-specific and non-antigen-specific activation of various cells including T-lymphocytes, natural killer (NK) cells and macrophages. T-lymphocytes comprise two functionally distinct subpopulations distinguishable by their surface phenotypes (Lillehoj, 1998). Cytotoxic T-lymphocytes ($CD8^+$) recognize foreign antigen presented by MHC (major histocompatibility complex) class I molecules, while T-helper cells ($CD4^+$) recognize antigen associated with MHC class II molecules. The importance of T-lymphocytes in immune responses to coccidia has been well documented (reviewed by Lillehoj and Trout, 1993, 1994, 1996), especially in relation to cytokine secretion, which will be addressed later.

NK cells are non-T, non-B, non-macrophage mononuclear cells capable of spontaneous cytotoxicitiy against a wide variety of target cells (Herbermann and Holden, 1978). Chicken intestinal intraepithelial lymphocytes (IEL) contain NK cells that mediate cytotoxic activity (Chai and Lillehoj, 1988), suggesting that NK cells may play a

role in local defense (Lillehoj, 1998). NK cell activity is also increased during the early stages of a coccidia infection (Lillehoj, 1989), suggesting that these cells may play a role in the control of parasite proliferation. Furthermore, due to the increased activity during infection, IEL NK cells may also be involved in defending the intestinal mucosa against coccidia.

Macrophages are white blood cells located within tissues and are a product of monocyte division. They are considered phagocytes and their primary function is to engulf and digest pathogens and stimulate further response by lymphocytes and other cells associated with the immune response. Trout and Lillehoj (1995) previously found that macrophage intake of sporozoites were increased following either primary or secondary infections with *Eimeria* spp. Previous research has shown that various cytokines are produced by activated macrophages, NK cells, and T-cells following a coccidial infection (Lillehoj and Trout, 1996; Lillehoj, 1998; Lillehoj and Lillehoj, 2000; Lillehoj et al., 2004).

Cytokine response. Cytokines are small peptides that regulate immunity and determine the nature of the inflammatory response. They are part of the normal physiological process and host response to injury and stimulation. Cytokines exhibit an assortment of activities ranging from activation of immune cells to the regulation of body temperature. Which cytokines are produced in response to an immune insult initially determines whether an immune response develops and subsequently determines whether that response is cytotoxic, humoral or cell-mediated. A cascade of responses can be seen in response to cytokines, and often several cytokines are required to synergize and express optimal function.

T-cell inflammatory responses are the major immune reaction in chickens infected with *Eimeria* and offer protection against re-infection (Lillehoj and Trout, 1996; Lillehoj and Lillehoj, 2000; Lillehoj et al., 2004). Previous research demonstrated that several pro- and anti-inflammatory cytokines are produced in response to a coccidial infection (Lillehoj and Choi, 1998; Min et al., 2001). Within the chicken, these cytokines include IL-1β, IL-6 and IL-17. IL-1β is important in the initiation of inflammation. Following a primary infection with *Eimeria,* IL-1β levels are up regulated or increased (Laurent et al., 2001; Hong et al., 2006). IL-17 is involved in inducing and mediating pro-inflammatory responses by stimulating the production of other cytokines and chemokines such as IL-1β, IL-6, IL-8, G-CSF, GM-CSF, TNF-α, and TGF-β (Veldhoen et al., 2006; Hong et al., 2008). IL-6 is important as both a pro-inflammatory and anti-inflammatory cytokine. As a pro-inflammatory, IL-6 is secreted by either T-cells or macrophages to stimulate further immune response at the site of inflammation. It acts as an anti-inflammatory by inhibiting the effects of TNF-α and IL-1

Coccidial infections also activate both humoral and cell-mediated immune responses. Research indicates that cytokines are imperative in controlling host immune response to these infectious agents. Th1 cells, which secrete IFN-γ, IL-2, IL-10, IL-12, IL-15, IL-16, and IL-18, are primarily responsible for cell-mediated immunity and delayed hypersensitivity (Mossman and Coffman, 1989). Alternatively, Th2 cells are important in inducing the humoral response to combat parasite invasion and produce IL-4, IL-5, and IL-13 (Avery et al., 2004; Mowen and Glimcher, 2004; Degen et al., 2005).

Th1 cytokines. In chickens, interferon (IFN) production has been used to measure T-cell response to coccidial antigens (Prowse and Pallister, 1989; Martin et al.,

1994). IFN-γ activates the phagocytic potential of macrophages and the cytotoxic potential of NK cells and cytotoxic T lymphocytes, which are important in host defense against coccidia (Lillehoj and Trout, 1996; Lillehoj and Choi, 1998). Generally, lymphocytes of uninfected chickens produce lower levels of IFN-γ, when compared with those that are infected with *Eimeria* spp. (Martin et al., 1994). The higher levels of IFN-γ are associated with a protective immune response to the coccidia infections (Lillehoj and Trout, 1996; Choi et al., 1999; Min et al., 2003; Hong et al., 2006).

IL-2 and IL-15 are structurally homologous cytokines produced by mononuclear phagocytes and other cell types in response to viral or protozoan infection (Sundick and Gill-Dixon, 1997; Lillehoj et al., 2001). Both play a central role in adaptive immunity and stimulate the proliferation of T lymphocytes and NK cells in the chicken (Choi and Lillehoj, 2000; Lillehoj et al., 2001). IL-12 is an important regulatory cytokine required for the initiation and regulation of cellular immunity (Mossman and Sad, 1996). It regulates the differentiation of naïve T cells into TH-1 type cells, which is crucial for resistance to many microbial pathogens (Eldaghayes et al., 2006).

IL-18 is structurally homologous to IL-1β, and as such it, plays a critical role in inflammation. In combination with IL-12, IL-18 induces cell-mediated immunity and is primarily associated with responses to intracellular pathogen infections (Biet et al., 2002; Dinarello and Fantuzzi, 2003). Finally, IL-10 plays an important immunoregulatory role in the intestine, especially as a differentiation factor for a subset of T cells with suppressor function (Rothwell et al., 2004). IL-10 inhibits the synthesis of pro-inflammatory cytokines, including IL-1β and IL-6, which causes a down regulation (decrease) in the Th1 inflammatory response (de Waal Malefyt et al., 1991; Groux and

Powrie, 1999).

Th2 cytokines. Similar to mammals, the chicken contains a cluster of Th2 cytokine genes containing IL-3, IL-4, IL-13, and GM-CSF, all of which are expressed in the lymphoid tissues and are often referred to as lymphokines (Avery et al., 2004). GM-CSF is associated with the down regulation of the Th1 inflammatory response and an increase in Th2 cell development (Hong et al., 2006). IL-3 stimulates the proliferation and differentiation of hematopoietic stem cells (Martinez-Moczygemba and Huston, 2003). IL-13 reduces macrophage activity, causing a reduction in the production of the pro-inflammatory cytokines (Zurawski and de Vries, 1994). In addition, IL-13 can also induce production of IgM and IgG.

Chemokines. Chemokines are cytokines with chemotactic activity that act primarily by attracting leukocytes to sites of inflammation and facilitate their migration from circulation into infected tissue to mediate host defense mechanisms (Ebnet and Vestweber, 1999). Chemokines are grouped into four subfamilies that are differentiated by the position of their amino-terminal cystine residues (Zlotnik and Yoshie, 2000). As of 2006, 23 chemokines had been identified in the chicken (Hwang et al., 2005; Kaiser et al., 2005; Wang et al., 2005; DeVries et al., 2006). In general, chicken chemokines play an important role in the gut by influencing the balance between T lymphocyte subpopulations (Siveke and Hamann, 1998).

2.5 Research Objectives

Following a preliminary experiment to establish whether flesh and muscle removal made a difference for determination of tibia breaking forces, subsequent

experiments discussed within this dissertation were designed to evaluate the effects of dietary P concentrations on live performance and tibia bone strength and to determine if the utilization of a phytase enzyme(s) would have a positive impact on these measures under normal and/or infectious conditions.

The first experiment was a 4-week grow-out trial utilizing diets formulated with marginal or standard levels of non-phytate P. The feeds were administered to broilers hatched from breeder flocks differing in age. The effect of maternal flock age was examined along with any advantage in performance and skeletal strength provided from the dietary concentrations of P.

The next two experiments consisted of broilers receiving commercially available phytase enzymes included in a low P diet. The objective of experiment 2 was to investigate the ability of three different enzymes to replace 0.2% dietary npP without negatively impacting growth performance and bone strength. Based on results from Experiment 2, Experiment 3 investigated the effects of two phytase enzymes in combination with marginal levels of Ca and P on broilers from fast and slow growing genetic strains.

The final experiments were aimed at analyzing the effects phytase enzyme inclusion on live performance and bone strength in birds challenged with coccidiosis. Experiment 4 compared the effects of a live, attenuated vaccination vs. ionophore inclusion in the feed in combination with a phytase-containing diet on performance and on coccidiosis control in the live bird. Experiment 5 further evaluated the effects of phytase in combination with a live, attenuated coccidiosis vaccine on live performance, coccidiosis control, and effects of the immune function.

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3.0 Effects of Flesh Attachment on Bone Breaking Strength and of Phosphorus Concentration on Performance of Broilers Hatched from Young and Old Flocks

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3.1 Abstract

Two experiments were conducted to 1) assess any differences in breaking force in bones with or without flesh attachment and 2) determine the effects of dietary non-phytate phosphorus (npP) concentration, maternal flock age, and chick sex on live performance and tibia strength of broilers. For experiment 1, 60 chicks were placed in battery cages and selected weekly for removal of both tibiae (15 chicks/wk). Raw flesh was either completely cut from the bone or left intact and broken using a texture analyzer. For experiment 2, Ross 708 chicks (1220) were hatched of 25- and 65-wk-old breeder flocks, separated by sex, vaccinated, and placed on used bedding across 64 floor pens (18 males or 17 females/pen, 8 reps/trt). Through 28 d all birds were fed corn-soybean meal diets (22% CP, 3086 kcal/kg) adequate in all nutrients but npP, which was included at either 0.35 or 0.50%. Individual BW and pen feed consumption (FC) were recorded weekly and corrected for mortality. Each wk, 24 birds/trt were killed for tibia evaluation. Experiment 1 resulted in no differences in breaking force, whether flesh remained or was removed from the bone. In experiment 2, BW was increased with an increase in npP ($P<0.001$) at the end of the experiment. Both BW and FC were increased ($P<0.001$) from 0-28 d of age in chicks from the 65-wk-old breeder flock. Males had increased ($P<0.001$)

final BW, FC, and tibia breaking forces. Breaking forces were also improved ($P<0.001$) when npP was increased or chicks were hatched from older breeder flocks. Interactions were present ($P<0.05$) for npP concentration x 25-wk breeder flock 7-21d BWG and 0-28 d FC, npP x chick sex 7-14 d BWG and 21-28d FCR, and breeder flock age x chick sex 0 d BW and 7-14 d BWG. These results indicate that broiler growth and performance can be affected by maternal flock age, chick sex, and dietary npP.

3.2 Introduction

Poultry skeletal strength and development has been of interest in research for many years. As commercial broiler strains continue to be pushed for increased meat production and maturity, bone development fails to keep pace with overall growth, resulting in excess physical load which may lead to bone deformity and fragility. Phosphorous (P) is one of the major minerals involved in the formation of inorganic matrix in bone (Ali et al., 1992; Watkins, 1992; Rath et al., 2000). In addition to the development of skeletal deformities, failure to supply adequate amounts of P in the diet may lead to reduced performance and excessive mortality (Waldroup, 1999).

Breeder flock age is known to have an effect on egg weight, chick weight and chick growth (Pinchasov, 1991; Peebles et al., 1999; Yalcin et al., 2001; O'dea et al., 2006). Egg size increases as the age of the breeder hen increases, resulting in improved chick weights at hatch (Vieira and Moran, 1998a,b). Hatching weight can be a major predictor of final body weight in broilers. Each 1 g increase in body weight at hatch can lead to an 8-13 g increase in final body weight (Wilson, 1991). While improvements in final body weight are associated with heavier hatch weight, mixed results have been

reported with regard to body weights during the first few weeks of grow-out. For instance, one study showed that a young breeder flock (35 weeks) produced chicks with increased body weight gain than chicks of an old flock (51 weeks) through the first 21 d of grow out, however the old flock chicks had improved weight gain in the three weeks that followed (Peebles et al., 1999). Another study reported that the older breeder flock produced heavier chicks throughout the entire experiment (Pinchasov, 1991).

Little research is available with regard to whether age of the maternal parent has an effect on bone breaking strength. Due to the increased body weight associated with an aging breeder flock, it is assumed that an improvement in skeletal strength would be present. However, Yalcin et al. (2001) reported no difference in bone breaking strength as a result of maternal flock age. This particular study included two bird ages, a young flock ranging from 32-35 wks old and an old flock ranging in age from 56-58 wks.

Difference in chick sex has been shown to affect body weight and bone growth. In comparison with females, body weights tend to be greater for male broilers as the bird ages (Applegate and Lilburn, 2002). Bond et al. (1991) reported an increase in bone weight and length for male chicks. These two anatomical measures may influence the strength of the bone and increases in these measures have often been associated with an increase in the percentage of bone ash (Rath et al., 2000; Yalcin et al., 2001).

The need to quantify bone mineralization has long been recognized by researchers in poultry. Tibia bone ash, as described by the Association of Official Analytical Chemists (2000), has been the most common method of doing this. While successful, this assay is labor intensive, resulting in increased time and cost to obtain results. As an alternative method, bone breaking strength via shear force has been utilized to assess

bone mineralization. In general, shear force values are well correlated with bone ash, though they are liable to variation based on bone storage, handling, and site of shearing (Wilson and Mason, 1992; Orban et al., 1993). This method, much like preparation for bone ashing, involves the complete removal of any associated flesh prior to analysis. The literature fails to explain whether flesh is removed to avoid problems with shear force measurements or for some other reason.

The aim of the preliminary experiment was to establish whether flesh and muscle removal made a difference in tibia breaking forces. The objective of the main experiment was to determine the effects of dietary concentrations of non-phytate phosphorus (npP), maternal flock age, and chick sex on live performance and tibia bone strength of broilers from hatch to 28 d of age.

3.3 Materials and Methods

Preliminary Experiment

A total of 60 male broiler chicks of unknown genetic origin were obtained from a commercial hatchery and placed in battery cages on day of hatch. These chicks were exposed to continuous lighting with temperatures maintained according to standard commercial practices. All chicks were fed a standard mash corn-soybean meal starter diet (21.5% CP, 3142 kcal/kg; Table 3.1) for 28 days.

On a weekly basis, 15 chicks were randomly selected for the removal of both tibiae. The right tibia of each bird was excised and cleaned of muscle and connective tissues, while adhering tissues remained fully intact on the left tibia. Muscling and flesh were removed directly following excision from the bird by cutting and manually pulling

off the majority of the raw flesh and then cleaning the bone with gauze to ensure that no flesh remained. Tibia samples were stored in plastic bags and frozen at -20^{o}C until analysis for breaking strength was conducted. Each tibia was brought to room temperature before being broken in the center using a TA-HDi texture analyzer (Texture Technologies, Scarsdale, NY). Each tibia was supported by a fulcrum with a width of 1.0, 2.5, 3.0, 4.0, and 4.5 cm at 0, 1, 2, 3, and 4 weeks, respectively. Fulcrum width was altered on a weekly basis to accommodate bone length. A probe with a round base was attached to a 50 kg load cell with a crosshead speed of 1 mm/s.

Data were statistically evaluated using the JMP software, version 5.1.2 (SAS, 2005). All data were subjected to an analysis of variance and means were separated by Tukey's Honestly Significant Difference procedure. Means were considered significantly different when the p-value was less than 0.05.

Main Experiment

A total of 2000 Ross-708 eggs were obtained from 25 and 65 week old commercial broiler breeder flocks (1000 eggs/flock). Both flocks were managed by the same commercial complex and were fed similar feeding programs, though the actual farms that eggs were obtained from and nutrient composition of diets for the breeder flocks is unknown. Thirty eggs were randomly selected from each flock, individually weighed, and broken out to obtain yolk, albumen and shell weights. The remaining eggs were incubated at the Auburn University Poultry Science Research Unit and were feather sexed at hatch. A total of 1220 (610/flock) chicks were randomly allotted to one of eight treatment groups with 18 males (32 pens total) or 17 females (32 pens total) assigned to each pen (8 reps/treatment) in a 2 x 2 x 2 factorial arrangement. All birds were placed in

4.18 m^2 area floor pens containing used pine shaving litter. All pens were in an open-sided house with cross ventilation and temperature control. Prior to placement, all birds were spray vaccinated with Coccivac-D® (Schering-Plough Animal Health Corporation, Summit, NJ). Chicks were kept on a continuous 24-h lighting program and provided *ad libitum* access to feed and water throughout the experiment. Animal handling procedures during experimentation were in accordance with guidelines of Auburn University's Institutional Animal Care and Use Committee.

The corn-soybean meal starter diets (Table 3.1) used for this experiment were formulated to include either 0.35 or 0.50% non-phytate phosphorous (npP) to create a marginal (MP) and standard (SP) P diet. The remainder of the diet was formulated in accordance with Ross 708 broiler nutritional recommendations (Aviagen, 2003). Both experimental diets were provided as a mash from day-of-hatch to 28 d of age.

Individual body weights (BW) and pen feed consumption (FC) were recorded weekly and feed:gain (F:G) was corrected for mortality on a "bird day" basis. On a weekly basis, three birds per pen were randomly selected for the collection of both tibiae (flesh remained intact), for a total of 48 tibiae (right and left) per treatment (192 total birds or 384 tibiae per week). The right and left tibia of 15 chicks of the same hatch for each sex and flock age, not allotted to a treatment, were collected at hatch to provide a base line for the breaking strengths in the weeks to follow. All tibiae were stored and analyzed in the same manner as in the preliminary experiment.

Data were analyzed using Fit Least Squares of the JMP software version 5.1.2 (SAS, 2005) as a factorial. The ANOVA for live performance included the main effects of parental flock age (2), % phosphorus (2), and sex (2), as well as any 2- and 3-way

interactions that may have occurred between these factors. Bone breaking force data was analyzed via ANCOVA with BW as a covariate and includes the main effects of % phosphorus (2), parental flock age (2), sex (2), and leg (2) in addition to 2-, 3-, and 4-way interactions. The experimental unit for both analyses was pen of birds. The data were assumed to be statistically significant when $P < 0.05$ and mean values were separated by Tukey's Honestly Significant Difference procedure.

3.4 Results and Discussion

Preliminary Experiment

This experiment was conducted to determine whether the attachment of flesh on the tibia would alter the breaking force when compared with tibiae without flesh. The results for this four-week experiment are shown in Figure 3.1. Removal of the muscling and flesh did not alter the breaking forces during any collection period ($P>0.05$). This suggests that removal of flesh from the bone is not required to achieve accurate data for bone breaking strength. This allows for a decrease in bone collection and preparation time, which is of great significance when high numbers of bones are being analyzed.

Main Experiment

Egg Composition. Egg weight was increased as breeder age increased (Table 3.2), because an increase in egg size is often associated with an increase in chick size at hatch (Wilson, 1991; Vieira and Moran, 1998a,b; O'dea et al., 2006). Eggs from the 65-week-old breeder flock had a larger proportion of yolk and decreased proportions of albumen and shell compared to the 25-week-old flock, in agreement with other studies (Marion et al., 1964; Vieira and Moran, 1998a). Vieira and Moran (1998a) also provided

evidence suggesting that while at hatch the size of the retained yolk sac is not decreased in proportion to bird size, P proportions are decreased in chicks of younger flocks due to the decreased size of the yolk sac in comparison with chicks of older breeder flocks.

Effect of legs. No differences were detected for breaking forces between the right and left tibia of birds (P>0.05; data not shown). Lack of difference in breaking strength between each tibiae aids in validating the information presented in the preliminary experiment where the left tibia remained fleshed while associated tissues were removed from the right tibia. This shows that both tibiae grow and mineralize at the same rate. Therefore, only one leg need be collected per bird in order to accurately represent bone breaking strength.

Effect of P Concentrations. Birds fed the SP diet had an improvement in body weight gain (BWG) compared to those provided the MP treatment (Table 3.3). The difference in gain was low during the first week, likely due to the amount of nutrients supplied by the retained yolk sac. However, as the requirement for P obtained from the feed increased differences in weight gain were increased.

Feed consumption was greater for birds fed the SP diet only during the second week. The overall lack of difference in FC indicates that the decrease in npP was not enough to affect the amount consumed by the MP birds. A difference in F:G was present during the 14-21d period where the chicks from the 65-wk flock had a lower F:G in comparison to those of the young flock. No differences in F:G were detected between the two npP treatments throughout the remainder of the experiment.

With the exception of d 7, the MP treatment resulted in decreased tibia breaking strength throughout the experiment (Table 3.4). Similar results were found by Burnell et

al. (1990), where bone breaking strength was found to increase as dietary P concentration increased. In both cases, a reduction in the concentration of npP was expected to cause a decrease in tibia breaking strength.

Effect of Breeder Flock Age. It should be noted that only one breeder flock per age is represented in this experiment, thus results should be interpreted with that in mind. There were differences in body weight among chicks from the different aged breeders (Table 3.4). Chicks obtained from the 25-week-old breeder flock were 29% lighter at hatch and 14% lighter at the end of the experiment than those from the 65-week-old flock. This is due to egg and chick size being dependent upon breeder flock age. As birds approach market weight, BWG increases for chicks hatched from a larger egg, which is more consistently produced as the age of the breeder hen increases (Sklan et al., 2003; Hulet et al., 2007).

In addition to being a smaller bird, chicks of the young flock had a decrease in FC from 0-21 d, resulting in an overall decrease in FC at the end of experimentation (Table 3.3). Despite these differences, F:G did not differ between the chicks hatched from young and old breeder flocks at any time during the experiment. Previous work comparing breeder age has produced variable results with regards to affecting F:G. Most other research has resulted in either an improved F:G for chicks of older flocks (Proudfoot et al., 1982; Hulet et al., 2007), or the opposite effect (Wyatt et al., 1985; Hearn, 1986; O'dea et al., 2006).

At day of hatch, chicks of the young flock had about half of the tibia breaking strength values in comparison with chicks hatched from older hens (Table 3.4). In the weeks that followed, the difference in flock age continued to affect tibia breaking

strength through 21 d of age. By d 28, however, the tibia breaking strength was not different between birds hatched from either young or old breeder flocks. Yalcin et al. (2001) previously found no differences in breaking strength between broilers with a maternal flock age of 32 to 35 and 56 to 58 weeks of age. The findings of these results in combination with those of the current study suggest that maternal flock age may have an influence on chick bone strength through the beginning of the grow-out period, but should not effect the final tibia bone strength.

Effect of Sex. Though no differences in body weight were detected at day of hatch, males weighed significantly more than the females in the subsequent weeks (Table 3.5). This was associated with an advantage in BWG during the same time period for males, as well as increased FC from 14-28 d (Table 3.3). No differences were noted for F:G throughout the experiment.

On a week-to-week basis, the results varied for bone breaking force among sexes, however males had a bone that was 8% stronger than females upon conclusion of the experiment (Table 3.4). Significant effects between sexes have previously been reported for both bone anatomy and mineralization (Bond et al., 1991; Rath et al., 1999; Yalcin et al., 2001). The improvement in tibia breaking strength for males is likely a function of increased body weight and sexual dimorphism

Effect of P Concentration x Breeder Flock Age. Significant results due to the npP concentration by breeder flock age interaction are shown in Tables 3.5 and 3.6. Despite the lack of difference in body weight, BWG was less in chicks of the young breeder flock fed the MP diet compared with those on the SP diet during the 7-14 d growth period. Given that this difference was only noted for a single period of time and

had no effects on overall BWG, this incidence may be the result of the individuals used in this particular experiment and may not be typical for other flocks given the same concentrations.

Differences were noted in FC for chicks from the young breeder flock during the 0-28 d period, with no differences among chicks of the older flock. The younger flock chicks provided with a MP diet consumed 8% less feed overall than their older counterparts, resulting in a total decrease in P consumption of 35.5% (5.95g vs. 9.23g total P consumed, respectively). This difference in FC resulted in a 105 g decrease in final body weight for MP birds. This data suggests that chicks hatched from the younger flock are more susceptible to the lower npP concentrations. No interactions were found for F:G during the entire production period. With regard to breaking strength, differences were not detected between the breeder flock age and concentration of dietary npP. The lack of difference suggests that maternal age does not have an effect on the utilization of npP for bone mineralization, despite its obvious effects on body weight. Though the study did not assess npP concentrations, Yalcin and collegues (2001) also noted little to no interaction between maternal age and other main effects with regard to tibia breaking strength.

Effect of P Concentration x Sex. Significant results due to the npP concentration by chick sex interaction are in Tables 3.5 and 3.6. There was an 18g difference in body weight between males and females of the MP treatment at 14 d of age. This was associated with a 7% increase in BWG for the MP males over females during the 7-14 d growth period. These results indicate that females may be more sensitive to decreases in dietary npP concentrations as they adjust from utilization of nutrients from the retained

yolk sac. Furthermore, because the MP concentrations did not result in further decreases in growth beyond 14 d of age, females are capable of overcoming this sensitivity as they age.

Throughout the experiment, no effects were noted for FC as a result of the npP x sex interaction. Males supplied with the SP treatment had an improvement in F:G in comparison with females of the same treatment. Male broilers are known to be more efficient at converting feed into body mass, however this data also shows that the concentration of P included into the diet can influence F:G as well. The dietary inclusion concentration of npP at 0.35% had no effect on bone breaking forces for chicks of different sexes.

Effect of Breeder Flock Age x Sex. At placement, a difference was seen between the sexes of chicks hatched from both the 25- and 65-week-old breeder flocks. On average, males of the young flock were 7.7% heavier than their female counterparts (Table 3.6). Within the old flock, the opposite effect was noted, where females were approximately 7% larger than males. By d 7 weights between both sexes within each breeder flock age were similar and remained as such through the end of the experiment.

During the 7-14 d growth period, males from the 25-week old flock had a 12 g increase in BWG in comparison with the females of the same hatch. This improvement did not follow through in to the following weeks, nor did it have an effect overall BWG. There were no effects of the interaction on FC or F:G during the entire experiment.

Differences in tibia breaking force were not detected for the interaction between breeder age and sex (Table 3.6). These findings coincide with the results of Yalcin et al.

(2001) who found no differences in breaking force at either 16 or 32 d of age due to a breeder flock age by sex interaction.

3.5 Conclusions

Data from these experiments suggest that standard concentrations of dietary npP increase total gain and improved tibia breaking strength at 4 weeks of age in comparison with a diet containing only 0.35% npP. Sexual dimorphism plays an important role in the amount of weight gained and feed consumed, as well as the breaking force of the tibia bone. Maternal flock age also has a large influence on final body weights and total feed consumed. Tibia breaking forces are also affected by the age of the maternal flock, with chicks hatched from younger flocks having decreased tibia breaking strength. There were, however, no effects of maternal age in combination with any of the other main effects. And finally, when analyzing tibia strength via breaking forces, flesh attachment did not affect the results of the analysis.

3.6 References

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Table 3.1 Ingredient and calculated nutrient composition (as fed) of broiler diets for the preliminary and main experiments, each fed from 0-28 d of age

	Preliminary Experiment	Main Experiment	
		Marginal npP	Standard npP
Ingredients		(%)	
Ground yellow corn (7.5% CP)	55.85	55.14	54.46
Soybean meal (48% CP)	35.09	36.59	36.70
Poultry fat	4.53	3.87	4.11
Dicalcium phosphate (21.5%P; 18.5%Ca)	1.73	1.17	1.98
Ground limestone (38% Ca)	1.23	1.64	1.13
Sodium chloride	0.45	0.45	0.48
Trace-mineral premix[1]	0.25	0.25	0.25
Vitamin premix[2]	0.50	0.50	0.50
L-lysine (98.5%)	0.10	0.12	0.12
DL-methionine (99.9%)	0.27	0.27	0.27
Total	100.00	100.00	100.00
Calculated Analysis (%)			
Metabolizable energy, kcal/kg	3142.15	3086.00	3086.00
Crude protein	21.5	22.00	22.00
Calcium	0.94	1.00	1.00
Non-phytate phosphorus	0.45	0.35	0.50
Total Phosphorus (Analyzed)	-----	0.67	1.24
Sodium	0.20	0.21	0.21
Lysine	1.27	1.35	1.35
Methionine	0.63	0.63	0.63
Methionine + Cystine	0.97	0.97	0.97

[1]npP: non-phytate phosphorus

[2] Supplied the following per kg of complete feed: 125 mg manganese; 1 mg iodine; 55 mg iron; 6 mg copper; 55 mg, zinc; 0.3 mg selenium.

[3] Supplied the following per kg of complete feed: 8,000 IU vitamin A (retinyl palmitate); 2,000 IU cholecalciferol; 8 IU vitamin E (dl-tocopheryl acetate); 2 mg menadione; 5.5 mg riboflavin; 13 mg pantothenic acid; 36 mg niacin; 500 mg choline; 0.02 mg vitamin B_{12}; 5 mg folic acid; 1 mg thiamin; 2.2 mg pyridoxine; 0.05 mg biotin; 125 mg ethoxyquin.

Table 3.2 Characteristics of eggs produced by 25- and 65-week old breeder hens used in experimentation[1]

Measurement	25 Weeks	65 Weeks	*SEM*	*Significance*
Egg weight (g)	47.2	69.2	*0.75*	***
Yolk weight (g)	13.0	23.8	*0.28*	***
Yolk (%)	27.5	34.5	*0.45*	***
Albumen weight (g)	30.0	39.5	*0.61*	***
Albumen (%)	63.6	57.0	*0.47*	***
Shell weight (g)	4.2	5.9	*0.09*	***
Shell (%)	9.0	8.6	*0.12*	*

[1]Values are the means of 30 measurements.
*P<0.05; ***P<0.001.

Table 3.3 Main effects of male and female broilers hatched from 25- and 65-week old breeder hens and fed diets differing in non-phytate phosphorus (npP) from 0-28 days of age on body weight gain (BWG), feed consumption (FC), and feed conversion (F:G)[1]

	0-7 Days			7-14 Days			14-21 Days			21-28 Days			0-28 Days		
	BWG (g)	FC (g)	F:G (g/g)	BWG (g)	FC (g)	F:G (g/g)	BWG (g)	FC (g)	F:G (g/g)	BWG (g)	FC (g)	F:G (g/g)	BWG (g)	FC (g)	F:G (g/g)
npP Level															
.35%	80	130	1.65	194 [b]	294 [b]	1.51	328 [b]	612	1.86	472 [b]	846	1.80	1074 [b]	1881	1.75
.50%	84	123	1.51	205 [a]	315 [a]	1.53	363 [a]	604	1.67	501 [a]	870	1.75	1153 [a]	1912	1.67
Breeder Age															
25wks	67 [b]	110 [b]	1.67	179 [b]	263 [b]	1.47 [b]	316 [b]	537 [b]	1.71	470 [b]	863	1.85	1033 [b]	1773 [b]	1.72
65 wks	96 [a]	142 [a]	1.48	220 [a]	346 [a]	1.57 [a]	375 [a]	679 [a]	1.82	503 [a]	853	1.70	1194 [a]	2020 [a]	1.70
Chick Sex															
Female	80	124	1.60	196 [b]	296	1.51	323 [b]	559 [b]	1.74	448 [b]	803 [b]	1.80	1048 [b]	1783 [b]	1.71
Male	84	127	1.55	203 [a]	313	1.53	368 [a]	657 [a]	1.80	524 [a]	912 [a]	1.76	1179 [a]	2010 [a]	1.71
SEM[2]	*1.4*	*4.7*	*0.073*	*2.0*	*6.8*	*0.030*	*6.0*	*18.6*	*0.058*	*6.8*	*26.3*	*0.057*	*10.93*	*35.7*	*0.035*
	Significance														
npP Level	NS	NS	NS	***	*	NS	***	NS	NS	**	NS	NS	***	NS	NS
Breeder Age	***	***	NS	***	***	*	***	***	NS	**	NS	NS	***	***	NS
Chick Sex	NS	NS	NS	*	NS	NS	***	*	NS	***	**	NS	***	***	NS

[1] Values represent grand means involving a total of 64 pens, each with 18 male or 17 female chicks at start of experimentation.
[2] SEM: Pooled standard error of the mean.
[a,b] Means with different superscripts in a column differ.
*P<0.05; **P<0.01; ***P<0.001; NS, P>0.05.

Table 3.4 Main effects of male and female broilers hatched from 25- and 65-week old breeder hens and fed diets differing in non-phytate phosphorus (npP) from 0-28 days of age on individual body weight (g) and tibia breaking force (kg)[1]

	Day 0		Day 7		Day 14		Day 21		Day 28	
	Weight	Force	Weight	Force	Weight	Force	Weight	Force	Weight	Force
npP Level										
.35%	39.5 [a]	---	120	1.6	315 [b]	3.7 [b]	642 [b]	9.1 [b]	1115 [b]	15.5 [b]
.50%	38.8 [b]	---	124	1.8	330 [a]	4.8 [a]	693 [a]	11.0 [a]	1194 [a]	16.7 [a]
Breeder Age										
25 wks	32.4 [b]	0.5 [b]	100 [b]	0.8 [b]	280 [b]	4.0	596 [b]	9.4 [b]	1066 [b]	16.1
65 wks	45.9 [a]	0.8 [a]	145 [a]	2.1 [a]	365 [a]	4.5	740 [a]	10.7 [a]	1242 [a]	16.1
Sex										
Female	39.4	0.6 [b]	121 [a]	1.8	317 [b]	4.3	640 [b]	9.4 [b]	1089 [b]	15.5 [b]
Male	39.0	0.7 [a]	124 [b]	1.6	327 [a]	4.2	695 [a]	10.7 [a]	1220 [a]	16.7 [a]
SEM[2]	*0.15*	*0.10*	*1.3*	*0.15*	*2.7*	*1.18*	*7.6*	*2.73*	*10.9*	*3.40*
					Significance					
npP Level	*	---	*NS*	*NS*	***	***	***	***	***	*
Breeder Age	***	***	***	*	***	*NS*	***	**	***	*NS*
Sex	*NS*	*	***	*NS*	*	*NS*	***	**	***	*NS*

[1]Day 0 force values represent 15 birds of each sex and age; all other force values represent a mean of 48 tibiae (right and left) per treatment. Body weight values represent means involving a total of 64 pens, each with 18 male or 17 female chicks at start of experimentation.
[2]SEM: Pooled standard error of the mean.
[a,b] Means with different superscripts in a column differ.
*P<0.05; **P<0.01;***P<0.001; NS, P>0.05.

Table 3.5 Interactive effects of male and female broilers hatched from 25- and 65-week old breeder hens and fed diets differing in non-phytate phosphorus (npP) from 0-28 days of age on body weight gain (BWG), feed consumption (FC), and feed conversion (F:G)[1]

		0-7 Days			7-14 Days			14-21 Days			21-28 Days			0-28 Days		
		BWG (g)	FC (g)	FCR (g/g)	BWG (g)	FC (g)	FCR (g/g)	BWG (g)	FC (g)	FCR (g/g)	BWG (g)	FC (g)	FCR (g/g)	BWG (g)	FC (g)	FCR (g/g)
npP x Breeder Age																
.35%	25 wks	66	111	1.72	170 ^{c}	252	1.48	296 ^{bc}	509	1.73	449	827	1.85	980	1700 ^{c}	1.74
.35%	65 wks	94	147	1.57	218 ^{a}	336	1.54	359 ^{a}	714	2.00	495	864	1.75	1167	2062 ^{a}	1.77
.50%	25 wks	69	109	1.61	188 ^{b}	274	1.46	336 ^{c}	564	1.69	491	898	1.85	1085	1846 ^{b}	1.71
.50%	65 wks	98	137	1.40	222 ^{a}	356	1.60	390 ^{ab}	644	1.64	510	842	1.66	1221	1979 ^{ab}	1.62
npP x Sex																
.35%	Female	77	123	1.64	187 ^{b}	288	1.54	307	565	1.85	443	758	1.72 ^{ab}	1013	1734	1.72
.35%	Male	83	135	1.64	200 ^{a}	300	1.49	349	659	1.89	501	934	1.88 ^{a}	1134	2028	1.79
.50%	Female	83	126	1.56	206 ^{a}	303	1.47	339	554	1.64	454	849	1.87 ^{a}	1082	1832	1.70
.50%	Male	84	120	1.46	205 ^{a}	327	1.58	387	654	1.70	547	892	1.66 ^{b}	1224	1993	1.63
Breeder Age x Sex																
25 wks	Female	65	111	1.75	173 ^{c}	258	1.50	289	503	1.74	435	820	1.88	962	1692	1.76
25 wks	Male	70	110	1.59	185 ^{b}	268	1.44	343	571	1.68	505	906	1.81	1103	1855	1.69
65 wks	Female	95	139	1.46	220 ^{a}	333	1.52	356	616	1.74	462	787	1.70	1133	1875	1.66
65 wks	Male	97	146	1.51	221 ^{a}	358	1.62	394	743	1.91	543	920	1.70	1254	2166	1.73
	SEM	*2.0*	*6.6*	*0.103*	*6.6*	*9.6*	*0.043*	*8.5*	*26.3*	*0.082*	*9.6*	*37.2*	*1.85*	*15.5*	*50.5*	*0.049*
								Significance								
npP x Breeder Age		NS	NS	NS	*	NS	NS	*	NS	NS	NS	NS	NS	NS	*	NS
npP x Sex		NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS
Breeder Age x Sex		NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1] Values represent grand means involving a total of 64 pens, each with 18 male or 17 female chicks at start of experimentation.
[2] SEM: Pooled standard error of the mean.
[a,b] Means with different superscripts in a column differ.
*P<0.05; NS, P>0.05

Table 3.6 Interactive effects of male and female broilers hatched from 25- and 65-week breeder old hens and fed diets differing in non-phytate phosphorus (npP) from 0-28 days of age on individual body weight (g) and tibia breaking force (kg)[1]

		Day 0		Day 7		Day 14		Day 21		Day 28	
		Weight	Force	Weight	Force	Weight	Force	Weight	Force	Weight	Force
npP x Breeder Age											
.35%	25 wks	31.5 [d]	---	99	0.7	268	3.4	564	8.6	1014	15.4
.35%	65 wks	47.6 [b]	---	143	2.5	361	4.0	720	9.5	1216	15.6
.50%	25 wks	33.4 [c]	---	103	0.9	291	4.6	627	10.3	1118	16.8
.50%	65 wks	44.2 [a]	---	146	2.7	368	5.0	759	11.7	1269	16.5
npP x Sex											
.35%	Female	38.5 [b]	---	119	1.6	306 [b]	3.7	612	8.6	1055	15.3
.35%	Male	40.6 [a]	---	123	1.6	324 [a]	3.7	673	9.5	1174	15.7
.50%	Female	40.3 [a]	---	124	2.0	329 [a]	4.9	668	10.1	1122	15.7
.50%	Male	37.4 [b]	---	125	1.5	330 [a]	4.6	718	11.9	1265	17.6
Breeder Age x Sex											
25 wks	Female	31.3 [d]	0.5	99	0.9	271	4.0	561	8.8	996	15.6
25 wks	Male	33.7 [c]	0.5	103	0.7	288	4.0	631	10.2	1136	16.6
65 wks	Female	47.5 [a]	0.8	144	2.7	364	4.6	719	9.9	1182	15.4
65 wks	Male	44.3 [b]	0.8	145	2.4	366	4.3	759	11.2	1303	16.7
	SEM	*0.19*	*0.10*	*1.82*	*0.38*	*3.9*	*2.60*	*10.8*	*4.12*	*15.4*	*4.50*
						Significance					
npP x Breeder Age		***	---	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
npP x Sex		***	---	*NS*	*NS*	*	*NS*	*NS*	*NS*	*NS*	*NS*
Breeder Age x Sex		***	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

[1]Day 0 force values represent 15 birds of each sex and age; all other force values represent a mean of 48 tibiae (right and left) per treatment; weight values represent means involving a total of 64 pens, each with 18 male or 17 female chicks at start of experimentation.
[2]SEM: Pooled standard error of the mean.
[a,b] Means with different superscripts in a column differ.
*P<0.05; ***P<0.001; NS, P>0.05

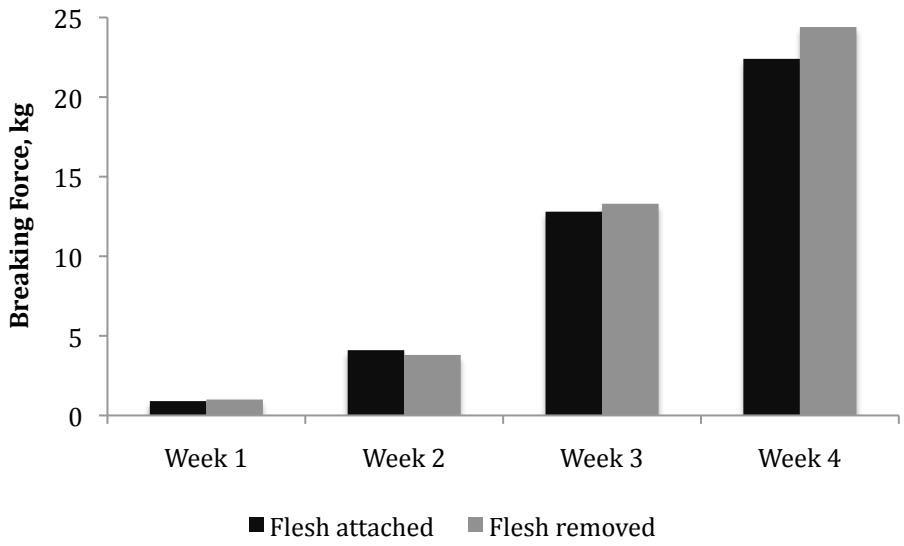

Figure 3.1 Breaking forces for tibia bones with and without muscling and flesh attached.

4.0 Evaluation of Commercial Phytase Enzymes on Performance and Tibia Breaking Strength of Male Broiler Chicks

4.1 Abstract

An experiment was conducted to evaluate the effects of three commercial phytase enzymes on performance and bone strength of broilers when included into a diet with 0.25% non-phytate phosphorus (npP). Day-old male broiler Ross 708 chicks (960) were placed on fresh bedding in 48 pens (20 birds/pen with 8 reps/treatment). All birds were fed a corn-soybean meal diet adequate in all nutrients but npP. Dietary treatments were created using 3 npP levels and 3 phytase enzymes: 1) standard P (SP, 0.45% npP), 2) marginal P (MP, 0.35% npP), 3) low P (LP, 0.25% aP), 4) LP + phytase A, 5) LP + phytase B, and 6) LP + phytase C. All 3 enzymes are classified as a 6-phytase with optimal activity occurring at pH 2.5-3.5, 5.0-5.5, and 4.5, respectively. Phytases A and B were of bacterial origin, while phytase C was fungal derived. All diets were pelleted at 79^{o}C, crumbled and provided through 35 days of age. Individual body weights and group feed consumption were recorded weekly and feed:gain was corrected for mortality. Weekly, 24 birds per treatment were sacrificed for tibia removal to evaluate bone strength. Supplementation of the LP diet with phytases A and C improved body

weight (P<0.001) similar to birds on the SP diet, while phytase B supplemented birds led to weights closely resembling the MP level. Generally, feed consumption from 0-35 d was only reduced (P<0.01) for birds receiving the low P diet. Broiler tibia strength most resembled (P<0.001) birds on the SP diet when phytases A and C were included in the LP treatment while birds given phytase B had strengths corresponding to the MP treatment through week 4 which rose to the SP treatment in the final week. These results suggest that broiler performance and bone mineralization responded best to either phytase A or C given the parameters of this study.

4.2 Introduction

The majority of broiler diets utilized in the industry are corn and soybean meal based, both of which are important for supplying the protein and energy requirements of the bird. Since the bulk of poultry feed is plant-based, up to 70% of the dietary phosphorus (P) can be in the form of phytate P. Phytate P is known to contain bound P that is unavailable to the bird, resulting in an increased requirement for the addition of inorganic P. Dietary additions of feed phosphates not only increase the feed and production cost, but may also lead to an increased excretion of soluble P.

Phytase is the only enzyme that has been recognized to initiate the release of phosphates from the phytate molecule, making it available for absorption and utilization (IUB, 1979; Zwart, 2006). In addition to improving P utilization, supplementation with a phytase enzyme can improve body weight, feed

consumption and bone strength, though these improvements may be dependent on the type of enzyme used and the degree of non-phytate phosphorus (npP) being replaced (Bedford, 2000; Panda et al., 2007; Liebert et al., 2008). Several phytase enzymes are produced and may be derived from plants, fungi or bacteria. Commercially available phytase enzymes are often differentiated by their original point of hydrolysis, pH profile, stability within the digestive tract and resistance to pelleting temperatures.

The point at which the phytase hydrolyzes the first phosphate from the phytic acid molecule is typically dependent on the source. Most plant-based phytases will first attack the phosphate molecule at the C_6 position (6-phytase), while the majority of microbial phytases are considered a 3-phytase and will initially hydrolyze phosphate at the C_3 position (Pallauf and Rimbach, 1995). In either case, once the molecule has been dephosphorylated, it can rebind to the phytase enzyme to sequentially release all phosphate groups except for the axial phosphate at the C_2 position.

Phytase enzymes may have up to two pH optima, though the number of optima each enzyme has is dependent on the original source. These differences in pH optima can result in varied effectiveness per unit of activity (Eeckhout and De Paepe, 1996). Regardless of the source or number of pH optima, phytase enzymes are generally active at a pH found within the crop, proventriculus and gizzard, with a tendency to lose activity within the small intestine.

Within the scientific literature, the activity of a phytase enzyme has been expressed as an FYT, FTU, PU, or U. All of these units have similar meaning in that

one unit of phytase can be defined as the amount of enzyme that liberates 1 micromole of inorganic phosphorus per minute from an excess of sodium phytate at pH 5.5 and 37^{o}C. Unless otherwise noted, the abbreviation FTU will be utilized to describe phytase activity within the context of this paper.

Bone status is commonly used as an indicator of adequate P in the diet because of its role in the formation of the inorganic matrix of the bone (Ali, 1992; Watkins, 1992; Rath et al., 1999). Historically bone ash has been used to evaluate P requirements based on the degree of mineralization. Typically, increased bone ash is associated with increased amounts of available phytate phosphorus and higher body weights (Mitchell and Edwards, 1996). Reichmann and Connor (1977) determined that bone mineral content has a direct effect on bone strength, thus bone breaking strength or shear force may be used as an alternative to ashing.

Phosphorous availability, known to be essential for growth and skeletal development in chickens, can be increased via the use of phytase enzymes, however few studies have shown whether they are capable of replacing dietary npP at greater than 0.10% of dietary needs. Therefore, the objective of the current study was to investigate the ability of three commercial phytase enzymes to replace 0.2% dietary npP while monitoring growth performance and bone strength.

4.3 Materials and Methods

Animals and Procedures

A total of 960 (Ross 708) male chicks were obtained from a commercial hatchery, spray vaccinated for coccidiosis (Coccivac D™) and randomly allotted to

one of six treatment groups with 20 birds assigned to each of 48 floor pens (8 reps/treatment) containing fresh pine shavings. Chicks were kept on a 24 h/d light program and had free access to feed and water throughout the trial. Individual body weights and feed consumptions were recorded each week and feed efficiency was corrected for mortality on a "bird day" basis. At placement, the right and left tibia of 20 chicks of the same hatch and not allotted to a treatment were collected to provide a base breaking strength. On a weekly basis, three birds per pen were randomly selected for the collection of both tibiae, for a total of 48 tibias (right and left) per treatment. Tibia samples were stored in plastic bags and frozen at -20^{o}C until analyzed for breaking strength. Each tibia was brought to room temperature before being broken in the center using a TA-HDi texture analyzer[1]. Each tibia was supported by a fulcrum with a width of 2.0, 2.5, 2.5, 3.0, 3.0, and 4.0 cm from 0 to 5 weeks, respectively. Fulcrum width was altered on a weekly basis to accommodate the shortest bones. A probe with a round base was attached to a 50 kg load cell with a crosshead speed of 1 mm/s.

Experimentation was carried out in the facilities of the Auburn University Poultry Science Research Unit and animal handling procedures were in accordance with guidelines of Auburn University's Institutional Animal Care and Use Committee.

Treatments

The corn-soybean meal basal diets (Table 4.1) included low (0.25%, LP), marginal (0.35%, MP) or standard (0.45% SP) levels of npP. The remainder of the

[1] Texture Technologies, Scarsdale, NY

diet was formulated in accordance with Ross broiler nutritional recommendations (Aviagen, 2003). The LP diet was supplemented with 0 units of phytase per kg (FTU), or 500 FTU of Phytase A, 500 FTU of Phytase B or 750 FYT of Phytase C based on manufacturer's recommendations. All experimental diets were pelleted at 79 ^{0}C (175 ^{0}F), crumbled, and provided from 0-35 days of age.

Phytase Enzymes

All three of the phytase enzymes utilized in this experiment were a 6-phytase. Both phytase A[2] and phytase B[3] were originally derived from *Escherichia coli* and mass-produced into *Pichia pastoris* and *Schizosaccharomyces pombe,* respectively. Phytase C[4] originated from the fungus *Peniophora lycii* and was spliced into *Aspergillus oryzae* for enzyme production. The pH for the optimal activity of each enzyme, according to their respective manufacturer, was 2.5-3.5, 4.5-5.0 and 4.5 for phytase A, B, and C respectively.

Statistical Analysis

Statistical analyses were performed on all data using JMP software version 5.1.2 (SAS, 2005). Data were subjected to a one-way analysis of variance and means were separated by Tukey's Honestly Significant Difference procedure at a probability level of 0.05. Analyses of percentages involving mortality were performed after transformation using arcsine of their square root.

[2] Optiphos; JBS United, Sheridan, IN
[3] Phyzme XP; Danisco, Denmark
[4] Ronozyme $P_{(CT)}$; DSM Nutritional Products, Inc., Parsippany, NJ

4.4 Results

Each level of phytase enzyme supplementation was used at recommended rates of addition and phytase activity was measured by each respective manufacturer (1150, 847, and 7438 units/kg for phytase A, B, and C respectively) and the activity rate of all three was well above expectations. Native phytase activity in the LP diet was also analyzed by each company and was determined to be 110 FTU/kg.

There were no differences in body weight detected at hatch among the male chicks allotted to the six dietary treatments (Table 4.2). However, in the weeks that followed, chick weight was improved in birds fed the SP treatment in comparison with either the unsupplemented MP (14 to 35d) or LP (7 to 35d) treatments. Body weight gain followed similar trends in body weight differences for the three treatment groups throughout the experiment (data not shown). At 35-d, supplementation with phytase A and C resulted in body weights similar to the SP treatment group, while phytase B had body weights similar to the MP treatment.

Differences in cumulative FC between the LP and all other treatments were detected during the 0-35 d experimental period (Table 4.2). In this case, the LP fed birds consumed significantly less feed than the birds provided a diet higher in npP or birds provided the LP treatment with the addition of a phytase enzyme. Despite differences in BW and FC, there was no effect of dietary treatment on overall F:G. In comparison with the other five treatments, mortality was up to 57% greater for the LP treatment by day 35 (Table 4.3).

Breaking force of the tibia bone was found to decrease as the level of dietary npP decreased (Table 4. 3). The inclusion of phytase A into the LP diet resulting in breaking

forces similar to SP fed birds during each week of the experiment. Breaking strengths were significantly reduced from 7 to 28d for the LP + phytase B treated birds in comparison with the SP chicks, though they had bones of similar or better strength than MP treated birds. Overall, birds supplemented with phytase C had tibia-breaking strength comparable to the SP treatment.

4.5 Discussion

Diets not supplemented with a phytase enzyme exhibited low levels of phytase activity, which is in line with the native phytase activity associated with the particular feed ingredients utilized. The in-feed value for the three phytase enzymes was high relative to the expectation of 500 FTU or 750 FYT per kg of feed. Due to variation in the biochemical nature of phytases, there are several different methods for analyzing phytase activity rather than one international standard (Selle and Ravindran, 2006). Thus, the use of different methods is one likely reason for the high variation among the three enzyme activities. Zwart (2006) stated that the analyzed values of phytase activity do not necessarily predict its efficacy in the animal. This certainly held true with regard to the current study since neither phytase B or C had an advantage over phytase A as a result of their 0.7 to 8.8 fold increase in analyzed activity.

Failure to supply adequate amounts of npP in the diet often leads to a reduction in performance (Waldroup, 1999). Results from the three control treatments (SP, MP, and LP) display this very well. A reduction of npP by 0.1% (MP) caused a decrease in body weight during the second week of growth, which the

birds were unable to overcome in the following weeks. The decreased body weights were accompanied by a reduction in feed intake. Loss of appetite has previously been reported to coincide with a dietary P deficiency (Underwood and Suttle, 1999; Leeson and Summers, 2001). The symptoms of deficiency were dramatically amplified with a 0.2% reduction in npP from the norm. Chick activity was reduced and mortality rate was also greatly increased with the larger reduction in npP, another symptom associated with a deficiency in dietary P (Waldroup, 1999).

To date, phytase studies show that supplementation of 500 FTU in the feed replaces approximately 0.1% npP in a corn-soybean meal diet. In the current study, the three enzymes were included into a diet with a 0.2% reduction in npP. With inclusion of these enzymes into the LP diet, the symptoms associated with an extreme P deficiency disappeared. Phytase B was able to improve the utilization of phytate P by 0.1%, resulting in a live performance similar to the MP treatment. Both phytase A and C exceeded expectations for the utilization of phytate P, restoring performance of low npP fed chicks to that of birds fed a standard npP diet. These results imply that with addition of either phytase A or C dietary npP could be reduced as much as 0.2% below recommended levels, without causing adverse affects in performance or livability. An additional decrease in the use of inorganic P sources to meet P requirements could be beneficial from an economic standpoint.

In addition, bone strength was reduced as dietary npP decreased. Bone formation is important during early growth and is highly dependent on nutrition (Rath et al., 2000). At day 7 there was a reduction in bone strength by 24% as dietary npP was decreased from 0.45 to 0.35%. This 0.1% decrease in npP resulted

in a peak reduction in breaking strength by 50% at 3 weeks of age. Following this peak, the reduced npP of the MP birds resulted in a decreased strength by 38 and 33% during weeks 4 and 5, respectively, suggesting that bone mineralization had begun to improve in MP birds. Despite these possible improvements in mineralization, bone strength was not able to fully recover as a result of the lower P level during the first 3 weeks of growth. A reduction in npP by an additional 0.1% (to 0.25%) resulted in a peak reduction of 77% breaking force for LP chicks in comparison to the SP control at 3 weeks of age. The inability of LP chicks to begin to improve bone mineralization as the MP chicks had following the peak reduction suggests that such a reduction in npP is too low for birds to attempt a recovery in bone strength.

Bone strength is proportional to bone mass, which is associated with growth (Frost, 1997; Seeman, 1999). Though physical measurements of the bone (ie shape, size and mass) were not evaluated for this study, distinct differences in bones were visually noted. For instance, tibiae from the LP birds were markedly smaller than those from either the MP or SP treatment. The results for body weight coincided with the visual differences in bone size, which had a result on breaking forces.

Much like the data for live performance, the addition of any phytase into the LP diet resulted in a reduction of the smaller, weaker legs associated with the P deficiency. The breaking forces for Phytase A suggest that the enzyme was able to extract the additional 0.2% P from the bound phytate to maintain a bone strength comparable to birds of the SP treatment. Analysis of bone strength for the phytase C treatment differed from the SP birds only at d 28, so the significance of these results

is likely minimal since bone strength was not affected in the previous or following week. With the exception of this collection date, phytase C was able to liberate enough P from phytate to overcome the 0.2% reduction in dietary npP without adverse effects on bone strength.

Overall, a failure to supply adequate amounts of npP in the diet can lead to reduction in performance**,** bone strength and livability. These adverse affects can be avoided with supplementation of phytase enzymes in diets with reduced levels of npP. This study reiterated what previous research has already shown, that regardless of source or manufacturer, phytase enzymes are capable of replacing at least 0.1% npP within the diet. Furthermore, given the parameters of this study, the utilization of either Phytase A or Phytase C may allow for the replacement of up to 0.2% dietary npP without causing negative implications.

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Table 4.1 Ingredient and calculated analysis of broiler diets containing varied levels of non-phytate phosphorus (npP), fed from 1-35 d of age

	Low npP	Moderate npP	Standard npP
Ingredients		(%)	
Ground yellow corn (7.5% CP)	55.53	55.11	54.67
Soybean meal (48% CP)	36.52	36.59	36.66
Poultry fat	3.72	3.87	4.03
Dicalcium phosphate (21.5% P; 18.5% Ca)	0.63	1.17	1.72
Ground limestone (38% Ca)	1.98	1.64	1.30
Sodium chloride	0.48	0.48	0.48
Trace-mineral premix[1]	0.25	0.25	0.25
Vitamin premix[2]	0.50	0.50	0.50
L-lysine (98.5%)	0.12	0.12	0.12
DL-methionine (99.9%)	0.27	0.27	0.27
Total	100.00	100.00	100.00
Calculated Analysis (%)			
Metabolizable energy, kcal/kg	3086.00	3086.00	3086.00
Crude protein	22.00	22.00	22.00
Calcium	1.00	1.00	1.00
Non-phytate phosphorus	0.25	0.35	0.45
Total phosphorus (analyzed)	0.55	0.68	0.78
Sodium	0.21	0.21	0.21
Lysine	1.35	1.35	1.35
Methionine	0.63	0.63	0.63
Methionine + Cystine	0.97	0.97	0.97

[1]Supplied the following per kg of complete feed: 125 mg manganese; 1 mg iodine; 55 mg iron; 6 mg copper; 55 mg, zinc; 0.3 mg selenium.

[2]Supplied the following per kg of complete feed: 8,000 IU vitamin A (retinyl palmitate); 2,000 IU cholecalciferol; 8 IU vitamin E (dl-tocopheryl acetate); 2 mg menadione; 5.5 mg riboflavin; 13 mg pantothenic acid; 36 mg niacin; 500 mg choline; 0.02 mg vitamin B_{12}; 5 mg folic acid; 1 mg thiamin; 2.2 mg pyridoxine; 0.05 mg biotin; 125 mg ethoxyquin.

Table 4.2 Weekly body weight (BW, g) overall feed consumption (FC, g), feed:gain (F:G, g/g), and mortality of male broilers fed diets varying in levels of non-phytate phosphorus (npP), which may or may not have contained a phytase source, from 0-35 days of age[1]

npP Level	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	0-35 days		
& Enzyme	BW	BW	BW	BW	BW	BW	FC	F:G	% Mortality
0.45% (SP)[2]	36.4	142 [a]	392 [a]	817 [a]	1329 [a]	1891 [a]	2833 [a]	1.53	0.62 [b]
0.35% (MP)	36.1	131 [ab]	292 [c]	608 [d]	1008 [b]	1464 [b]	2542 [a]	1.78	1.86 [b]
0.25% (LP)	36.2	111 [c]	228 [d]	385 [e]	590 [c]	1028 [c]	1783 [b]	1.82	20.63 [a]
+ Phytase A	36.1	141 [ab]	378 [a]	780 [ab]	1286 [a]	1823 [a]	2880 [a]	1.61	1.86 [b]
+ Phytase B	36.5	127 [b]	334 [b]	667 [c]	1071 [b]	1564 [b]	2553 [a]	1.68	2.52 [b]
+ Phytase C	36.4	134 [ab]	373 [a]	756 [b]	1225 [a]	1777 [a]	2835 [a]	1.63	3.14 [b]
SEM[3]	*0.38*	*2.9*	*5.1*	*9.6*	*24.8*	*31.5*	*152.9*	*0.122*	
Significance	*NS*	***	***	***	***	***	***	*NS*	***

[1] Values represent grand means involving a total of 48 pens, each with 20 chicks at start of experimentation
[2] SP, MP, and LP refer to standard, marginal, and low levels of npP, respectively
[3] SEM: Pooled standard error of the mean
[a,b] Means with different superscripts in a column differ.
NS $P>0.05$; *** $P<0.001$

Table 4.3 Tibia breaking force (kg) of male broilers fed diets varying in levels of non-phytate phosphorus (npP), which may or may not have contained a phytase source, from 0-35 days of age[1]

npP Level & Enzyme	Day 7	Day 14	Day 21	Day 28	Day 35
0.45% (SP)[2]	1.74 [a]	7.9 [a]	18.2 [a]	28.8 [a]	33.5 [a]
0.35% (MP)	1.27 [c]	4.1 [c]	9.1 [c]	18.0 [b]	22.3 [b]
0.25% (LP)	0.80 [d]	2.2 [d]	4.1 [d]	9.2 [c]	9.4 [c]
+ Phytase A	1.67 [a]	7.0 [a]	16.1 [ab]	29.7 [a]	33.1 [a]
+ Phytase B	1.33 [bc]	5.5 [b]	10.3 [c]	18.6 [b]	27.7 [ab]
+ Phytase C	1.54 [ab]	6.8 [ab]	15.4 [b]	25.7 [a]	32.8 [a]
SEM[3]	*0.057*	*0.31*	*0.60*	*1.14*	*1.56*
Significance	***	***	***	***	***

[1]Day 0 force = 0.46 kg (mean of 20 chicks, with both legs per chick represented); all other force values represent a mean of 48 tibiae (right and left) per treatment

[2] SP, MP, and LP refer to standard, marginal, and low levels of npP, respectively

[3] SEM: Pooled standard error of the mean

[a,b] Means with different superscripts in a column differ.

*** $P<0.001$

5. Evaluation of Commercial Phytase Enzymes on Performance and Skeletal Strength of Two Broiler Strains

5.1 Abstract

An experiment was conducted to evaluate the effects of two commercial phytase enzymes on performance and bone mineralization of male broilers of two commercial strains when included in a diet marginal in both calcium (Ca) and non-phytate phosphorus (npP). Male Ross 708 and Cobb 700 chicks (320 of each) were placed on fresh bedding across 64 pens (10 birds/pen with 8 reps/trt). All birds were fed a corn-soybean meal diet (22% CP, 3086 kcal/kg) adequate in all nutrients but Ca and npP. Dietary treatments were created using 2 npP/Ca combinations and 2 phytase enzymes: 1) standard (0.45% npP, 1.0% Ca), 2) marginal (0.35% npP, 0.87% Ca), 3) marginal + phytase A, and 4) marginal + phytase B. Both enzymes were of bacterial origin, classified as a 3-phytase, and had optimal activity at pH 2.5-3.5 and 4.5-5.0, respectively. All diets were pelleted at 79^{O}C, crumbled and provided through 21 days of age. Individual body weights and feed consumption were recorded weekly and feed efficiency was corrected for mortality. At 21 d of age, 24 birds per treatment were sacrificed for tibia breaking strength.

Ross chicks were 1.7g heavier ($P<0.05$) than Cobb chicks at hatch, however there were no differences ($P>0.05$) in body weight between the two strains at 21 d of age. Birds

fed either the control or phytase-inclusion diets had an improved body weight gain ($P<0.05$) in comparison with birds fed the marginal npP-Ca diet. Feed consumption was also decreased ($P<0.05$) in birds consuming the marginal npP-Ca diet in comparison with the remaining treatments. Bone mineralization, as measured by tibia breaking strength, declined ($P<0.05$) by approximately 4 – 4.5 kg in birds fed a marginal npP-Ca diet not supplemented with a phytase enzyme. Overall, this study concluded that live performance and bone mineralization are not affected by genetic strain or type of phytase utilized in the diet. Furthermore, these two genetic strains were determined to elicit similar responses to phytase supplementation.

5.2 Introduction

Phytase enzymes are often included into the diet of broiler chickens to alleviate some of the reliance on inorganic phosphorus (P) inclusion in the diet. Within the literature, research suggests that diets supplemented with phytase enzymes should be formulated with a proper reduction in calcium (Ca) and non-phytate phosphorus (npP). Phytate hydrolysis decreases with a high inclusion of inorganic P (Ballam et al., 1984; Karim, 2006). Wise (1983) suggested excess intake of inorganic P may inhibit the catalytic activity of microbial phytase within the avian digestive tract. High dietary concentrations of Ca also affect the hydrolysis of phytate due to the formation of insoluble Ca-phytate complexes (Nelson, 1980; Sands et al., 2003; Tamin et al., 2004), suggesting that phytate hydrolysis is inversely related to dietary Ca levels (Singh, 2008). Furthermore, high Ca:npP ratios antagonize the digestibility and absorption of inorganic

soluble forms of P due to increased precipitation of insoluble Ca:P complexes (Hurwitz and Bar, 1971).

Bone is a living tissue and its strength can be influenced by a number of variables. Genetic factors can determine the way a bone grows and remodels. Bone density is a heritable trait in mammals and laying hens (Whitehead et al., 1994; Boskey et al., 1999). Williams et al. (2000a,b) showed that continued selection for faster growth rate can result in greater cortical porosity and can increase the Ca:P ratio in bone mineral. There have been reports of differences in bone strength between egg and meat-type birds (slow vs. fast growth), however few studies have compared commercial broiler strains (Pitsillides et al., 1999; Williams et al., 2000a). Yalcin et al. (2001) found no effect on bone strength for two commercial broiler strains beyond two weeks of age, however they neglected to mention how the strains differed. With the exception of this study, little research has been published to show whether bone mineralization, which is directly related to bone strength, differs between commercial broiler strains.

The objective of the current study was to investigate the effects of two commercial phytase enzymes when included into a diet marginal in both calcium and non-phytate phosphorus, with an appropriate reduction in the Ca:npP ratio for enzyme inclusion, on performance and bone mineralization of male broilers from two genetically different commercial strains.

5.3 Materials and Methods

Animals and Procedures

A total of 640 male chicks (320 of Ross 708; 320 of Cobb 700) from 38 wk-old

hens were obtained from a commercial hatchery, vaccinated for Marek's disease, spray vaccinated with Coccivac D™, and randomly allotted to one of eight treatment groups with 10 birds assigned to each of 64 floor pens (8 reps/treatment) containing fresh pine shavings. The chicks were kept on a continuous lighting program and provided free access to feed and water throughout the trial. Individual body weights and feed consumption were recorded at 21 d of age and feed efficiency was corrected for mortality on a "bird day" basis. At placement, three birds per pen were randomly selected for the collection of both tibiae, for a total of 48 tibias (right and left) per treatment. Tibia samples were stored in plastic bags and frozen at -20^{o}C until analyzed for breaking strength. Each tibia was brought to room temperature before being broken in the center using a TA-HDi texture analyzer[1]. A fulcrum with a width of 3.0 cm, chosen to accommodate the shortest bones, supported each tibia. A probe with a round base was attached to a 50 kg load cell with a crosshead speed of 1 mm/s. Experimentation was carried out in the facilities of the Auburn University Poultry Science Research Unit and animal handling procedures were in accordance with Auburn University's Institutional Animal Care and Use Committee (IACUC).

Treatments

The corn-soybean meal basal diets (Table 5.1) included marginal (0.35%, 0.87%) or standard (0.45%, 1.0%) levels of npP and Ca. The remainder of the diet was formulated to meet or exceed all nutrient requirements set forth by the individual breed standards (Aviagen, 2007; Cobb-Vantress, 2008). The marginal diet was supplemented with 0 units of phytase per kg (FTU), 500 FTU of Phytase A[2], or 500 FTU of Phytase B[3]

[1] Texture Technologies, Scarsdale, NY
[2] Optiphos; JBS United, Sheridan, IN

based on manufacturer's recommendations. All experimental diets were pelleted at 79°C (175 °F), crumbled, and provided from days 0-21 of age.

Phytase Enzymes

Both phytase enzymes utilized in this experiment were a 3-phytase and were originally derived from *Escherichia coli* and mass-produced into *Pichia pastoris* and *Schizosaccharomyces pombe,* respectively. The pH for the optimal activity of each enzyme, according to their respective manufacturer, is 2.5-3.5 and 4.5-5.0 for phytase A and B, respectively.

Statistical Analysis

Statistical analyses were performed on all data using JMP® software (SAS, 2005). Data were subjected to a one-way analysis of variance and means were separated by Tukey's Honestly Significant Difference procedure at a probability level of 0.05. Analyses of percentages involving mortality were performed after transformation using arcsine of their square root.

5.4 Results

Initial chick weight differed ($P<0.001$) between the two genetic strains, where Cobb chicks were, on average, 1.7 g lighter than Ross chicks at time of placement (Table 5.2). By 21 d of age, body weights did not differ ($P>0.05$) between the two strains.

Regardless of genetic strain, at 21 d, birds fed the marginal npP-Ca diet in combination with either Phytase A or B were heavier ($P<0.05$) than those birds fed the marginal (0.35% npP, 0.87% Ca) dietary treatment with no enzyme, while the standard diet was intermediate. Total weight gained from 0-21 d was greater ($P<0.05$) for the

[3] Phyzyme XP; Danisco, Denmark

marginal diet supplemented with either enzyme and the standard npP-Ca diet, as compared to the unsupplemented marginal diet. Feed consumed was also significantly decreased ($P<0.01$) for birds on the unsupplemented marginal diet in comparison with the standard and marginal + Phytase B diets. Despite the differences in body weight gain and feed consumed at 21 d of age, no differences ($P>0.05$) were detected among the dietary treatments for feed:gain. Bone breaking strength was decreased ($P<0.001$) for birds fed the non-supplemented, marginal npP-Ca diet in comparison with the remaining three dietary treatments. No differences were detected ($P>0.05$) for bone strength due to genetic strain or enzyme type. There were also no interactive effects between chick strain and diet upon the end of the experiment for any of the main effects.

5.5 Discussion

Both the Cobb 700 and Ross 708 broiler strains are marketed for their production of high meat yield. Initial chick weight differed ($P<0.001$) between the two genetic strains, where Cobb 700 chicks were on average 1.7 g lighter than Ross 708 chicks at time of placement (Table 5.2). By 21 d of age, body weights did not differ ($P>0.05$) between the two strains.

The results indicate a 7% decrease in body weight gain between the marginal and standard npP-Ca diets. This was expected due to the known negative implications on weight gain associated with lowering P levels (Waldroup, 1999). Bone breaking strength was also found to differ between diets, where birds fed either the the phytase-supplemented diets or the standard npP-Ca diet had a tibial bone approximately 4.5 kg stronger than birds fed the unsupplemented marginal npP-Ca diet. It is well known that

Ca and P are the major minerals involved in bone mineralization (Leeson and Summers, 2001). It was expected that by not providing these minerals, a decrease in bone mineralization would result, leading to decreases in bone breaking strength values. The lack of significant difference between breaking strength for the two broiler strains was expected since both strains performed equally well.

Neither phytase enzymes lead to statistical improvements in live performance when compared with each other. Phytase A did, however, result in a decreased FC (50 g less/bird than phytase B). This, in combination with the comparable BWG, resulted in birds that had an FCR that was a minimum of 0.05 FCR points lower than either the standard or phytase B supplemented treatment. This improvement in feed efficiency could lead to improvements in overall production costs due to the decreased amount of feed required.

Overall, neither broiler strain or phytase enzyme source was found to have a true effect on bone mineralization, though phytase A did lead to slightly more positive effects on live performance and bone strength in the strains evaluated. The only negative effect on bone strength or live performance parameters was due to decreased Ca and P inclusion into an otherwise adequate diet.

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Table 5.1 Ingredient and calculated analysis of broiler diets containing two levels of non-phytate phosphorus/calcium combinations, fed from 0-21 d of age

	Marginal	Standard
Ingredients	(%)	
Ground yellow corn (7.5% CP)	55.71	54.67
Soybean meal (48% CP)	36.49	36.66
Poultry fat	3.65	4.03
Dicalcium phosphate (21.5% P; 18.5% Ca)	1.17	1.72
Ground limestone (38% Ca)	1.36	1.30
Sodium chloride	0.48	0.48
Trace-mineral premix[1]	0.25	0.25
Vitamin premix[2]	0.50	0.50
L-lysine (98.5%)	0.12	0.12
DL-methionine (99.9%)	0.27	0.27
Total	100.00	100.00
Calculated Analysis (%)		
Metabolizable energy, kcal/kg	3086.00	3086.00
Crude protein	22.00	22.00
Calcium	0.87	1.00
Non-phytate phosphorus	0.35	0.45
Sodium	0.21	0.21
Lysine	1.35	1.35
Methionine	0.63	0.63
Methionine + Cystine	0.97	0.97

[1] Supplied the following per kg of complete feed: 125 mg manganese; 1 mg iodine; 55 mg iron; 6 mg copper; 55 mg, zinc; 0.3 mg selenium.
[2] Supplied the following per kg of complete feed: 8,000 IU vitamin A (retinyl palmitate); 2,000 IU cholecalciferol; 8 IU vitamin E (dl-tocopheryl acetate); 2 mg menadione; 5.5 mg riboflavin; 13 mg pantothenic acid; 36 mg niacin; 500 mg choline; 0.02 mg vitamin B_{12}; 5 mg folic acid; 1 mg thiamin; 2.2 mg pyridoxine; 0.05 mg biotin; 125 mg ethoxyquin.

Table 5.2 Live performance and bone breaking strength of male broilers from two broiler strains fed diets with either standard or marginal non-phytate phosphorus (npP), of which the latter may have contained a phytase source, from 0-21 days of age[1]

	Day 0	Day 21		Day 0-21		
	Weight (g)	Weight (g)	Force (kg)	BWG (g)	FC (g)	F:G (g/g)
Diet						
Standard	47.2	783 [ab]	16.0 [a]	736 [ab]	1068 [a]	1.46
Marginal	47.8	733 [b]	11.5 [b]	685 [c]	984 [b]	1.43
+ Enzyme A	47.3	797 [a]	16.6 [a]	750 [a]	1052 [ab]	1.41
+ Enzyme B	47.1	795 [a]	16.0 [a]	748 [ab]	1102 [a]	1.48
SEM	*0.31*	*17.0*	*0.70*	*16.4*	*22.4*	*0.028*
Strain						
Cobb 700	46.5 [b]	774	14.8	728	1047	1.44
Ross 708	48.2 [a]	780	15.2	732	1055	1.45
SEM	*0.23*	*13.0*	*0.53*	*13.0*	*15.2*	*0.023*
npP-Ca x Strain						
0.45% Cobb	----	779	15.3	732	1081	1.48
0.45% Ross	----	788	16.8	741	1053	1.43
0.35% Cobb	----	728	11.8	682	968	1.43
0.35% Ross	----	738	11.1	689	1000	1.45
Enz. A Cobb	----	785	16.6	739	1041	1.41
Enz. A Ross	----	810	16.5	761	1062	1.40
Enz. B Cobb	----	806	15.6	759	1099	1.45
Enz. B Ross	----	769	16.3	737	1106	1.50
SEM		*23.4*	*1.10*	*23.3*	*30.6*	*0.040*
			Significance			
npP-Ca level	*NS*	*	***	*	**	*NS*
Strain	***	*NS*	*NS*	*NS*	*NS*	*NS*
npP-Ca x Strain	----	*NS*	*NS*	*NS*	*NS*	*NS*

[1]All values represent contrasts involving 64 pens, each with 10 chicks at start of experimentation.
[2] Enzyme A and B refer to phytase enzymes, OptiPhos and Phyzyme XP, respectively.
[3] SEM: Pooled standard error of the mean
NS, $P>0.05$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$

6. Does Phytase Enzyme Supplementation Reduce the Effects of an *Eimeria* Challenge?

6.1 Abstract

A series of two experiments were conducted to evaluate the effects of feeding a phytase enzyme to broiler chickens subjected to a coccidiosis challenge. For both experiments, commercial broiler chicks (1008) were placed across 48 floor pens (21 birds/pen, 6 reps/trt) on either fresh or seeded bedding. An additional 120 chicks were placed across 12 Petersime grow-out battery pens (10/pen, 6 reps/trt) to serve as negative controls for Exp 1. Treatments for Exp 1 involved phytase-supplemented diets containing two different Ca-npP levels (0.5% Ca, 0.25% npP or 0.7% Ca, 0.35% npP), three coccidiosis control strategies (vaccination, in-feed coccidiostat or none), and two coccidia challenges (unchallenged or challenged). Experiment 2 treatments were created using a combination of two Ca-npP levels (0.9% Ca, 0.45% npP vs. 0.7% Ca, 0.25% npP and 500 FTU phytase), two coccidia challenges (unchallenged vs. challenged), and two vaccination strategies (unvaccinated vs. vaccinated). On d 11 and 20 (Exp 1) and 10, 18, and 21 (Exp 2) bodyweight (BW) and feed consumption (FC) were recorded for each pen. Five birds/trt were sacrificed and intestinal samples were obtained for visual and microscopic lesion scoring on these days. At 11 and 20 (Exp 1) or 21 d (Exp 2) 30 and 18 birds/trt (Exp 1 and 2, respectively) were selected for removal of the left tibia to assess bone

strength. On d 10 and 18 of Exp 2, tissues from the duodenum and ceca were also collected from 5 birds/trt to assess immune response via cytokine production.

BW, FC, and bone strength were unaffected ($P>0.05$) by diet in both experiments, though control methods had an affect on both body weight gain (BWG) and FC from 0-20 d in Exp 1. The coccidia challenge led to a decline ($P<0.05$) in overall BWG for either experiments. Upon conclusion of the Exp 2, birds exposed to coccidia also had a lower FC ($P<0.01$), higher feed conversion ($P<0.001$), and decreased bone strength ($P<0.01$) in comparison to those not challenged. Regardless of treatment, visual and microscopic scoring of the duodenum and ceca showed few differences ($P>0.05$) in either experiment. The percentage of birds having lesions associated with *Eimeria* was increased ($P<0.05$) on d 20 (Exp 1) for birds reared in floor pens. Expression of IFN-γ and IL-17 were not up regulated during d 10 in either intestinal section. On d 18 IFN-γ gene expression was increased in vaccinated or challenged chickens in response to *E. tenella*, and IL-17 expression was increased in challenged birds in response to *E. acervulina*.

6.2 Introduction

Coccidiosis is one of the most common diseases to affect poultry. It is caused by intracellular protozoan parasites of the genus *Eimeria*, which multiply in the intestinal lumen and cause tissue damage. Coccidial infections can lead to reductions in performance (Matthews and Southern, 2000), lower intestinal pH (Stephens et al., 1974; Fox et al., 1987; Giraldo et al., 1987), and altering absorption of certain nutrients (Willis and Baker, 1981). Several studies demonstrated that birds undergoing a coccidiosis

infection have decreased absorption for calcium (Ca) and/or phosphorus (P) (Takhar and Farrell, 1979; Turk, 1973; Giraldo et al., 1987) and a decline in tibia ash, which is often associated with a decline in mineral absorption (Willis and Baker, 1981, Watkins et al., 1989; Ward et al., 1990).

Phytic acid, which is present in high amounts in the industry-typical corn-soybean meal diet, is also known to bind Ca and P. The enzyme phytase is able to initiate the release of phosphates from phytic acid, making it available for absorption and utilization (IUB, 1979; Zwart, 2006). Releasing these phosphate groups eliminates the ability of phytic acid to complex with Ca, thus avoiding the decline in the mineral's intestinal absorption. Watson and colleagues (2005) previously reported that both body weight gain and feed consumption were improved in chicks fed a phytase enzyme in a low Ca, low non-phytate P (npP) diet (0.80% and 0.25%, respectively) during a coccidiosis challenge. Furthermore, the percentage of tibia ash was improved in these infected chicks.

While coccidiosis must be controlled, consumer preference has the poultry industry moving away from the use of antibiotics in the feed, thus other means of coccidiosis control must be used. Live coccidia vaccines provide an opportunity to reduce the negative impact of coccidiosis. They enhance the natural immunity of the chicken by recycling very low doses of coccidial oocysts (Chapman et al., 2002; Williams, 1998, 2002, 2003). The disadvantage to live vaccination is that it can lead to reductions in early growth, which is generally associated with increased incidence of secondary enteritis (Chapman et al., 2002). An alternative to vaccination is the use of coccidiostats in the feed. Ionophores, which include monensin and salinomycin, have are

commonly used for the control of coccidiosis in broiler chickens. These products act through general mechanisms to alter ion transport and disrupt osmotic balance, which causes damage to the coccidia sporozoites during their release into the lumen.

Following either natural exposure or vaccination to *Eimeria* spp., both antibody and cell-mediated immune responses are activated. Live parasites in the intestinal epithelial cells induce the secretion of cytokines and chemokines by T-cells, which are critical in mounting protective immunity (Kaiser et al., 2005). Among these are interferon gamma (IFN-γ), interleukin (IL) -4, IL-6 and IL-17. IFN-γ is an important Th1 cytokine that activates the phagocytic potential of macrophages and cytotoxic potential of natural killer cells and cytotoxic T lymphocytes, which are important in the host's defense against coccidia (Lillehoj and Trout, 1996; Lillehoj and Choi, 1998). The production of IFN-γ has been used to measure the T-cell response to coccidial antigens (Prowse and Pallister, 1989; Martin et al., 1994). In general, IFN-γ production is lower in uninfected chickens in comparison with those infected with *Eimeria* spp., and higher levels of IFN-γ coincide with a protective immune response to the infection (Lillehoj and Trout, 1996; Choi et al., 1999; Min et al., 2003; Hong et al., 2006).

Interleukin-4 is an important Th2 cytokine expressed by the lymphoid tissue in response to mucosal infection. Th2 cells are important for inducing a humoral response to combat parasite invasion (Avery et al., 2004; Mowen and Glimcher, 2004; Degen et al., 2005). Both IL-6 and IL-17 are inflammatory cytokines, which are produced in response to a coccidial infection and also offer protection against re-infection in the future (Lillehoj and Trout, 1996; Lillehoj and Choi, 1998; Lillehoj and Lillehoj, 2000; Min et al., 2001; Lillehoj et al., 2004). IL-17 is involved in inducing and mediating pro-

inflammatory responses by stimulating the production of other cytokines and chemokines such as IL-1β, IL-6, IL-8, G-CSF, GM-CSF, TNF-α, and TGF-β (Veldhoen et al., 2006; Hong et al., 2008). IL-6 is important as both a pro-inflammatory and anti-inflammatory cytokine and is essential for the transition from acute inflammation to either acquired immunity or chronic inflammatory disease. As a pro-inflammatory, IL-6 is secreted in combination with TNF-α and IL-1 by either T-cells or macrophages to stimulate further immune response at the site of inflammation. It inhibits the effects of other pro-inflammatory cytokines in order to control the extent of tissue inflammation, thus serving as an anti-inflammatory as well (Xing et al., 1998).

Based on the information referenced, it was of interest to determine the effects of feeding a phytase enzyme to broiler chickens undergoing a coccidiosis challenge on live performance and bone mineralization. The first experiment was aimed at assessing any differences in birds fed low and marginal Ca-npP diets supplemented with phytase in combination with two coccidiosis control strategies. The second experiment was implemented to further evaluate the effects of phytase in a marginal Ca-npP diet in combination with a live, attenuated coccidiosis vaccination on bird performance and immune function.

6.3 Materials and Methods

General procedures

Litter seeding. Prior to two separate main experiments, a total of 240 (120/experiment) commercial broiler chicks were placed across 24 floor pens (5 birds/pen) containing fresh pine shavings. Chicks were provided ad libitum access to

water and a corn-soybean meal diet similar to the Standard diet of Table 6.1. At 12 days of age, all chicks were administered a 1 ml cocktail of coccidia via oral gavage. The cocktail was composed of *Eimeria acervulina* and *E. tenella* with concentrations of approximately 100,000 and 5,000 sporulated oocysts per milliliter, respectively. Both *Eimeria* species had been obtained previously from field isolates, which were maintained in 2.0% potassium dichromate in the laboratory. Each species was passed through young chicks prior to preparation of the cocktail to ensure the availability of viable sporulated oocysts. Birds remained in the pens through 28 days of age to seed the bedding with coccidia. After removal and euthanization of all birds via carbon dioxide inhalation, the pens remained empty for three days.

Animals. For the main experiments, mixed-sex commercial broiler chicks were obtained and randomly allocated to treatments in a randomized incomplete block design. All chicks were kept on a 24 h/d light program and had free access to feed and water throughout the trial. Body weights and feed consumption were recorded per pen at 11 and 20 (Experiment 1) or 10, 18, and 21 d (Experiment 2) of age and the calculated feed conversion was corrected for mortality on a "bird day" basis. Animal handling procedures during experimentation were in accordance with guidelines of Auburn University's Institutional Animal Care and Use Committee (IACUC).

At 11 and 20 (Experiment 1) or 10 and 18 d (Experiment 2) of age five birds per treatment were randomly chosen and sacrificed for the collection of intestinal samples. From these birds, gross lesions due to *Eimeria* infection were sought and scored in accordance with Johnson and Reid (1970), with scores ranging from 0 (no gross lesions) to 4 (most severe gross lesions). Samples were also collected from the descending loop

of the duodenum and from the center of the cecal tonsil, placed in 10% buffered formalin phosphate and sent to the diagnostic lab[1] for hematoxylin and eosin staining. Following staining, these samples were subjected to microscopic scoring for lesions via a light microscope with a 40x objective. *E. acervulina* lesions were scored on a scale of 1-4 as follows: 1 = 0 parasites, 2 = 1-2 parasite clusters, 3 = 3-5 parasite clusters, and 4 = >5 parasite clusters. From the intestinal cross-section, a cluster was considered to be a single villous or adjacent villi having multiple *E. acervulina* parasites present in the epithelial lining of each villous (Figure 6.1). Lesions associated with *E. tenella* were scored on a 1-4 scale as well, with 1 = 0 parasites, 2 = 1-30 parasites, 3 = 31-100 parasites, 4 = >100 parasites. Individual parasites were counted if they stained purple and had an outer ring of white (Figure 6.2).

After collecting the required tissue samples, fecal matter was collected from the latter half of the intestine and pooled into 2% potassium dichromate in order to enumerate sporulated *Eimeria* oocysts. These samples were placed in a gyrating water bath for a period of 48 hours at 37^{o}C. Following this incubation period, 1 ml of the sample was diluted and placed on a hemocytometer to determine the number of sporulated oocysts. Enumeration was replicated four times and the amount of live oocyst/ml was determined by the following equation: (cell count/5) * dilution factor * 10^4.

At 11 and 20 d (Experiment 1) or 21 d (Experiment 2) of age, birds were randomly selected for the collection of the left tibia, for a total of 30 or 24 tibias per treatment (Experiment 1 and 2, respectively). Tibia samples were stored in plastic bags and frozen at -20^{o}C until analysis for breaking strength was conducted. Each tibia was

[1] Thompson Bishop Sparks State Diagnostic Laboratory, Auburn, Alabama

brought to room temperature before being broken in the center using a TA-HDi[2] texture analyzer. A fulcrum with a width of 3.0 cm, chosen to accommodate the shortest bones, supported each tibia. A probe with a round base was attached to a 50 kg load cell with a crosshead speed of 1 mm/s.

Phytase enzyme. The phytase enzyme utilized in the present experiments is commercially available and is marketed as Optiphos[3] and is the same product as Phytase A mentioned in previous chapters. Optiphos is a 3-phytase and was originally derived from *Escherichia coli* and mass-produced in the yeast species *Pichia pastoris.* According to the manufacturer, the pH range for the optimal activity of this enzyme within the digestive tract was 2.5-3.5.

Statistical analysis. Statistical analyses were performed on all performance, coccidiosis and cytokine data using JMP® software (SAS, 2005). Data were subjected to a one-way analysis of variance and means were separated by Tukey's Honestly Significant Difference procedure at a probability level of 0.05. Analyses of percentages were performed after transformation using arcsine of their square root.

Experiment 1

Animals. For Experiment 1, 1008 chicks were allocated to eight treatments. Each of these treatments was replicated six times with 21 chicks assigned to each of 48 floor pens. Two additional treatments, which contained an additional 120 chicks, were placed across 12 pens of a Petersime[4] grow-out battery with raised wire floors to serve as negative controls for each of the diets.

[2] Texture Technologies, Scarsdale, NY
[3] Optiphos; JBS United, Sheridan, IN
[4] Petersime Incubator Company, Gettysburg, OH

Treatments. Upon arrival from the hatchery 504 of the chicks were spray vaccinated with a commercial coccidia vaccine, Coccivac-B[5]. Of these, 252 were placed across 12 floor pens containing fresh shavings, and the remaining vaccinated chicks were placed on the used litter of 12 pens that had been seeded previously. Five hundred four unvaccinated chicks were placed across the remaining floor pens in a similar manner, with half placed on fresh shavings and the remaining half placed on the used, seeded litter.

Two corn-soybean meal basal diets were formulated to contain marginal (0.35%, 0.80%) or low (0.25%, 0.70%) levels of npP and Ca (Table 6.1). The remainder of the diet was formulated to meet or exceed all nutrient requirements set forth by the NRC (1994). Both diets were supplemented with 500 FTU of the phytase enzyme Optiphos according to the manufacturer's recommendations. Both basal diets were divided evenly and the coccidiostat Coban 90[6] was included into one batch of low and marginal npP-Ca diets. Each diet was pelleted at 79°C (175°F), crumbled, and provided from days 0-20 of age. Diets containing Coban 90 were fed to the unvaccinated chicks, while chicks that were vaccinated at hatch or unvaccinated and placed in battery pens were provided diets free of a coccidiostat.

Experiment 2

Animals. For Experiment 2, 1008 chicks were allocated to eight treatments. Each treatment was replicated six times with 21 chicks assigned to each of 48 floor pens. An additional 30 chicks were placed across three pens of a Petersime[7] grow-out battery with raised wire floors to serve as negative controls.

[5] Intervet/Schering-Plough Animal Health, Millsboro, DE
[6] Elanco, Indianapolis, IN
[7] Petersime Incubator Company, Gettysburg, OH

Treatments. Half of the chicks were spray vaccinated at day of hatch with Coccivac-B. Of these, 252 were placed across 12 floor pens containing fresh shavings, and the remaining vaccinated chicks were placed on the previously seeded litter. The unvaccinated chicks were placed across the remaining pens.

Two corn-soybean meal diets were formulated to contain marginal (0.35%, 0.87%) or standard (0.45%, 1.0%) levels of npP and Ca (Table 6.1). The remainder of the diet was formulated to meet or exceed all nutrient requirements set forth by the NRC (1994). The marginal diet was supplemented with 500 FTU of the phytase enzyme Optiphos, according to the manufacturers recommendations. All diets were pelleted at 79^{o}C (175^{o}F), crumbled, and provided from days 0-21 of age. Each diet was allotted to a pen to ensure that both the vaccinated and unvaccinated chicks on either new or seeded bedding received one of each.

Procedures. In addition to the general procedures mentioned previously, another five birds per treatment and five from the battery pens were sacrificed for the collection of additional intestinal samples. Tissue samples were collected from one cecal tonsil and the duodenal loop for cytokine analysis. The remaining cecal tonsil and its contents were placed in pre-weighed bag and weights taken. Saline solution (0.85%), which contained 8.5g of sodium chloride[8] per liter, was added to the bag containing the ceca at a ratio of 10:1. All samples were mixed in a stomacher[9] for 90 s and then diluted out to 10^{-3}. Duplicate plates were streaked onto tryptose sulfite cycloserine (TSC)[10] agar for each dilution and incubated in an aerobic chamber at 37 C for 24 hours for the detection of *C. perfringens*. Due to low colonization, the original 10^{-1} dilutions were incubated in

[8] Fisher Scientific, Fairlawn, New Jersey
[9] Mix 1, AES Laboratoire, Combourg, France
[10] Merck, Darmstadt, Germany

cooked meat broth[11] at 37 C for 24 hours and then plated onto TSC agar and incubated for an additional 24-h period.

RNA extraction and cDNA synthesis. Tissue samples, approximately 100 mg in weight, were collected from the cecal tonsil and duodenal loop and immediately submerged in a micro-centrifuge tube containing 1 ml of Tri Reagent[12]. Approximately 100 mg of 0.5mm zirconium oxide beads were included into the centrifuge tube and the contents were homogenized at maximum speed for 20 minutes using a CDX24 Bullet Blender[13]. The samples were isolated according to the manufacturer's protocol (Appendix 1), then stored at -80 C. To ensure samples were free of extraneous DNA, they were subjected to treatment with Turbo DNA-free[14] according to the manufacturer's protocol (Appendix 2). Two microliters of total RNA from each sample were reverse transcribed into cDNA with the use of qScript One-Step SYBR Green qRT-PCR kit for iQ[15] according to manufacturer's protocol (Appendix 3).

Quantitative RT-PCR. Oligonucleotide primers for the chicken cytokines and β-actin control were designed based on sequences available from public databases or selected from the literature (Table 6.2). Primers were first run with lymphocytes stimulated with the lectin protein Concavalin A (ConA) to determine the proper protocol for detection of each cytokine. Protocols for each cytokine evaluated are presented in Table 6.3. Amplification and detection were carried out using approximately 5 ng/µl of RNA using the BioRad CFX96 real-time PCR detection system[16]. Standard curves were

[11] Cooked Meat Medium, Becton, Dickinson and Co. (BD), Franklin Lakes, NJ
[12] Molecular Research Center, Inc., Cincinati, OH
[13] Next Advance, Inc., Averwill Park, NY
[14] Applied Biosystems/Ambion, Inc., Austin, TX
[15] Quanta Biosciences, Inc., Gaithersburg, MD
[16] Bio-Rad Laboratories, Hercules, CA

generated with the use of $\log_{10}$ dilutions of standard RNA by CFX manager software. The levels of individual transcripts were normalized by those of β-actin using the mean threshold cycle value (C_t) of each: $\left| C_{t\ (\beta\text{-actin})} - C_{t\ (\text{cytokine})} \right|$.

6.4 Results

Experiment 1

Live performance and bone strength data for Experiment 1 is presented in Table 6.4. Live performance was not affected by dietary Ca-npP levels throughout the experiment (P>0.05). Birds provided no method of coccidiosis control due to their placement in battery pens had a lower feed consumption (FC; P<0.001) than birds reared in floor pens, which resulted in a decrease (P<0.05) in body weight gain (BWG) at 11 and 20 d of age. Also, the decline in FC and BWG led to a lower feed:gain ratio during the 0-11 d feeding period. During both feeding periods, BWG was affected by the presence or absence of an *Eimeria* challenge, where unchallenged birds gained an average of 20 and 35 g more (P<0.05) during each period, respectively. While there were no differences due to the main effects for bone breaking strength at either d 11 or 20, there was a diet*challenge interaction on day 11 (Figure 6.3). This interaction suggests that unchallenged chicks fed a marginal Ca-npP diet exhibited an increased bone strength (P<0.05) in comparison with challenged chicks fed the same diet.

There was no presence of live oocysts in birds for any treatment at d 11 or 18, and though there was presence of unsporulated oocysts on d 18, no differences were detected between treatments (data not shown). The percentage of birds positive for gross lesions differed at 20 d of age (Table 6.5). The data indicate that birds vaccinated or

administered a coccidiostat had an increased incidence (P<0.05) of *E. tenella* invasion, which was indicated by a greater incidence of severe lesions. Only vaccinated birds were found to have an increased (P<0.05) incidence of *E. acervulina* at the same time point, though the scores between control strategies did not differ (P>0.05). There were no differences (P>0.05) in microscopic lesion scores at either point of tissue collection.

Experiment 2

Data for both live performance and bone breaking strength are presented in Table 6.6. Neither live performance nor bone mineralization was affected (P >0.05) by the main effects of diet or vaccination strategy throughout the entirety of the experiment (Table 6.6). The *Eimeria* challenge caused a 90g reduction in BWG (P<0.001) and reduced feed intake by 5.3% (P<0.01) in comparison with birds not challenged. Bone mineralization was also negatively affected (P<0.01) by the *Eimeria* challenge, resulting in a 2.3 kg reduction in bone breaking strength.

Following multiple incubation methods of cecal samples for the detection of *C. perfringens*, colonies were present at less than detectable limits (<30 cfu/ml) even after enrichment and were considered nonsignificant across treatments, thus this data is not shown. The presence of oocysts was not found to differ (P>0.05) across treatments on d 10, however on d 18 birds that were vaccinated tended to have a lower incidence (P<0.05) of oocysts present in comparison with those birds that remained unvaccinated (Table 6.7). Likewise, birds fed a phytase-supplemented diet had decreased (P<0.05) incidence of cocci oocysts. Regardless of treatment, gross lesion scores did not differ (P>0.05) at 10 d of age, though there was an increase (P<0.05) in the microscopic scores for both *E. acervulina* and *E. tenella* (Tables 6.8). Birds that were not supplemented

with phytase or were not vaccinated had higher microscopic lesion scores for *E. tenella* ($P<0.01$) at 18 d of age. In addition, challenged birds had an increase in the lesion scores for *E. tenella,* both gross and microscopic, and displayed a trend towards increased number of birds having lesions present under both scoring systems.

The IL-6 primers used proved to be unpredictable in combination with these samples, resulting in multiple melt peaks that differed from that of the ConA stimulated cells even at concentrations as high as 100 ng/ul. Therefore, results for IL-6 are not presented. Preliminary data from RT-PCR analysis with IL-4 suggested there was little to none of this cytokine present, so analysis of the remaining samples was not conducted.

Analysis of IFN-γ from intestinal tissue samples of the duodenum and ceca resulted in no difference ($P>0.05$) in gene expression between the main effects of diet, vaccination, or challenge at 10 d of age, though birds that were vaccinated had an increased expression of IFN-γ ($P<0.10$) in the duodenum (Figure 6.4). At 18 d of age there was no difference in IFN-γ in the duodenum, however gene expression of this cytokine was increased ($P<0.05$) in the ceca of birds that were vaccinated or challenged. Figure 6.5 displays a significant enzyme * challenge interaction ($P<0.05$), wherein birds fed the phytase enzyme had an increase in the up-regulation of IFN-γ if they were exposed to the pre-seeded litter.

IL-17 was not found to differ in the duodenum or ceca ($P>0.05$) due to the main effects at 10 d of age, though challenged birds had an increase in IL-17 expression ($P<0.10$) in the duodenum in comparison with the unchallenged birds (Figure 6.6). IL-17 gene expression was significantly increased ($P<0.05$) in challenged birds that were not fed the enzyme, (Figure 6.7). At 18 d of age, birds that were vaccinated had higher levels

of IL-17 gene expression ($P<0.05$) in the duodenum, though no other differences were detected.

6.5 Discussion

Live Performance

Both studies were implemented to assess the effects of phytase supplementation on commercial broilers undergoing an *Eimeria* challenge. The lack of difference between the supplemented and unsupplemented diets of Experiment 2 reiterates that addition of the enzyme to a marginal npP diet results in the release of P from phytic acid so that the bird may utilize it for proper growth and formation of the skeleton. Furthermore, the lack of difference between the low and marginal Ca-npP phytase-supplemented diets of Experiment 1 suggest that the Optiphos phytase enzyme is capable of releasing an equivalent of up to 0.20% of dietary npP from phytic acid without ill effects on bird performance.

The live performance data from these two experiments further suggests supplementation with a phytase enzyme is able to provide added improvements to birds infected with coccidiosis, enabling them to perform as well as birds that are not infected. Data from the present experiments differ from results previously published by Watson and collegues in 2005, who found that phytase was effective in improving growth and bone performance in birds infected with coccidia, though the response was not as great as in healthy chicks. From the current results, it may be speculated that supplementing the diet with phytase enzymes benefits the *Eimeria* infected bird, enabling it to make up for the decreased mineral absorption generally associated with coccidiosis.

Overall body weight gain was lower in birds that were undergoing a coccidiosis challenge for both experiments. In addition, both feed consumption and feed:gain ratio were affected by the coccidiosis challenge. These reductions in live performance were expected and produced results similar to that of other researchers (Matthews and Southern, 2000).

The diet*challenge interaction seen in experiment 1 suggests that bone-breaking strength is affected by marginal dietary Ca-npP levels in birds undergoing a coccidiosis challenge. The meaning of these results is unclear, however, considering birds on the low Ca-npP diet did not show the same affect. Mortality was not found to play a role in the lack of difference in the interaction, since there were no differences in mortality for unchallenged and challenged birds fed low (0 vs. 3.6% mortality, respectively) or marginal (0 vs. 5.5% mortality, respectively) phytase-supplemented npP-Ca diets. Regardless of the cause, no differences were seen as a result of either main effect at the next collection date, suggesting that any differences due to an interaction of diet and challenge are overcome with age.

Similar to other reports in the literature, birds of experiment 2 challenged with coccidia were found to have a decreased bone breaking strength (Willis and Baker, 1981, Watkins et al., 1989; Ward et al., 1990), which is highly correlated with percent tibia ash (Wilson and Mason, 1992; Orban et al., 1993). The decreased bone mineralization seen in the infected birds is evidence to further validate the decreased absorption of Ca and P that is often seen in birds undergoing a coccidiosis infection. The lack of difference for bone strength in experiment 1 between challenged and unchallenged birds may be

attributed to the supplementation of the diets with a phytase enzyme, which likely led to improvements in mineral absorption for challenged birds.

Presence of Coccidiosis

The lack of oocysts present in Experiment 1 suggests that the coccidiosis control methods implemented were able to disrupt the coccidia lifecycle, resulting in little coccidia invasion in the intestinal tract at 11 and 20 d of age. In Experiment 2, birds fed a diet supplemented with phytase had a reduction in the number of oocsyts, signifying that phytase may aid in the birds ability to combat *Eimeria* parasites, though the mechanism by which this may occur cannot be explained via the data presented in this experiment. Vaccination also led to a reduction in the number of oocysts at 18 d of age. This was expected since the purpose of vaccination is to provide low doses of *Eimeria* spp. at an early age to enhance the birds' natural immunity. Numerically, there was a 5500 oocsyt/ml difference between challenged and unchallenged birds, though these values did not differ statistically. Unchallenged treatments were placed in floor pens resulting in some exposure to any *Eimeria* spp. naturally present in the litter, though challenged birds had a higher incidence of oocyst invasion as would be expected.

In general, the percentage of birds having microscopic lesions present was greater than those with gross lesions, though the lesion scores for both methods were comparable. Previous research found that gross and microscopic lesion scores do not correlate and overall microscopic lesion scores are higher than gross lesion scores (Idris et al., 1997; Goodwin et al., 1998). Overall, these researchers showed that a combination of both forms of scoring provides the best representation for the presence of coccidiosis.

The percentage of birds positive for lesions, either microscopic or gross, did not differ at 11 d of age in Exp. 1 or at either time point in Exp. 2 and the scores across all treatments suggests that there was a low presence of coccidia, which corresponds with the low live oocysts counts. At 20 d of age (Exp. 1) vaccinated or challenged birds had a higher incidence of gross lesions for *E. acervulina*. In addition, birds either vaccinated or fed a coccidiostat had an increased incidence of *E. tenella* lesions, which corresponded with a higher lesion score in comparison to birds provided no form of control. Birds that did not receive a control method were placed on wire floor pens and did not have access to their fecal matter, which is how coccidia cycles through the bird, thus it was expected that these birds should not have coccidiosis or lesions associated with the *Eimeria* parasites. Furthermore, an incidence of coccidiosis was anticipated in challenged birds since they were placed on preseeded litter, so the increased percentage of birds positive for either *E. acervulina* or *E. tenella* was in line with expectations when compared with birds that were not exposed to coccidia.

Cytokines

The oligonucleotide primers for chicken cytokines were selected from the literature due to others' success in detecting differences in gene expression via their use. During the preliminary analyses, IL-4 gene expression was present at extremely low levels, suggesting it would be difficult to detect differences in gene expression across treatments. The primers for IL-6 were found to be very unpredictable in combination with the samples used in Experiment 2. Both of these specific IL-4 and IL-6 primer sets, as well as others published in the literature, have been successful in detecting gene expression differences in response to infection (Abdul-Careem et al., 2006; Hong et al.,

2006; Park et al., 2008). The manner in which tissue samples were collected and prepared for RNA extraction differed between the previously referenced papers and the current experiment, and is a reasonable explanation for low or improper gene expression. In general, other researchers have gone through a series of procedures to isolate intraepithelial lymphocytes from specific tissues for RNA extraction. The methods of RNA extraction employed in Experiment 2 involved the extraction of RNA from an entire portion of the intestinal tissue. Therefore, results indicate that lymphocytes need to be isolated to achieve proper gene expression in chickens exposed to low doses of coccidiosis.

Overall, supplementation of the diet with phytase had no affect on cytokine expression. There was, however, an interactive effect of enzyme and challenge on IL-17 gene expression in 10 d old chicks. During this time period, challenged birds fed an unsupplemented diet had an increased expression of IL-17 in response to *E. acervulina*, suggesting that the challenged birds have a higher immune response when not provided enzyme supplementation in comparison with unchallenged chickens. The interaction between enzyme and challenge at 18 d of age suggests that challenged chickens supplemented with phytase have an increased expression of IFN-γ in response to *E. tenella*. The influence of phytase supplementation on cytokine secretion is still unclear due to significant up-regulation of gene expression for IFN-γ and IL-17 occurring in response to different coccidia species at different time points while under different enzyme supplementation conditions.

Previous research detected increased gene expression of IFN-γ and IL-17 in broilers up to 10 d following inoculation with E. acervulina and E. tenella in comparison

with uninnoculated chickens (Hong et al., 2006). In the present experiment, neither IFN-γ nor IL-17 were up-regulated in response to either *Eimeria* spp. at 10 d of age. However at 18 d, IFN-γ gene expression was increased in response to *E. tenella* for vaccinated or challenged birds, and the expression of IL-17 was increased in response to *E. acervulina* in challenged birds. This delay in up-regulated gene expression in comparison to the study by Hong and colleagues (2006) is likely due to the way in which birds were exposed to coccidia. Hong et al. (2006) inoculated their birds directly, via oral gavage, while the present study exposed the birds to coccidia in a typical production scenario. Because of the difference in original exposure, it would take several lifecycles for the parasites to induce an immune response. Thus the induced secretion of cytokines to mount an immune response would be delayed in comparison.

IFN-γ was only found to have higher gene expression in response to *E. tenella* in the ceca at 18 d of age, suggesting the immune response to this parasitic species was still a primary response (Lillehoj, 1998) and a memory response had not yet been initiated. Likewise, the only significant response of IL-17 secretion was in response to *E. acervulina*. Since IL-17 is secreted by activated T-cells (Yao et al., 1995; Broxmeyer, 1996; Fossiez et al., 1996) the secretion of IL-17 in response only to *E. acervulina* suggests the infection was strong enough to induce production of memory T-cells, while the exposure to *E. tenella* was not. Overall, these cytokine responses coincided with the time period required for lesions to form in the intestinal tract due to these two coccidia species. *E. acervulina* typically produces lesions 2-4 d earlier than *E. tenella*, which may have provided enough time for the immune system to mount both an initial and memory response to *E. acervulina*, but only an initial response to *E. tenella*.

6.6 Conclusions

Data from both experiments confirm that supplementation of the diet with Optiphos allows for the release of an equivalence of 0.20% npP when dietary Ca and npP are adjusted accordingly. Birds undergoing a coccidiosis challenge have reductions in live performance and decreased absorption of Ca and P, which leads to decreased bone strength, when not provided phytase supplementation. In addition, coccidiosis control programs can provide improvements in bird performance and decreases in the incidence of coccidiosis. Finally, IFN-γ gene expression was up-regulated by 18 d of age in response to *E. tenella* in vaccinated or challenged birds, indicative of a primary immune response occurring at that time point, while IL-17 gene expression was up-regulated in response to *E. acervulina* in challenged birds, indicating an immune response involving memory T-cells.

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Table 6.1 Ingredient and calculated analysis of broiler diets containing three non-phytate phosphorus– calcium combinations for both experiments[1]

	Low	Marginal	Standard
Ingredients		(%)	
Ground yellow corn (7.5% CP)	58.23	56.65	55.07
Soybean meal (48% CP)	36.09	36.34	36.60
Poultry fat	2.71	3.30	3.89
Dicalcium phosphate (21.5% P; 18.5% Ca)	0.62	1.16	1.71
Ground limestone (38% Ca)	0.71	0.92	1.12
Sodium chloride	0.48	0.48	0.47
Trace-mineral premix[2]	0.25	0.25	0.25
Vitamin premix[3]	0.50	0.50	0.50
L-lysine (98.5%)	0.13	0.12	0.12
DL-methionine (99.9%)	0.27	0.27	0.27
Optiphos[4]	0.01	0.01	----
Total	100.00	100.00	100.00
Calculated Analysis (%)			
Metabolizable energy, kcal/kg	3086.00	3086.00	3086.00
Crude protein	22.00	22.00	22.00
Calcium	0.50	0.70	0.90
Non-phytate phosphorus	0.25	0.35	0.45
Sodium	0.21	0.21	0.21
Lysine	1.35	1.35	1.35
Methionine	0.63	0.63	0.63
Methionine + Cystine	0.97	0.97	0.97

[1]For experiment 1, both the low and marginal diets were duplicated to include Coban 90 at 0.05% of the diet, replacing corn.

[2]Supplied the following per kg of complete feed: 125 mg manganese; 1 mg iodine; 55 mg iron; 6 mg copper; 55 mg, zinc; 0.3 mg selenium.

[3]Supplied the following per kg of complete feed: 8,000 IU vitamin A (retinyl palmitate); 2,000 IU cholecalciferol; 8 IU vitamin E (dl-tocopheryl acetate); 2 mg menadione; 5.5 mg riboflavin; 13 mg pantothenic acid; 36 mg niacin; 500 mg choline; 0.02 mg vitamin B_{12}; 5 mg folic acid; 1 mg thiamin; 2.2 mg pyridoxine; 0.05 mg biotin; 125 mg ethoxyquin.

[4]JBS United, Sheridan, IN

Table 6.2 Sequence of primers used in quantitative RT-PCR, Experiment 2

RNA target	Primer Sequences	
	Forward	Reverse
β-actin[1]	5'-ATCGTACTCCTGCTTGCTGAT-3'	5'-CAACACAGTGCTGTCTGGTGG-3'
IFN-γ	5'-GTCATTCAGATGTAGCTGACGGTGGA-3'	5'-CGCCATCAGGAAGGTTGTTTTTCA-3'
IL-4[2]	5'-ACCCAGGGCATCCAGAAG-3'	5'-CAGTGCCGGCAAGAAGTT-3'
IL-6[1]	5'-CAGGACGAGATGTGCAAGAA-3'	5'-TAGCACAGAGACTCGACGTT-3'
IL-17[2]	5'-CTCCGATCCCTTATTCTCCTC-3'	5'-AAGCGGTTGTGGTCCTCAT-3'

[1] Obtained from Abdul-Careem, M.F., B.D. Hunter, A.J. Sarson, A. Mayameei, J. Zhou and S. Sharif. 2006. Marek's disease virus-induced transient paralysis is associated with cytokine gene expression in the nervous system. Viral Immunol. 19:167-176.

[2] Obtained from Hong, Y.H., H.S. Lillehoj, S.H. Lee, R.A. Dalloul and E.P. Lillehoj. 2006. Analysis of chicken cytokine and chemokine expression following *Eimeria acervulina* and *Eimeria tenella* infections. Vet. Immunol. Immunopathol. 114:209-223.

Table 6.3 Protocols for each cytokine sought via quantitative RT-PCR, Experiment 2

RNA target	RT reaction phase		Denaturation, Annealing, and Extension Phases
β-actin	50°C for 10 min	95°C for 5 min	95°C for 30 s, 50°C for 30 s, and 72°C for 30 s – 40 cycles
IFN-γ	50°C for 10 min	95°C for 3 min	95°C for 30 s, 58°C for 45 s, and 72°C– 40 cycles
IL-4	50°C for 10 min	95°C for 3 min	95°C for 30 s, 56°C for 30 s, and 72°C for 1 min – 45 cycles
IL-6	50°C for 10 min	95°C for 3 min	95°C for 30 s, 56°C for 30 s, and 72°C for 1 min – 45 cycles
IL-17	50°C for 10 min	95°C for 3 min	95°C for 30 s, 55.6°C for 30 s, and 72°C for 30 s – 40 cycles

Table 6.4 Body weight gain (BWG), feed consumption (FC), feed conversion ratio (F:G), and bone breaking forces of birds that may or may not have been exposed to *E. acervulina* and *E. tenella,* Experiment 1[1]

	0-11 Day			0-20 Day			11 Day	20 Day
	BWG (g)	FC (g)	F:G (g/g)	BWG (g)	FC (g)	F:G (g/g)	Breaking Force (kg)	
Diet[2]								
Low	229	307	1.35	737	988	1.34	3.7	13.5
Marginal	236	312	1.32	758	1005	1.33	3.2	14.1
Control method								
None	219 [b]	256[b]	1.18[b]	703[b]	939[b]	1.34	3.2	14.8
Coccidiostat[3]	246 [a]	347[a]	1.41[a]	789[a]	1040[a]	1.32	3.5	13.9
Vaccination[4]	232[ab]	326[a]	1.42[a]	751[a]	1010[a]	1.34	3.4	12.6
Challenge								
Unchallenged	243[a]	318	1.32	765[a]	1013	1.32	3.7	13.2
Challenged[5]	222[b]	301	1.35	730[b]	979	1.34	3.2	14.4
SEM[6]	*5.58*	*6.87*	*0.023*	*9.51*	*13.01*	*0.009*	*0.18*	*0.72*
	Significance							
Diet	*NS*	*NS*	*NS*	*NS*[†]	*NS*	*NS*	*NS*[†]	*NS*
Control method	*	***	***	***	***	*NS*	*NS*	*NS*
Challenge	*	*NS*[†]	*NS*	*	*NS*[†]	*NS*	*NS*[†]	*NS*

[1] Besides a Diet*Challenge interaction (d 11; Figure 2), there was no significance between the interactions of main effects
[2] Low: Ca=0.50% and P=0.25%; Marginal: Ca=0.70% and P=0.35%; both diets contained Optiphos
[3] Birds provided Coban 90 in the feed
[4] Birds vaccinated at placement (0d) with Coccivac B
[5] Birds were placed on pre-seeded litter to mimic natural exposure to *Eimeria* spp.
[6] SEM: Pooled standard error of the mean
[a,b] Means with different superscripts in a column differ.
NS $P>0.05$; *$P<0.05$; *** $P<0.001$; [†] P-value falls between 0.05 and 0.10

Table 6.5 Percentage of birds positive for gross (G) and microscopic (M) lesions, and the average score of each, associated with *E. acervulina* and *E. tenella* in the duodenum and ceca, Experiment 1[1,2]

	Day 11								Day 20							
	% Positive				Average Score				% Positive				Average Score			
	acervulina		*tenella*		*acervulina*		*tenella*		*acervulina*		*tenella*		*acervulina*		*tenella*	
	G	M	G	M	G	M	G	M	G	M	G	M	G	M	G	M
Diet																
Low	16	4	12	0	0.1	1.0	0.1	1.0	12	16	24	0	0.2	1.1	0.4	1.0
Marginal	12	0	8	0	0.3	1.0	0.1	1.0	16	8	36	0	0.1	1.1	0.2	1.0
Control method																
None	20	0	10	0	0.4	1.0	0.1	1.0	0 [b]	0	0 [b]	0	0.2	1.1	0.0 [b]	1.0
Coccidiostat[4]	13	5	13	0	0.0	1.0	0.1	1.0	6 [b]	20	53 [a]	0	0.1	1.0	0.6 [a]	1.0
Vaccination[5]	20	0	13	0	0.1	1.1	0.1	1.0	40[a]	10	40 [a]	0	0.2	1.2	0.3[ab]	1.0
Challenge																
Unchallenged	6	0	6	0	0.0	1.0	0.1	1.0	3 [b]	30	23	0	0.1	1.0	0.3	1.0
Challenged[6]	20	3	15	0	0.3	1.0	0.1	1.0	50 [a]	0	40	0	0.2	1.2	0.2	1.0
SEM[7]	---	---	---	---	*0.10*	*0.02*	*0.07*	*0.0*	----		----	----	*0.08*	*0.08*	0.08	*0.0*
	Significance															
Diet	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
Control method	*NS*[†]	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*	*NS*	*	*NS*	*NS*	*NS*	*	*NS*
Challenge	*NS*[†]	*NS*	*NS*	*NS*	*NS*[†]	*NS*	*NS*	*NS*	*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

[1]All values represent 5 birds/trt/intestinal section.
[2] No significant differences between the interactions of main effects were found
[3] Low: Ca=0.50% and P=0.25%; Marginal: Ca=0.70% and P=0.35%; both diets contained Optiphos
[4]Birds provided Coban 90 in the feed
[5] Birds vaccinated at placement (0d) with Coccivac B
[6] Birds were placed on pre-seeded litter to mimic natural exposure to *Eimeria* spp.
[7] SEM: Pooled standard error of the mean; Not available for values presented as a percentage
[a,b] Means with different superscripts in a column differ.
NS $P>0.05$; *$P<0.05$; [†] P-value falls between 0.05 and 0.10

Table 6.6 Body weight gain (BWG), feed consumption (FC), feed:gain (F:G), and bone breaking force of broilers fed a standard P or marginal P + Optiphos diet from 0-21 days of age, Experiment 2 [1,2]

	0-10 Day			0-18 Day			0-21 Day			21 Day
	BWG (g)	FC (g)	F:G (g/g)	BWG (g)	FC (g)	F:G (g/g)	BWG (g)	FC (g)	F:G (g/g)	Force (kg)
Diet[3]										
No Enyzme	220	262	1.19	574	762	1.33	767	1037	1.36	16.8
Enzyme	224	267	1.19	584	771	1.33	797	1060	1.34	15.3
Vaccination[4]										
Unvaccinated	222	267	1.21	576	770	1.34	789	1052	1.34	15.6
Vaccinated	223	262	1.18	582	763	1.31	775	1046	1.35	16.1
Challenge										
Unchallenged	226	263	1.16 [b]	618 [a]	791 [a]	1.28 [b]	827 [a]	1077 [b]	1.31 [b]	17.0 [a]
Challenged[5]	218	267	1.22 [a]	539 [b]	742 [b]	1.38 [a]	736 [b]	1020 [a]	1.39 [a]	14.7 [b]
SEM[6]	*4.35*	*4.94*	*0.010*	*8.78*	*10.74*	0.015	*13.93*	*14.59*	*0.014*	*0.522*
	Significance									
Diet	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
Vaccination	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
Challenge	*NS*	*NS*	***	***	**	***	***	**	***	**

[1] All values represent contrasts involving 48 pens, each with 21 chicks at start of experimentation.
[2] No significant differences between the interactions of main effects were found
[3] Enzyme: Optiphos (500 FTU), Ca=0.7% and P=0.35%; No enzyme: Ca=0.9% and P=0.45%
[4] Birds vaccinated at placement (0d) with Coccivac B
[5] Birds were placed on pre-seeded litter to mimic natural exposure to *Eimeria* spp.
[6] SEM: Pooled standard error of the mean
[a,b] Means with different superscripts in a column differ.
NS $P>0.05$; ** $P<0.01$; *** $P<0.001$

Table 6.7 Enumeration of oocysts/ml present in the intestinal tract on d 10 and 18, Experiment 2

	Day 10	Day 18
Diet[1]		
Standard	3500	7000 [a]
Marginal	857	2857 [b]
Vaccination[2]		
Unvaccinated	1847	9230 [a]
Vaccinated	3714	2857 [b]
Challenge		
Unchallenged	3230	3077
Challenged[3]	2428	8571
SEM[4]	*969.7*	*1852.4*
	Significance	
Diet	*NS*	*
Vaccination	*NS*	*
Challenge	*NS*	*NS*

[1]Marginal: Optiphos (500 FTU), Ca=0.7% and P=0.25%; Standard: Ca=0.9% and P=0.45%
[2]Birds vaccinated at placement (0d) with CoccivacB
[3]Birds were placed on pre-seeded litter to mimic natural exposure to *Eimeria* spp.
[4]SEM: Standard error of mean
NS $P>0.05$; *$P<0.05$

Table 6.8 Percentage of birds positive for gross (G) and microscopic (M) lesions, and the average score of each, associated with *E. acervulina* and *E. tenella* in the duodenum and ceca, Experiment 2[1,2]

	Day 10								Day 18							
	% Positive				Average Score				% Positive				Average Score			
	acervulina		*tenella*		*acervulina*		*tenella*		*acervulina*		*tenella*		*acervulina*		*tenella*	
	G	M	G	M	G	M	G	M	G	M	G	M	G	M	G	M
Diet[3]																
Standard	15	30	25	15	0.2	1.3	0.3	1.3	20	15	50	70	0.3	1.2	0.5	2.8 [a]
Marginal	15	10	35	35	0.2	1.1	0.5	1.7	15	5	35	25	0.2	1.1	0.8	1.7 [b]
Vaccination[4]																
Unvaccinated	10	20	20	25	0.2	1.2	0.3	1.5	15	5	50	70	0.3	1.1	0.7	2.8 [a]
Vaccinated	20	20	40	25	0.2	1.2	0.5	1.5	20	15	35	25	0.1	1.2	0.6	1.8 [b]
Challenge																
Unchallenged	5	15	35	10 [b]	0.1	1.0 [b]	0.4	1.2 [b]	5	5	15 [b]	20	0.3	1.1	0.3 [b]	1.5 [b]
Challenged[5]	25	25	25	40 [a]	0.3	1.4 [a]	0.4	1.8 [a]	30	15	70 [a]	75	0.1	1.2	1.0 [a]	3.1 [a]
SEM[6]	---	----	---	----	*0.07*	*0.05*	*0.09*	*0.13*	---	----	---	----	*0.07*	*0.05*	0.10	*0.14*
	Significance															
Diet	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	***
Vaccination	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	***
Challenge	*NS*	*NS*	*NS*	**	*NS*[†]	**	*NS*	*	*NS*	*NS*	*	*NS*[†]	*NS*	*NS*	**	***

[1] All values represent 5 birds/trt/intestinal section.
[2] No significant differences between the interactions of main effects were found
[3] Enzyme: Optiphos (500 FTU), Ca=0.7% and P=0.35%; No enzyme: Ca=0.9% and P=0.45%
[4] Birds vaccinated at placement (0d) with Coccivac B
[5] Birds were placed on pre-seeded litter to mimic natural exposure to *Eimeria* spp.
[6] SEM: Pooled standard error of the mean; Not available for values presented as a percentage
NS $P>0.05$; * $P<0.05$;*** $P<0.001$; [†] P-value falls between 0.05 and 0.10

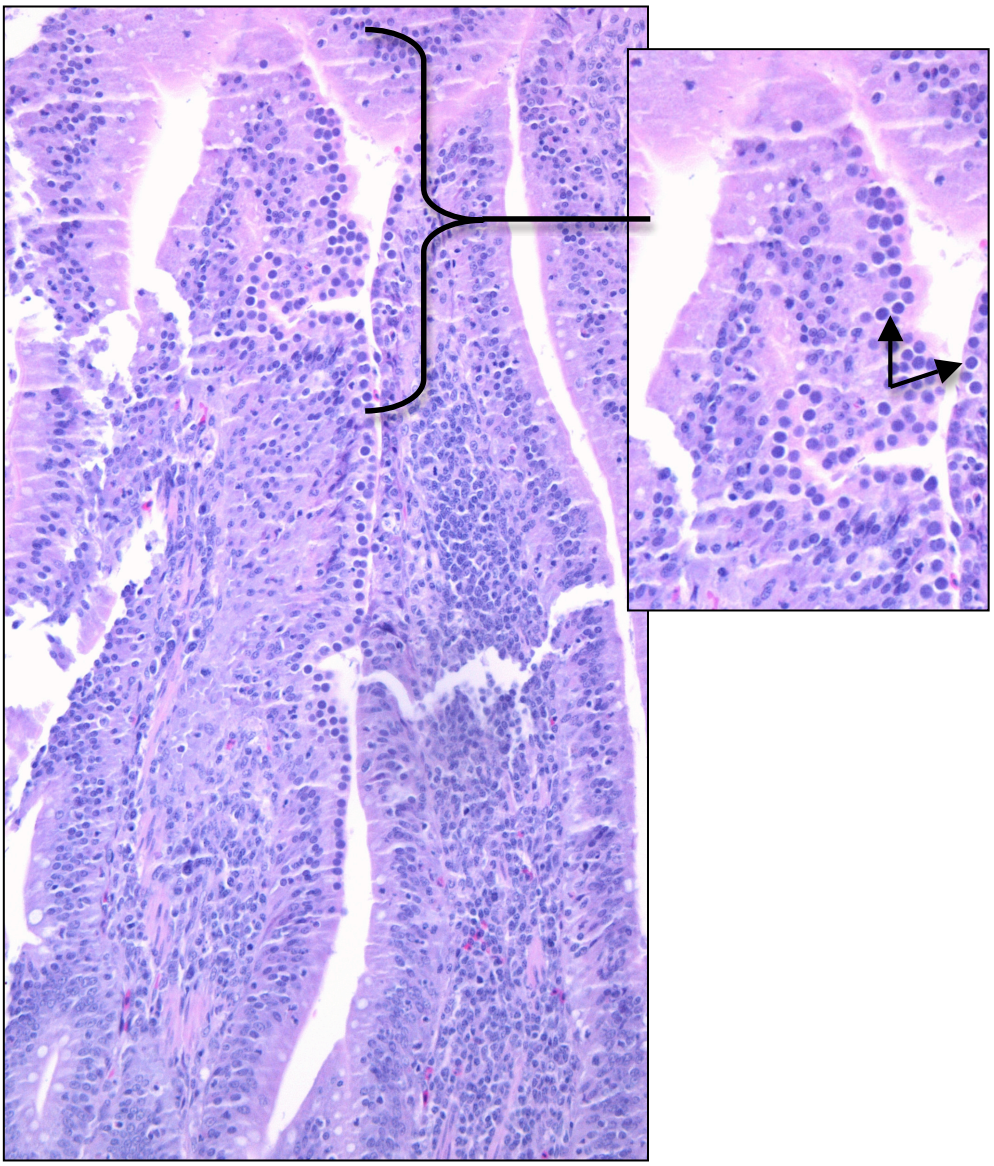

Figure 6.1 Villi of the duodenum containing *Eimeria acervulina* oocysts in the epithelial cells. Individual oocysts are seen in the enlarged picture to the right as dark purple stained circles, indicated by the arrows, on the outer edge of the villi. A cluster of parasites was considered to be any number of villi adjacent to one another that contained multiple oocysts within the epithelial lining of the villi.

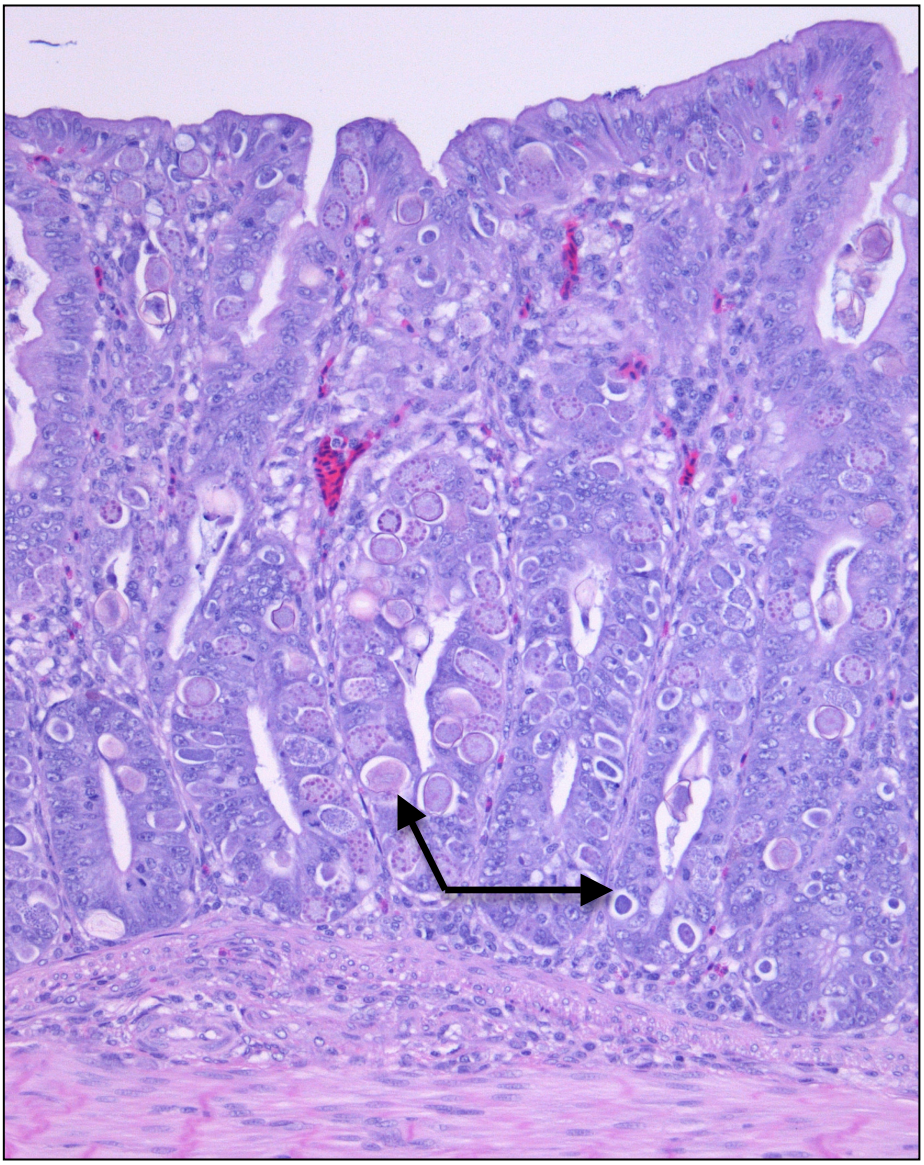

Figure 6.2 Villi of the cecum containing *Eimeria tenella* oocysts. Individual oocysts are indicated by the arrows and are stained purple with an outer ring of white.

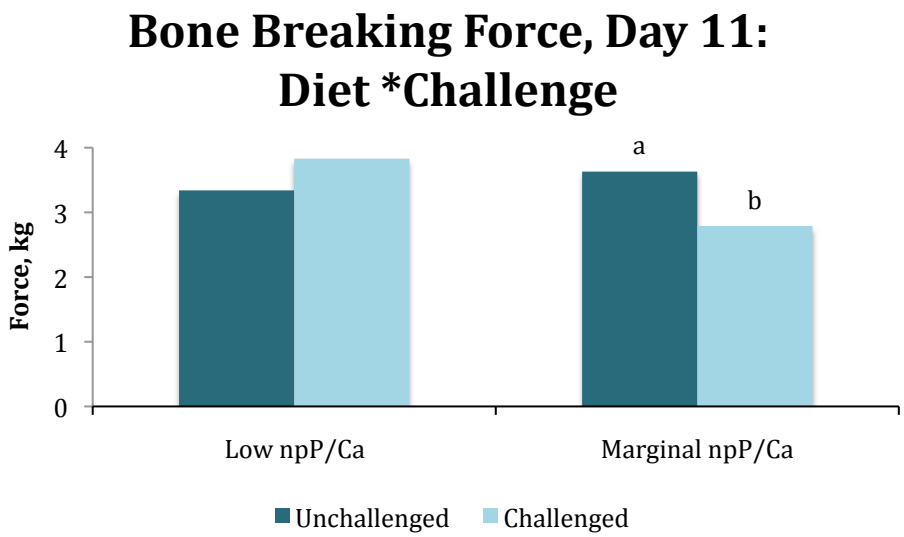

Figure 6.3 Significant dietary treatment (npP: non-phytate phosphorus; Ca: calcium) * *Eimeria* challenge interaction for bone breaking strength on day 11, Experiment 1.

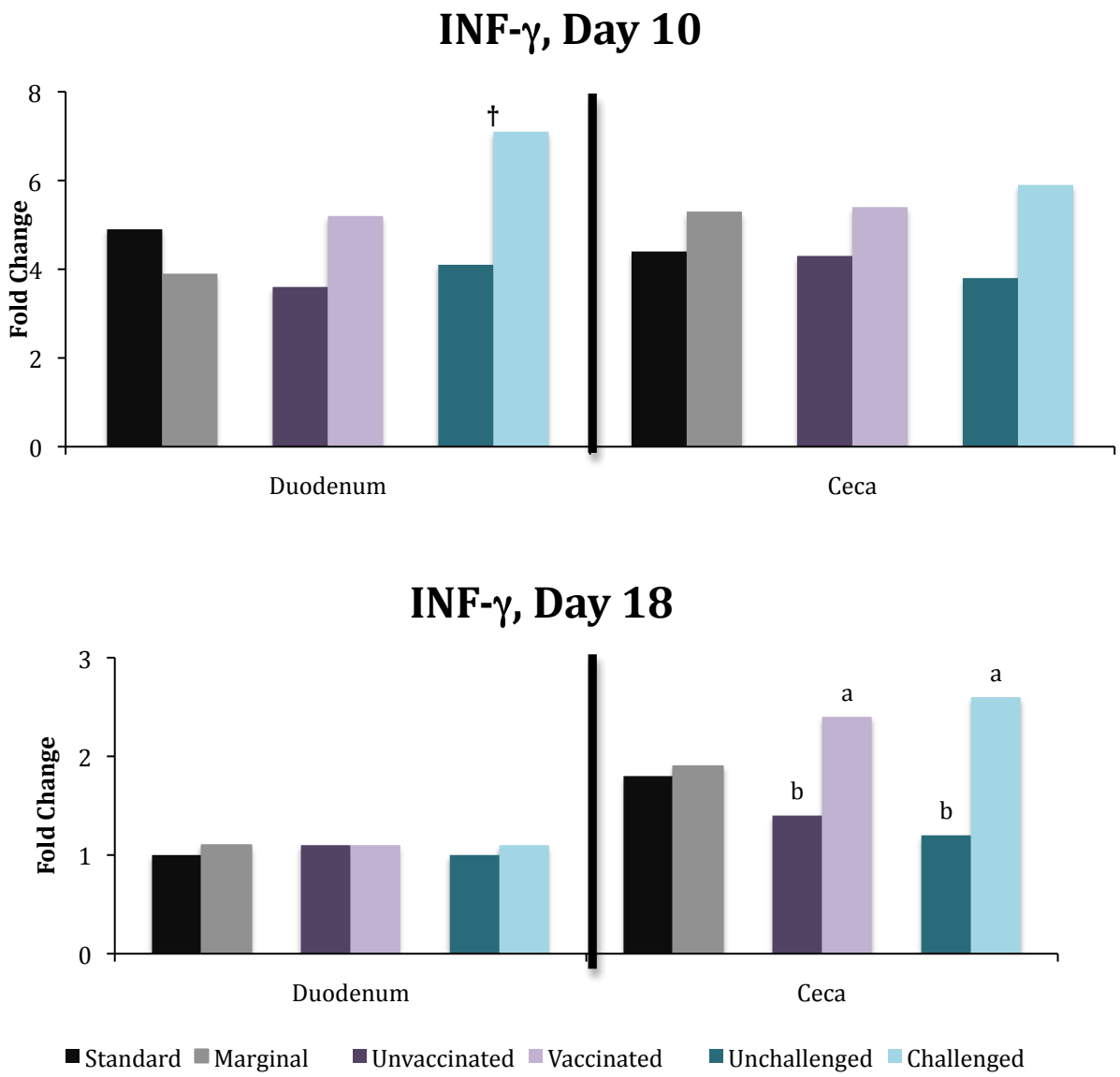

Figure 6.4 IFN-γ mRNA levels in chicken duodenum and ceca 10 and 18 days after a mimicked natural exposure to *Eimeria* spp. Birds were either placed on unseeded (unchallenged) or seeded (challenged; litter was preseeded with *E. acervulina* and *E. tenella*), unvaccinated or vaccinated with CoccivacB, and provided one of two diets (standard phosphorus diet or a marginal diet containing a combination of 0.35% available phosphorus + 500 FTU/kg Optiphos). RNA was isolated, reverse transcribed and used for RT-PCR analysis with primers listed in Table 2. Expression of target gene is normalized to β-actin and includes samples from five chickens per treatment. [a,b] indicates that the means with different superscripts differ ($P<0.05$). [†] indicates a P-value >0.05 and <0.10.

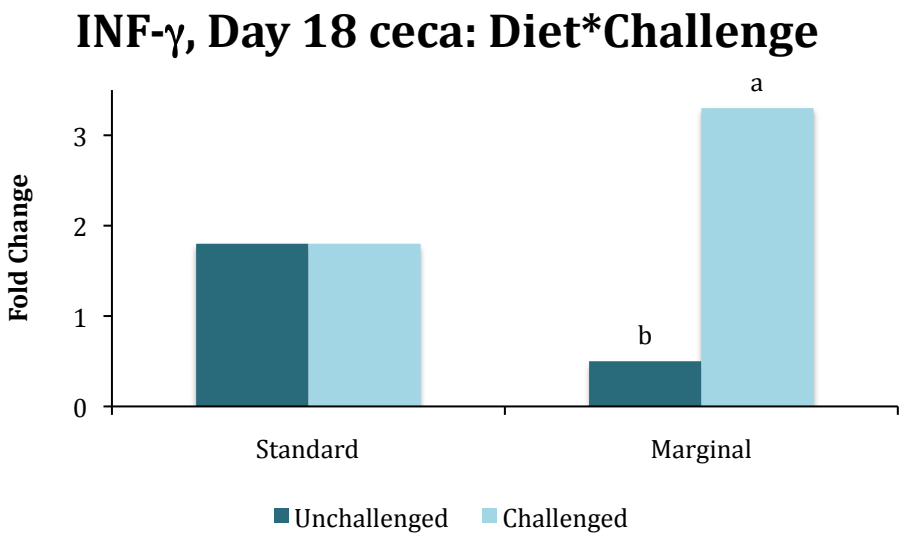

Figure 6.5 Significant Diet * *Eimeria* challenge interaction of IFN-γ mRNA levels in chicken ceca 18 days after a mimicked natural exposure to *Eimeria* spp. Birds were either placed on unseeded (unchallenged) or seeded (challenged; litter was preseeded with *E. acervulina* and *E. tenella*), unvaccinated or vaccinated with CoccivacB, and provided one of two diets (standard phosphorus diet or a marginal diet containing a combination of 0.35% available phosphorus + 500 FTU/kg Optiphos). RNA was isolated, reverse transcribed and used for RT-PCR analysis with primers listed in Table 2. Expression of target gene is normalized to β-actin and includes samples from five chickens per treatment. [a,b] indicates that the means with different superscripts differ ($P<0.05$).

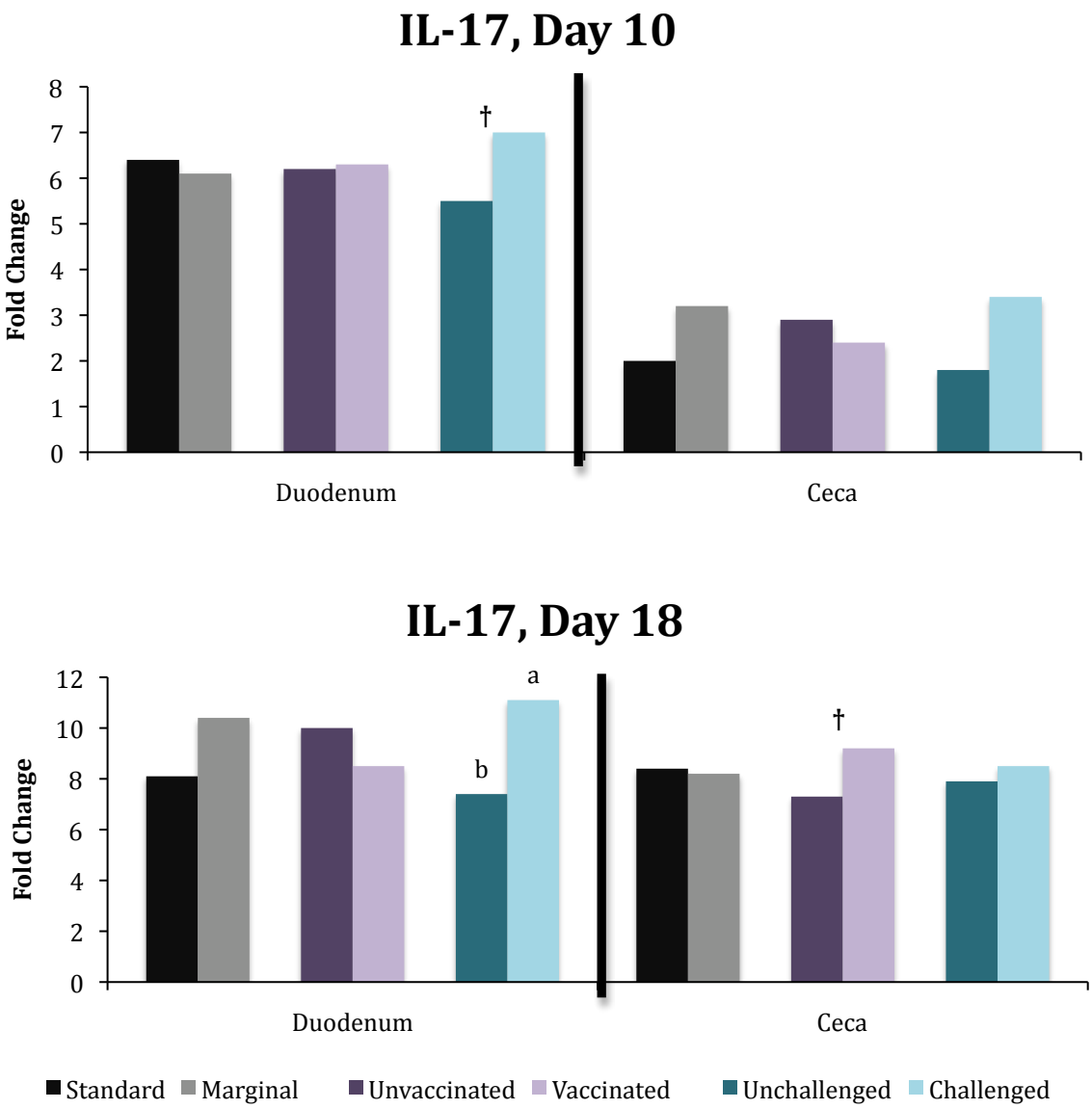

Figure 6.6 IL-17 mRNA levels in chicken duodenum and ceca 10 and 18 days after a mimicked natural exposure to *Eimeria* spp. Birds were either placed on unseeded (unchallenged) or seeded (challenged; litter was preseeded with *E. acervulina* and *E. tenella*), unvaccinated or vaccinated with CoccivacB, and provided one of two diets (standard phosphorus diet or a marginal diet containing a combination of 0.35% available phosphorus + 500 FTU/kg Optiphos). RNA was isolated, reverse transcribed and used for RT-PCR analysis with primers listed in Table 2. Expression of target gene is normalized to β-actin and includes samples from five chickens per treatment. ^{a,b} indicates that the means with different superscripts differ ($P<0.05$). † indicates a P-value >0.05 and <0.10.

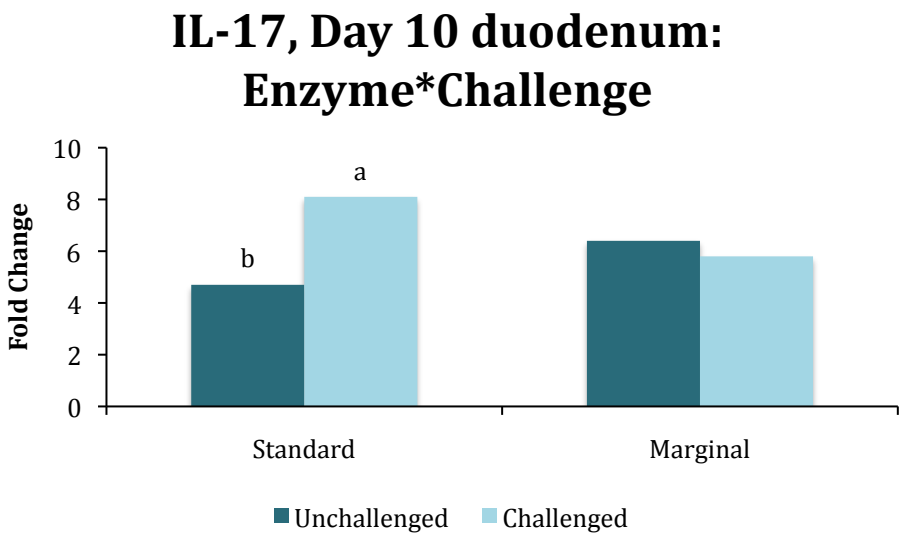

Figure 6.7 Significant phytase enzyme * *Eimeria* challenge interaction of IL-17 mRNA levels in chicken duodenum 10 days after a mimicked natural exposure to *Eimeria* spp. Birds were either placed on unseeded (unchallenged) or seeded (challenged; litter was preseeded with *E. acervulina* and *E. tenella*), unvaccinated or vaccinated with CoccivacB, and provided one of two diets (standard phosphorus diet or a marginal diet containing a combination of 0.35% available phosphorus + 500 FTU/kg Optiphos). RNA was isolated, reverse transcribed and used for RT-PCR analysis with primers listed in Table 2. Expression of target gene is normalized to β-actin and includes samples from five chickens per treatment. ^{a,b} indicates that the means with different superscripts differ ($P<0.05$).

7.0 Conclusions

Improving performance in poultry continues to be of importance in the poultry industry. It has been well established that phosphorus (P) is a major mineral involved in the formation of bone and that P has many functions outside of bone mineralization making it essential for day-to-day functions within the body. Therefore, including this mineral into the diet at inadequate levels may lead to reductions in performance and bone strength. Phytase enzymes are commonly included into reduced non-phytate phosphorus (npP) diets to eliminate the negative effects of inadequate dietary P levels. In addition to dietary P levels, both live and skeletal performance can be affected by a number of parameters including age of the breeder hen, bird sex, genetics, and health. The experiments outlined in the previous chapters were conducted to evaluate P levels in combination with phytase enzymes under different conditions.

In the preliminary experiment it was of interest to compare the removal of flesh and muscling from the tibia bone, as is commonly done prior to assessing values of shear force, to bones with flesh and muscling left intact. The resulting data suggested that accurate results for bone breaking strength could be obtained regardless of whether flesh was removed or intact. This lead to a reduction in bone collection and preparation time in the experiments that followed.

The first experiment was designed to determine the affects of npP levels, breeder hen age, and chick sex on both bird and skeletal performance. From an analysis of

unincubated eggs it was found that eggs from older breeder flocks have an increased size and weight in comparison with young breeder flocks. This egg sized directly related to chick size and bone strength at hatch and continued to have an affect on both parameters through four weeks of age. Birds provided a 0.35% npP diet also had lower body weights and bone breaking force values than birds fed a 0.50% npP diet. Body weight, feed consumption and bone strength were also reduced in females throughout the experiment.

Once determining that both live performance and bone strength are affected by breeder flock age, chick sex and dietary levels of npP, a second experiment was conducted to evaluate three commercial phytase enzymes for their ability to release an equivalence of 0.20% npP from the phytic acid molecule, which may bind P by up to 70% in a typical corn-soybean meal broiler diet, in comparison with unsupplemented diets in male broilers. Results indicated that reducing npP in the diet in 0.10% increments from 0.45-0.25% leads to a reduction in live performance and bone strength. In addition, supplying npP at only 0.25% causes mortality to significantly increase. Supplementing the 0.25% npP diet with phytase enzymes, however, resulting in live performance, bone breaking forces and mortality similar to an unsupplemented 0.45% npP diet.

From this information, two phytase enzymes, differing in the pH at which optimal performance occurs, were selected and evaluated in two broiler strains to determine if either enzyme or genetic selection had an affect on live performance or bone strength. Similar to the previous experiment, growth and skeletal performance were only affected by feeding an inadequate level of npP (0.35%), though enzyme supplementation alleviated these negative implications. Following day of hatch, body weight was not

found to differ between the two broiler strains and bone strength was unaffected by genetic differences at three weeks of age.

A single phytase enzyme was selected for Experiment 4 and was included into either a 0.25 or 0.35% npP diet which had calcium (Ca) levels adjusted to maintain a Ca:npP ratio of 2:1. Birds were subjected to coccidiosis control strategies (none, coccidiostat or vaccination) and a challenge with *E. acervulina* and *E. tenella* (unchallenged or challenged) via seeded litter. Phytase supplementation of either diet lead to no differences in live or skeletal performance. Live performance was improved with the implementation of coccidiosis control methods and was decreased in challenged birds, though bone strength was unaffected by either main effect. Incidence of coccidiosis was increased in birds that were challenged or provided a coccidiosis control method.

The final experiment served as a continuation of Experiment 4 and sought to determine the effects of phytase enzyme supplementation on live performance, bone breaking strength, incidence of coccidiosis infection and immune function. When the 0.35% npP diet was supplemented with phytase, bird performance did not differ from birds fed adequate npP levels (0.45%). Birds undergoing a coccidiosis challenge displayed reduced performance and a reduced absorption of Ca and P, which was accompanied by a decline in bone strength values. Vaccination was not shown to have an affect on performance or bone strength, which contradicted the results from Experiment 4. There was a response of the immune system due to vaccination and/or challenge, as indicated by an up-regulation in the gene expression of the cytokines IFN-γ and IL-17 at

18 d of age. The expression of these cytokines indicates a both an innate and memory immune response.

These experiments reiterate results found in the literature, where reduced levels of dietary npP and/or Ca levels can lead to negative implications on growth and bone performance. Supplementation of these low or marginal diets with phytase enzymes, however, leads to improvements in growth and skeletal performance. In addition, supplementation with phytase enzymes may have an affect on incidence of coccidiosis infection and immune function.

Appendix 1

RNA Isolation Procedures

1) HOMOGENIZATION:

- Homogenize tissue samples in TRI Reagent[1] (1 ml/50 - 100 mg tissue).

2) PHASE SEPARATION:

- Place 1ml aliquots of samples into microcentrifuge tubes.
- Supplement the aliquot with 0.2 ml chloroform per 1 ml of TRI Reagent, cover the samples tightly and shake vigorously for 15 seconds.
- Store the resulting mixture at room temperature for 10 minutes and centrifuge at 12,000 g for 15 minutes at 4 C.
- Following centrifugation, the mixture separates into a lower red phenol-chloroform phase, interphase and the colorless upper aqueous phase.

3) RNA PRECIPITATION:

- Transfer the aqueous phase to a fresh tube.
- Precipitate RNA from the aqueous phase by mixing with isopropanol. Use 0.5 ml of isopropanol per 1 ml of TRI Reagent used for the initial homogenization.
- Store samples at room temperature for 10 minutes and centrifuge at 12,000 g for 8 minutes at 4 C.
- RNA precipitate forms a gel-like or white pellet on the side and bottom of the tube.

4) RNA WASH:

- Remove the supernatant and wash the RNA pellet (by vortexing) with 1 ml 75% ethanol and subsequent centrifugation at 7,500 g for 5 minutes at 4 C.

5) RNA SOLUBILIZATION

- Remove the ethanol wash and briefly air-dry the RNA pellet for 3 - 5 min.
- Dissolve RNA in 10 μl of RNase-free water.

[1] Molecular Research Center, Inc., Cincinnati, OH

Appendix 2

DNase Treatment Procedures Using the Turbo DNA-free Kit[2]

1) Aliquot 50 μl of RNA to a new microcentrifuge tube.
2) Add 50 μl of 10X TURBO DNase Buffer and 1 μl TURBO DNase to 50 μl of RNA and mix gently.
3) Incubate at 37^{o} C for 20-30 minutes.
4) Add 5 μl resuspended DNase Inactivation Reagent.
5) Incubate at room temperature for 5 minutes, mixing occasionally.
6) Centrifuge at 10,000 g for 1.5 minutes
7) Avoiding the pellet, transfer RNA to a fresh tube.

[2] Applied Biosystems/Ambion, Inc., Austin, TX

Appendix 3

Reaction Assembly Using the qScript One-Step SYBR Green qRT-PCR Kit for iQ[3]

Component	Volume for 25μl reaction
One-step SYBR Green Master Mix for iQ	12.5 μl
Forward primer	1 μl
Reverse primer	1 μl
Nuclease free water	9 μl
RNA template	2 μl
qScript One-Step RT*	1 μl

*Omit addition of qScript One-Step RT in minus RT control reactions, replace with nuclease free water.

[3] Quanta BioSciences, Gaithersburg, MD