

EFFECTS OF DIETARY CALCIUM TO NON-PHYTATE PHOSPHORUS RATIO AND
PHYTASE SUPPLEMENTATION ON GROWTH PERFORMANCE, DIGESTIBILITY, AND
BONE CHARACTERISTICS OF BROILER CHICKENS

BY

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THESIS

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ABSTRACT

Three experiments were conducted to evaluate the interrelationships among Ca, non-phytate phosphorus (**NPP**), and phytase supplementation in diets fed to broiler chickens. Experiment 1 was conducted to evaluate the influence of dietary Ca concentrations by evaluating growth performance, tibia measurements, and apparent retention of broiler chicks. Dietary treatments consisted of 7 concentrations of Ca (0.4, 0.6, 0.8, 1.0, 1.2, 1.4, or 1.6% of the diet) and NPP concentrations were maintained at 0.3%. Growth performance and bone ash of broilers fed 0.6% Ca were improved compared with those fed higher Ca concentrations. Tibia ash and tibia break force were reduced in birds fed Ca inclusions above 0.6%. Tibia reference force indentation measurements exhibited quadratic responses as the Ca inclusion increased from 0.4 to 1.6%. Dietary treatment effects were observed for apparent retention of P and Ca, which decreased linearly or quadratically with increasing Ca concentrations. Experiment 2 evaluated effects of dietary Ca and NPP combinations to create distinct Ca-to-NPP ratios on growth performance, tibia measurements, and apparent nutrient retention in broiler chicks. Dietary treatments contained 3 concentrations of Ca (0.4, 1.0, or 1.6% of the diet) with NPP concentrations either constant at 0.45% or adjusted to maintain a Ca-to-NPP ratio of 2:1 (6 treatments total). No growth performance outcomes were influenced by either Ca concentration or the Ca-to-NPP ratio. Maintaining a constant 2:1 Ca-to-NPP ratio, tibia break force and ash improved as Ca and NPP concentrations increased. Moreover, apparent retention of Ca decreased with increasing Ca concentration and apparent retention of P reduced as the concentrations of Ca and NPP increased. In Experiment 3, the effects of supplementing exogenous phytase in diets of varying Ca and NPP concentrations on growth performance, tibia and organ P concentrations, and apparent nutrient digestibility and retention were evaluated in broiler chicks. Chicks received 1 of 6 dietary treatments that consisted of [control diet with 1.0% Ca and 0.5% NPP; mineral

matrix 1 with reductions of 0.15% NPP and 0.16% Ca compared with control; and mineral matrix 2 with reductions of 0.21% NPP and 0.23% Ca compared with control] and phytase supplementation (0 or 1,500 FTU/kg). Phytase supplementation increased BW gain, however, feed efficiency was not influenced by mineral matrix, phytase addition, or their interaction. Feed intake was quadratically influenced by the mineral matrix, but there was no effect of phytase or their interaction. The impact of phytase on tibia ash varied among the dietary mineral matrices, and tibia P content was highest in the control dietary treatment. In contrast to tibia P, concentrations of P in muscle, liver, and spleen were not influenced by dietary treatment. Various interactions among mineral matrix and phytase supplementation were observed for apparent ileal nutrient digestibility and apparent retention values. Overall, results of this research demonstrates that Ca and NPP concentrations, as well their ratio, influenced broiler growth performance, apparent nutrient digestibility and retention, and bone responses in broiler chicks.

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CHAPTER 1: INTRODUCTION

Phosphorus (P) is an essential mineral required for poultry because it is an important component in proper growth and feed efficiency, as well as bone and skeletal development. Poultry diets are primarily composed of plant based ingredients, and more than half of the total P in these corn-soybean meal diets is in the form of phytate. Phytate is considered to be an anti-nutrient because it can bind with other minerals and proteins, which make them unavailable, and cause a decrease in digestibility. Failure to meet the P requirements will decrease bird performance, therefore inorganic P is commonly added to poultry diets (Nelson, 1967). Due to the importance of P inclusion in poultry diets, it was common practice to over-supplement this mineral in the diet. However, over-supplementation of inorganic P to the diet resulted in large portions of dietary P not being utilized by the bird. Consequently, the excessive P concentrations in the diet were excreted, which raised environmental concerns (Waldroup, 1999). Poor utilization of phytate by poultry and its consequences on formulation costs, environment, and digestibility of minerals have led to extensive research toward improving phytate digestion.

Addition of feed enzymes has been commonly used in the commercial poultry industry. Microbial phytase is commonly added to poultry diets, which dephosphorylates phytate, and allows for the utilization and absorption of previously bound nutrients. Phytase inclusion increases the availability of P and other nutrients, as well as increases energy and amino acid digestibility (Waldroup, 1999). Numerous studies have been conducted to evaluate the effect of phytase in broiler chickens. However, the effectiveness of phytase in its ability to breakdown the phytate molecule may be affected by the mineral composition of the diet (Wise, 1983). Having correct nutrient matrix values allows for a more accurate formulation of diets and an overall improvement in phytase efficacy. Recently, interest in the benefits of applying higher phytase

doses to eliminate the anti-nutrient effect of phytate and the rephosphorylation of inositol is rapidly growing. Supplementing high doses of phytase to achieve near complete phytate degradation is beneficial in promoting growth and feed efficiency.

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CHAPTER 2: LITERATURE REVIEW

ROLE OF MINERALS IN POULTRY PRODUCTION

Minerals are essential for skeletal development, activation of enzymes and hormones, and for proper maintenance of homeostasis relationships that occur within the body of the bird. Minerals are classified in a number of ways to understand their requirements or nutritional roles. In poultry nutrition, minerals are typically classified as macro or micro-minerals. Two essential macro-minerals, calcium (Ca) and phosphorus (P), are acknowledged for their importance in skeletal formation and proper bird growth. To ensure birds are reaching peak performance, adequate inclusion of Ca and P in poultry diets is vital for production.

Phosphorus and Ca represent the most abundant mineral elements in the body, with 99% of Ca and 80% of P stored in the skeleton as hydroxyapatite, and both have an important role in bone development and mineralization (Veum, 2010). The remaining Ca is located in extracellular fluid and is essential for blood clotting, supporting normal heart functions, and muscle contraction. Beyond providing structural integrity in bone, P is required in the diet because it plays a role in the metabolism of fats and carbohydrates, along with regulating acid-base balance. The inclusion of Ca and P in the diet is crucial for growing poultry, not only because of the individual benefits of each mineral, but also because both minerals largely contribute to proper bone formation.

Due to increased efficiency in production and genetic advances, the growth rate of poultry has dramatically increased throughout the years. The National Chicken Council reported that 40 years ago (1975) it took approximately 56 days for a broiler to reach a live market weight of 3.76 pounds. Today's broiler (2015) can achieve a live market weight of 6.2 pounds in approximately 48 days (National Chicken Council, 2012). Such changes in genetic improvement

to elicit rapid growth creates a need for nutritionists to evaluate the inclusion of both Ca and P when formulating poultry diets for faster growing birds.

Calcium and P are often discussed together due to their interdependent functions for proper skeletal development during early stages of growth (Nelson et al., 1971; Waldroup, 1999). Skeletal abnormalities represent a great concern within the poultry industry because of their negative influence on growth performance and animal welfare. Supplying a diet that is inadequate in Ca and/or P causes skeletal issues, including abnormal bone development. The most common problems associated with Ca and P deficiencies, or imbalances, are rickets and tibial dyschondroplasia (Waldenstedt, 2006). Rickets is commonly observed in rapidly growing birds and is caused by bone mineralization abnormalities, which subsequently leads to flexibility of long bones (Pattison, 2008). Rickets attributed to Ca deficiency results in a thickening of the epiphyseal plate due to an accumulation of proliferating chondrocytes, which causes an increase in the length of perforating epiphyseal vessels. Similarly, rickets associated with a P deficiency commonly manifests as an accumulation of hypertrophic chondrocytes (Pattison, 2008). In tibial dyschondroplasia, there is a failure in the processes that are required for normal bone formation processes, including hypertrophy, mineralization, vascular invasion, and removal of growth plate cartilage (Leeson and Summers, 2009). Tibial dyschondroplasia is characterized by an abnormal cartilage mass in the proximal head of the tibiotarsus and is commonly seen when dietary Ca is low relative to available P, or when dietary P is high relative to Ca (Leeson and Summers, 2009).

Calcium and P are well known for their roles in bone development and inadequate inclusions can lead to numerous problems that can be detrimental to poultry production (Adedokun and Adeola, 2013). Therefore, when formulating poultry diets, it is essential to ensure birds are provided with adequate mineral concentrations to promote optimal growth and

performance (Applegate and Angel, 2004). The digestion, absorption, and metabolism of Ca and P are influenced by many factors including dietary phytic acid or phytate concentrations, Ca-to-P ratio, and sodium and vitamin D₃ concentrations of the diet (Adedokun and Adeola, 2013). These factors can create interactions or antagonisms between minerals, which either enhance or reduce bird performance. Therefore, these factors should be taken into consideration when establishing the Ca and P concentrations in poultry diets.

CALCIUM AND PHOSPHORUS METABOLISM

In poultry nutrition, Ca and P are major minerals in which poultry growth, performance, and production depend greatly upon. Phosphorus is considered the third most expensive component of poultry feeds, after energy and protein (Summers, 1997). While Ca is considered to be an inexpensive ingredient, it is a crucial for poultry performance, as well as its interrelationship with P. Therefore, it is necessary to discuss Ca and P together since the metabolism of these minerals are closely related. It is essential to realize how Ca and P are absorbed, metabolized and utilized by poultry to understand and establish mineral requirements.

Calcium is the most abundant mineral in the body and exists in three different forms hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$; Ca-to-P ratio of 2:1], in the extracellular matrix as ionized Ca and Ca bound to protein, or as Ca bound to anions (Matos, 2008). Calcium is important for the activity of enzymes and for function of muscle contraction. Phosphorus is required for the organic matrix formation of bone, approximately 80% stored in the bird's skeleton, with a 1:2 ratio with Ca in bone hydroxyapatite (Marks et al., 2010). In addition to serving as a structural component, P is an essential component of many metabolic processes. Phosphorus is a key element in phosphoproteins, phospholipids, and nucleic acids (Brody, 1994). Certain phosphates such as adenosine triphosphate, are present in cells and are vital for storage of energy as well as

energy utilization. Nucleic acids are essential for cell growth and phospholipid functions in membrane lipid bilayer stability, which play a role in fatty acid transport as well as amino acid synthesis.

Calcium is absorbed across the intestinal wall by both transcellular and paracellular routes (Auchère et al., 1998; Wasserman, 2004). Transcellular transport occurs largely in the upper section of the small intestine, more specifically referring to the duodenum and upper jejunum. The absorption of Ca by the transcellular route involves vitamin D₃ and occurs via three mechanisms: 1) epithelial Ca channels from the intestinal lumen into the enterocyte (Peng et al., 2008), 2) intracellular calbindins for the trans-cytosolic diffusion (Bronner et al., 1986), and 3) adenosine triphosphate-activated basolateral membrane Ca pump (Carafoli, 1991). Calcium is mainly absorbed via the paracellular route, which is non-saturable, and absorption occurs throughout the entire small intestine (Adedokun and Adeola, 2013).

These two pathways determine where the absorption of Ca and P take place in the small intestine. The transcellular pathway predominately allows the animal to absorb Ca and P in the duodenum and upper jejunum of the small intestine (Walling, 1997). However, Ca and P absorbed via the paracellular pathway showed a significant amount of both minerals being absorbed in the ileum (Adedokun and Adeola, 2013). When examining the small intestine, it is apparent that the length of the distal jejunum and ileum are significantly longer than that of the duodenum and upper jejunum, which allows a greater surface area of absorption for Ca and P through the vitamin D₃-dependent paracellular route (Fujita et al., 2008). It is also important to consider the amount of time that digesta spends in the jejunum and ileum. Differences in transit time through the digestive track are influenced by age of the bird, environmental temperature, and viscosity of the diet (Gordon and Roland, 1997b and Petersen et al., 1999).

Phosphorus is required for the organic matrix formation of bone, while Ca is needed for mineralization of that matrix (Underwood, 1999). The metabolic link between Ca and P is how the minerals are regulated. This process involves three hormones: parathyroid hormone (PTH), vitamin D₃ (25-OH-D₃) and calcitonin (Bronner et al., 1986). This regulation is critical for normal cell function, bone structure, and intracellular signaling.

The parathyroid gland produces PTH from chief cells when Ca concentrations become too low. Parathyroid hormone increases Ca mobilization from the skeleton by activating osteocytes to release Ca trapped in the bone matrix while also stimulating osteoclasts to reabsorb the bone tissue. Absorption of Ca from the digestive tract is promoted by PTH through regulating the production of 25-OH-D₃, the active form of vitamin D₃. Finally, PTH retains Ca through reabsorption by stimulating the kidneys to excrete P (Frandsen and Spurgeon, 1992). Overall, PTH allows for an increase in Ca concentration from bone absorption and renal reabsorption. Calcitonin is a peptide hormone secreted by C cells in the thyroid gland and has the opposite effect of PTH. Calcitonin inhibits the reabsorption of bone, as well as decreases the release of Ca and P from the bone into the blood (Frandsen and Spurgeon, 1992). Calcitonin in the kidney inhibits renal Ca reabsorption and increases Ca excretion.

Calcium and P are the most abundant mineral found in the body and have a major role in structural support. Calcium is essential for several metabolic processes, and it is especially important because of its interrelationship with P absorption and utilization. Poultry diets need to start being formulated based on digestible Ca and P since physiological factors, PTH and vitamin D₃ all play large roles in Ca and P metabolism (Adedokun and Adeola, 2013). Therefore, many different factors need to be considered when establishing the requirements of these minerals in poultry diets.

PHOSPHORUS AND CALCIUM REQUIREMENTS

Providing inadequate amounts of Ca and P in poultry diets can be detrimental to poultry production due to complications that can arise. Poultry diets are primarily composed of plant-based ingredients and plants mainly store P in the form of phytate P, which is not efficiently utilized by the bird. Therefore, it is essential to understand the terms used when describing the inclusion of P in a poultry diet. Total phosphorus is generally referred to as P and encompasses any and all forms of P, available phosphorus refers to P that is absorbed from the diet into the animal, and non-phytate phosphorus (**NPP**) can be chemically determined by the difference between total P and phytate P (Applegate and Angel, 2004). The main difference between available P and NPP is that available P includes the absorption of both inorganic and organic P, including phytate P, whereas NPP excludes any available phytate P. It is important to accurately determine nutrient requirements since it is well known that feeding inadequate inclusion of Ca and P can result in bone deformities as well as have an overall decrease in poultry performance.

Calcium and P play a key role in bone development and higher requirements of these minerals are desirable during periods of rapid growth in the bird. Mineral requirements in poultry diets, as a percentage, are higher during the first few weeks of life, compared to when the bird is older. Older birds are more developed and have larger intestinal tracts which retain feed longer and contain endogenous phosphatases. The nutritional requirements published in the National Research Council (NRC, 1994) for broilers during the starter phase, 0-3 weeks of age, is 0.45% NPP, 0.35% NPP for birds 3 to 6 weeks of age, and 0.30% NPP for birds 6 to 8 weeks of age. As the age of the bird increased, the requirement for NPP decreases. As the bird progressively ages, less bone development occurs, which lessens the need for NPP. Calcium requirements follow a similar decrease in inclusion with respect to bird age. The NRC (1994) recommends 1.00% Ca

for birds 0 to 3 weeks of age, 0.90% Ca for birds 3 to 6 weeks of age, and 0.80% Ca during weeks 6 and 7 of age.

Waldroup et al. (2000) conducted an experiment in 0-to-3-week-old broiler chicks to determine the demands for dietary P. Chicks were provided a high available P corn (0.27% total P and 0.17% NPP) in comparison with yellow dent corn (0.23% total P and 0.03% NPP). Regression analysis was used to determine the NPP requirement for maximum tibia ash, which was between 0.37% and 0.39% depending on the type of corn that included high or low concentrations of NPP. Regardless, these dietary inclusion are considerably lower than those published by the NRC (1994). Similar results were observed by Angel et al. (2000b), which included raising birds on floor pens to evaluate NPP concentrations during grower and finisher phases. Graded NPP concentrations for the grower and finisher diets were ranged from 0.28-0.45% and 0.19-0.34%, respectively. Adequate concentrations of NPP in the grower phase were between 0.32 and 0.28% NPP and adequate concentrations in the finisher phase were between 0.24 and 0.19% NPP.

Yan et al. (2001) conducted an experiment to investigate the NPP requirement for male broilers during the grower phase, 3 to 6 weeks. Broilers were fed diets that ranged in NPP concentrations from 0.10 to 0.45% NPP, in 0.05% increments, during the grower phase. Nonlinear regressions estimated optimal NPP concentrations of 0.33, 0.186 and 0.163% to maximize tibia ash content, body weight gain, and feed conversion ratio, respectively. The optimal tibia ash response achieved at the NPP concentration of 0.33% was similar to the requirement stated in the NRC (1994). The NPP concentrations for optimal bird performance were 0.186 and 0.163%, which is considerably lower than the NRC (1994) requirement of 0.35% NPP.

Additional research has been conducted to understand whether carry-over effects exist between feeding phases. Broilers were grown to 42 d on diets that contained low, medium, or high concentrations of Ca and NPP (Skinner et al., 1992b). At d 42, broilers continued to be fed low, medium or high concentrations of Ca and NPP, or they were fed diets with no Ca or NPP supplementation. Broilers fed diets in which Ca and NPP supplements were removed from 42 to 49 days did not differ significantly from those broilers fed diets where the concentrations of Ca and NPP were maintained. Skinner (1992b) reported that maintaining a 2:1 Ca-to-NPP ratio during the 42-to-49 day period enabled the removal of supplemental dicalcium phosphate with minimal effect on tibia strength. This finding has direct implications as removal of dietary components with little to no detriments on growth performance or bone parameters would certainly benefit the economics and environmental effects of poultry production.

Overall, previous research suggests that the NRC (1994) recommendation for NPP during the broiler grower and finisher phases may be overestimated. This idea is further supported by the genetic changes that the broiler market has seen throughout the year and especially since the last revision of NRC (1994). Modern broilers are more efficient in absorbing and retaining dietary nutrients, as well as have a faster growth rate when compared with the older broilers strains. Providing current and relative information about dietary requirements of broilers would allow for more accurate diet formulations and reduce diet costs.

PHYTATE PHOSPHORUS

The amount of P that is available for poultry utilization from cereal grains is not equal to the total amount of dietary P. Roughly 20 to 30% of P found in most cereal grains is available in a usable form for poultry (Sebastian et al., 1996). Poultry diets are primarily composed of plant based ingredients, with approximately two-thirds of the total P present as part of phytate, which

is poorly utilized by poultry (Viveros et al., 2002). Phytic acid (myoinositol hexaphosphate) is primarily found in plant seeds and is the main storage form of P, phytate accounts for approximately 50 to 80% of P in seed feedstuffs (Ravindran et al., 1995). Phytic acid is composed of six negatively charged phosphate groups bound to 12 hydrogens, which form an inositol ring. Phytate is a mixed cation salt of phytic acid, composed of myoinositol 1,2,3,4,5,6-hexakis dihydrogen phosphate, commonly referred to as IP6 (Angel et al., 2002). Phytate is considered an anti-nutrient due to its ability to bind mineral cations in the diet, which result in nutrients being partially or completely unavailable for digestion in poultry (Applegate and Angel, 2004).

Results of extensive studies have demonstrated the influence of phytate on the utilization of nutrients (Nelson et al., 1971; Ravindran et al., 1995; Veum, 2010). Findings from these studies indicate that as an anti-nutrient factor, phytate lowers the bioavailability of P, Ca, and other minerals, as well as functional capacity of digestive enzymes. Most cereal grains have a low Ca bioavailability and the binding of phytate has been shown to further decrease Ca availability. Therefore, Ca supplementation is required, however, since Ca supplements are relatively inexpensive, over-supplementation often occurs. Trace minerals such as zinc, iron and magnesium can be influenced by the presence of phytate by having a negative effect on absorption, retention, and metabolism (Couzy et al., 1993). Phytate can decrease the activity of digestive enzymes and the availability of dietary amino acids, as well as apparent metabolizable energy due to the formation of protein-phytate-mineral complexes (Cosgrove, 1980). Phytate may adversely affect starch digestion, which could inhibit amylase activity and reduce the glucose absorption rate (Selle and Ravindran, 2007). The negative effect of phytate is believed to be due to phytate's ability to directly bind with nutrients, reduce solubility, change the

conformation of the enzymes, and interfere with the exposure of active sites for digestion and absorption (Sebastian et al., 1997).

Previously, it was common practice for poultry nutritionists to create a safety margin when formulating poultry diets (Waldroup, 1999). Over supplementing P was commonly utilized until the cost of inorganic phosphate sources increased and the over supplementation of P led to an excessive amount of P being excreted in poultry litter. It has been estimated that up to 82% of the phytate consumed in poultry diets is recovered in the excreta (Cowieson et al., 2004). The reduced bioavailability of Ca, P, and other minerals by phytate leads to increased excretion of these nutrients. This raised environmental concerns and encouraged producers to explore alternative approaches that could reduce the concentration inclusion of total P in broiler diets (Ravindran et al., 1995). Even though poultry diets contain adequate amounts of organic P, it is in the molecule form of phytin, which results in a small amount of P that is accessible to poultry without making dietary modifications (Angel et al., 2002). There are several strategies for improving nutrient utilization and reducing nutrient excretion. Formulating diets based on accurate requirements and maintaining proper ratios between nutrients are ways to improve nutrient utilization and decrease waste. However, the most practical approach for improved nutrient utilization is the addition of enzymes in poultry diets.

PHYTASE

Poultry have insufficient endogenous phytase activity, therefore they do not have the ability to effectively digest phytate (Nelson et al., 1971). McCollum and Hart (1908) were one of the first authors to publish findings on phytase activity in animal tissues and this has been confirmed in virtually every decade since (Nelson, 1967). Therefore, the problem with phytate digestion is not a lack of compatible enzymes but instead, poor substrate solubility in the small

intestine. To alleviate this problem, exogenous enzymes are commonly used as feed additives to release phytate bound P. Enzymes are proteins, or protein-based substances, that catalyze chemical reactions (Applegate and Angel, 2004). Phytases (myoinositol hexaphosphate hydrolases) are the only enzymes that have the ability to initiate dephosphorylation of phytate P because they are capable of catalyzing the hydrolysis of one or more phosphate groups from IP₆, thus yielding inorganic P (Applegate and Angel, 2004).

Phytase consists of two main categories, fungal phytases, which come from *Aspergillus* or *Peniophora* species, and bacterial phytases, which come from *Escherichia coli*. The International Union of Biochemistry (1979) recognizes these categories as three-phytase and six-phytase. This classification is based on the location of the phosphate group, within the phytin molecule, that is hydrolyzed first. Three-phytase, such as *Aspergillus niger*, initiate dephosphorylation of the inositol ring at the three position, whereas six-phytases, mainly present in plants, such as fungal phytase *Peniophora lycii* or microbial phytase *Escherichia coli*, initiate dephosphorylation at the six position of the inositol ring. Microbial phytase, along with all other acid phosphatases, hydrolyzes phosphoesters in a two-step mechanism, with a phosphorylated active amino acid site as an intermediate (Shute et al., 1988). The amino acid region at the active site of phytase creates an electrostatic environment for binding the negatively-charged phytate substrate. During hydrolysis, a phosphate group is transferred from phytate to the active phytase site, and then from phytase to water. Once the initial phosphate group has been dephosphorylated, the other five phosphate groups are dephosphorylated in sequential order.

Phytases are a proteinaceous enzymes, which makes them highly susceptible to denaturation or destruction by digestive enzymes and harsh digestive environments. A change in enzyme structure can alter its performance, therefore, enzymes have ideal conditions,

temperature and pH, where they function at optimal performance. Plant phytases work better at 45 to 60°C, whereas microbial phytases work more readily at wider temperature ranges, 35 to 65°C (Wodzinski and Ullah, 1996). Enzyme activity is characterized as one phytase unit (FTU) which is defined as the quantity of enzyme required to liberate one μ mole of inorganic P/min, at pH 5.5, from an excess of 15 μ M, pH at 37°C (International Union of Biochemistry, 1979). The pH optima and range is highly important when determining phytase activity. In general, the pH throughout the digestive tract varies. In broilers, the pH of the crop is between 4 and 5, whereas the pH of the gizzard and proventriculus is much lower, around 2 to 5, and the small intestine, which includes the duodenal loop with a higher pH ranging from 5.5 to 8 (Simon and Igbasan, 2002). A three-phytase produced from the fungus *Aspergillus niger* has been reported to have an optima pH at 5.5 to 6.5 (Pasamontes et al., 1997). Six-phytases, such as that produced from *Peniophora lycii*, can have a pH optima at 4.0 to 4.5 (Lassen et al., 2001). Thus, microbial phytases may retain higher activity in the proventriculus and gizzard. Current phytases function best at a specific pH, which result in little phytase activity once it has passed through the proventriculus due to unfavorable pH values.

Augspurger and others (2003) reported *E. coli* phytases may have an advantage in young chicks when compared with fungal phytases. An experiment was conducted to investigate the P-releasing efficiency in two fungal-derived phytase enzymes compared with an *E. coli*-derived phytase. A significant improvement was reported in body weight gain, gain-to-feed, and tibia ash in those birds fed diets containing *E. coli* phytase. Previous research supports these findings, suggesting that bacterial phytases perform better than fungal phytases (Igbasan and Simon, 2000). This may be largely due to the fact that fungal phytase enzymes are susceptible to

inactivation in the proventriculus and gizzard, as well as sensitivity to heat treatments, while bacterial enzymes do not possess these characteristics (Igbasan and Simon, 2000).

The efficacy of phytase is influenced by many factors such as pH conditions and the physiological status of the bird, therefore the ideal phytase is defined as being able to remain active after heat treatment in feed processing, remain stable in the gastrointestinal tract, as well as have a low cost inclusion in the diet (Greiner and Konietzny, 2010). While the primary function of phytase is P release, additional benefits have been noted. Numerous experiments have been conducted to evaluate the efficacy of microbial phytase addition to poultry diets (Simons et al., 1990, Schoner et al., 1991; Bronz et al., 1994; Ravindran et al., 1995; Augspurger et al., 2003).

Growth Performance

Several experiments have been performed in broilers using phytase-supplemented feed. The inclusion of phytase in diets fed to poultry has focused on determining the ability of phytase to replace inorganic P supplementation, through release of phytate-bound P and making it available for absorption. The addition of phytase in poultry diets that are deficient in available P has been shown to improve body weight gain, feed intake, and feed efficiency (Simons et al., 1990; Denbow et al., 1995; Sebastian et al., 1996; Augspurger et al., 2003; Shirley et al., 2003; Dilger et al., 2004). High Ca inclusions in the diet interact with phytate and form insoluble complexes, which are resistant to phytase hydrolysis (Nelson et al., 1971; Wise, 1983; Waldroup et al., 2000; Weglarz and Angel, 2013). While research has reported the benefits of supplementing microbial phytase in poultry diets, the concentrations of Ca and P in poultry diets, as well as their ratio, can influence the efficacy of phytase.

Modal et al. (2007) evaluated the efficiency of microbial phytase on broilers fed diets that contained; 0.65% P (control), 0.52% P (low P), and low P plus phytase to determine growth performance. An increase in body weight gain was reported in birds fed the low P plus phytase diet, and the addition of supplemental phytase in the low P diet had comparable results to those birds fed the control P diet. The positive growth performance due to the addition of phytase may be credited to the release of minerals from the phytate complex and/or an increase in digestibility (Sebastian et al., 1996). These findings indicate that the addition of phytase improve the utilization of phytate-bound P for poultry.

The efficacy of phytase can also depend upon the inclusion of other minerals in the diet, such as Ca, and it has been suggested that Ca concentrations need to be decreased when phytase is added to the diet due to the ability for Ca to form insoluble complexes in the gut (Schoner et al., 1991; Sebastian et al., 1996; Qian et al., 1997). Sebastian et al. (1996) reported phytase supplementation to a low P diet increased Ca retention in broiler chickens. Furthermore, Schoner et al. (1993) reported that with the addition of phytase, a diet that contained 0.6% Ca resulted in a higher body weight gain than a diet with 0.9% Ca, which shows the negative effects that can occur due to improper Ca inclusions. These studies suggest that higher Ca inclusions in the diet may reduce phytate P hydrolysis due to the formation of insoluble Ca-phytate complexes. Increased inclusion of Ca in poultry diets have been shown to decrease phytase efficacy, therefore reducing the concentration of dietary Ca can improve phytase efficacy, however drastic Ca reductions can be damaging to the skeletal integrity (Schoner et al., 1991; Yan et al., 2003).

Yan and Waldroup (2006) conducted an experiment with diets that contained varying concentrations of Ca and NPP, with and without the addition of supplemental phytase. A basal diet was formulated that provided nutrients in excess of minimum NRC (1994) requirements

with 1.0% Ca and 0.50% NPP. Varying the inclusion of these minerals, along with washed sand as an inert ingredient, allowed for the formulation of the experimental diets. Increasing the Ca inclusion with low concentrations of NPP and without phytase supplementation resulted in a decreased body weight gain, however birds fed this diet with the addition of phytase exhibited an increase in body weight gain. Phytase supplementation to diets with NPP concentrations closer to the NRC (1994) requirement had a minimum effect on body weight, which suggests the primary function of phytase is P release, since P was not a limiting factor for growth in the higher NPP diets. This work is consistent with previous findings from Sebastian et al. (1996) who reported high dietary Ca concentrations had a significant negative effect on phytase activity which could be due to the pH in the small intestine of broilers, which makes the Ca and phytate complex unavailable for phytase to degrade. Qian et al. (1997) reported widening the Ca:P ratio from 1.4 to 2.0 resulted in a decrease in phytase efficacy, which led to negative broiler growth performance. In contrast, research conducted by Driver et al. (2005) reported higher Ca concentrations (0.86%) and lower NPP concentrations (0.20%) elicit a greater phytase response. Efficient use of supplemental phytase for improving utilization of phytate P and Ca in corn-soybean meal broiler diets has been reported to be a Ca:P ratio range of 1.1 to 1.4 (Qian et al., 1997). Imbalances between Ca and P can lead to a negative effect on poultry performance and Ca reductions should not be implemented to the diets without the addition of phytase.

Tibia Bone Ash

Abnormal bone development is one of the first and most obvious signs of a P deficient animal (Qian et al., 1996). The tibia has been used as a reliable marker of bone mineralization for many years. The addition of microbial phytase to broiler diets has been reported to increase the tibia bone ash content, due to the improvement in phytate P hydrolysis. An increase in tibia ash

content suggests an improvement in bone mineralization which resulted from an increase in Ca and P utilization (Perney et al., 1993). Since tibia ash is a sensitive measurement, it has been the most used common method to evaluate Ca and P requirements based on the degree of mineralization.

Addition of phytase to broiler diets leads to an improvement in tibia ash content (Nelson et al., 1971; Perney et al., 1993; Sebastian et al., 1996). An experiment was conducted by Sebastian et al. (1996) which used broiler chicks from day old to 21 d of age. Diets consisted of low P corn-soybean meal diet, varying Ca inclusions (low, recommended, and high) and the addition of microbial phytase. The NPP was below the NRC (1994) requirement at 0.3%, dietary inclusions of Ca were; 0.66% (low), 1.0% (recommended), 1.25% (high), and phytase was supplemented at 0 or 600 FTU/kg diet. The calculated Ca:P ratios of the diets containing 0.66, 1.0 and 1.25% Ca were 1.25, 2.08, and 2.60, respectively. The percentage of fat-free tibia ash significantly improved with the addition of phytase, regardless of the Ca concentration, which agrees with previous literature (Nelson et al., 1971; Perney et al., 1993; Bronz et al., 1994). This resulted in an improvement in tibia ash percent. The maximum ash content was observed at 1.0% dietary Ca concentration with phytase supplementation. Furthermore, phytase supplementation at the 0.66% Ca diet resulted in ash content of tibia shaft equivalent to that obtained with the 1.0 and 1.25% Ca diet plus phytase. Optimal growth performance, retention of P and Ca, and tibia ash content were all achieved at the dietary Ca inclusion of 0.66% (low) with the addition of microbial phytase. The improvement in P retention with phytase supplementation at the low Ca concentration suggests that there was less Ca-phytate complex formed; thus, more phytate molecules were exposed to phytase hydrolysis.

Powell et al. (2011) conducted a similar experiment to determine the effect of dietary Ca inclusion on the efficacy of phytase. Diets were corn-soybean meal based with a positive control containing 0.45% NPP and 1.00% Ca and negative control diets with 0.20% NPP with 0.67, 1.00 or 1.33% Ca, fed with or without 500 phytase units of a microbial *E. coli* phytase. Phytase supplementation, regardless of the mineral matrix, resulted in an increase in overall bird growth and performance. Increasing the Ca inclusion from 0.67 to 1.33% linearly decreased the percentage of tibia ash content. However, the decrease in tibia ash was quadratic because there was no difference in tibia ash of broiler fed diets containing 1.00 or 1.33% Ca. The experiments evaluating bone ash content involved broilers housed in battery cages, however it is important to mention there may be bone growth differences between broilers reared in cages versus floor pens (Bond et al., 1991).

Nutrient Utilization

Addition of microbial phytase has been shown to improve digestibility and retention of P and Ca (Schoner et al., 1991; Qian et al., 1996; Sebastian et al., 1996; Ravindran et al., 2000; Viveros et al., 2002; Shirley and Edwards, 2003; Dilger et al., 2004; Onyango et al., 2004; Silversides et al., 2004; Wu et al., 2004b). Silversides et al. (2004) reported broilers fed a P adequate diet had a higher Ca digestibility when compared with a deficient diet, however a P deficient diet with the addition of phytase at 1,250 U/kg resulted in Ca digestibility that was similar to the P adequate diet. Phosphorus and Ca availability can be further improved by the adding different concentrations of microbial phytase to broiler diets (Simmons et al., 1990). Denbow et al. (1995) concluded that increasing phytase inclusion from 250 to 1,000 units of phytase/kg diet resulted in a 31 to 58% release of P from phytate. Simmons et al. (1990) conducted an experiment to determine the availability of P with different concentrations of

microbial phytase. From 0 to 24 d of age birds were fed diets that contained 750, 1000 or 1,500 units microbial phytase/kg, the birds that consumed diets of 1,500 unit microbial phytase/kg had a significant improvement in performance. Wu et al. (2004b) concluded when phytase was added to a low P diet, the availability of phytate bound P increased over 60%, therefore, the addition of phytase to the low P diet, compared with the adequate P diet, resulted in improvements in the apparent retention of P which is consistent with the improvements in ileal phytate P hydrolysis and ileal P digestibility. This suggests that inclusion of phytase allow broilers to efficiently utilize dietary P, which decreases the amount of P being excreted.

The influence of supplemental phytase on the utilization of protein and amino acids has been conducted in several experiments (Ravindran et al., 1995; Sebastian et al., 1996; Ravindran et al., 2000; Selle and Ravindran, 2007). Phytate not only binds to mineral cations, but may also bind to nutrients, such as protein, and has also been known to inhibit digestive enzymes. The addition of phytase releases proteins that were previously bound to phytate, as well as reduces the inhibitory effects on digestive enzymes, pepsin and trypsin, which leads to an improvement in the availability of protein and amino acids for absorption (Sebastian et al., 1997). Energy utilization has also been improved with phytase supplementation, which could partially be a reflection of increased protein digestibility. However, phytase may act independently of energy effects. Energy utilization may be improved with the addition of phytase by preventing the formation of mineral-phytate complexes, which prevents the formation of insoluble metallic soaps in the gastrointestinal tract that constraint lipid utilization, and therefore enhance the utilization of energy derived from lipids (Ravindran et al., 2001).

However, there is conflicting data in the literature. Augspurger and Baker (2004) fed high concentrations of *E. coli* phytase and reported no improvements in protein utilization. Zhang et

al. (1997) observed no significant differences in amino acid and protein digestibility in broilers fed microbial phytase. These reported variations in digestibility could be influenced by factors such as source and concentration of phytate and protein in the diet, Ca and P concentrations in the diet, digestibility of the protein component, and the phytase inclusion rate (Selle et al., 2000). It has become common practice to add phytase to poultry diets to improve the digestibility of nutrients and ensure least-cost diet formulations. Thus, to generate additional cost savings in poultry production, the benefits of applying higher doses of phytase have been evaluated.

High Doses of Phytase

Interest in the benefits of supplementing higher phytase doses to further eliminate the anti-nutrient effect of phytate is rapidly growing. For poultry, supplementing high doses consists of the application, typically three to four times the standard dose, of an intrinsically thermostable, highly efficient phytase that is developed specifically to target near-complete phytate destruction (Bedford, 2014). Supplementing phytase at high doses allows for the breakdown of phytate IP₆, as well as the breakdown of lower anti-nutritive phytate esters. These lower esters, IP₅ through IP₁, have been linked with poor digestion of protein, energy, and minerals, therefore by not only releasing P, but also eliminating all inhibitors of digestion, enables poultry to grow more efficiently (Cowieson and Bedford, 2004).

Lower esters are either absorbed, or precipitate with cations and excreted from the bird (Cowieson and Bedford, 2004). Absorbed esters will be further dephosphorylated in mucosa, blood, and liver, while lower esters, free phosphate and inositol, are available for secondary metabolic processes such as the assembly of secondary messengers via phosphorylation (Cowieson and Bedford, 2004). Inositol is present in plant and animals cells, is an essential component for normal growth, and has a major role in lipid metabolism, as well as functions in

cell signaling and cell growth (Papaleo et al., 2009) Cowieson et al. (2013) reported dietary supplementation of 0.15% inositol was effective in improvement performance broiler chickens. Inositol is mainly in the form of IP6, therefore supplementing high doses of phytase for near complete phytate degradation, dephosphorylation of all six P ions, allows for the release of inositol (Cowieson and Bedford, 2004). High doses of phytase may elicit beneficial effects such as more complete and rapid destruction of phytate, the generation of inositol, and a more proportionate P/Ca release, all which can lead to an improvement in overall bird performance.

The effectiveness of applying high doses of supplemental phytase have been reported and lead to an overall improvement by increasing bird performance, bone ash, nutrient utilization and digestibility when phytase was included between 1,000 FTU/kg and as high as 12,000 FTU/kg in low P poultry diets (Shirley and Edwards, 2003; Augspurger et al., 2004; Cowieson et al., 2004; Selle and Ravindran, 2007). Previous research has reported that P utilization may plateau around 500 FTU when diets deficient in P are supplemented up to 2,000 FTU of phytase (Simons et al., 1990). Shirley and Edwards (2003) conducted an experiment to evaluate maximum performance in broilers fed diets that were deficient in P and reported that from 0 to 16 d of age broilers achieved maximum performance when phytase was supplemented at 12,000 FTU/kg diet. Bird response to supplemental phytase depends on Ca and P concentrations in the diet, therefore differences in diet composition or environmental and housing conditions may be the reason for these contradicting results of phytase efficiency (Driver, 2005).

Recent studies have investigated the beneficial effects associated with the inclusion of high doses of supplemental phytase in P adequate diets (Cowieson, 2011; and Olukosi and Fru-Nji 2014). An experiment conducted by Olukosi and Fru-Nji (2014) examined nutrient matrix values and their effect on phytase efficiency. Birds were fed experimental diets that had a narrow

or wide Ca:P ratio (2.0:1 and 2.5:1) and a full nutrient specification, meeting all nutrient requirements, or a reduced nutrient specification, formulated to be deficient in Ca and P. It was reported that a lower phytase supplementation (1,000 FTU/kg) had a better response with the narrow Ca:P ratio combined with reduced nutrient specifications, whereas higher phytase supplementation (2,000 FTU/kg) with the wide Ca:P ratio and full nutrient specifications had a better response. The ability of phytase to hydrolyze phytate has been shown to be negatively affected by high inclusions of Ca or a high Ca:P ratio and the importance of maintaining a narrow ratio of Ca to P is well recognized (Qian et al., 1997). This has been linked to the formation of extremely insoluble Ca-phytate complexes under intestinal conditions, which make the phytate molecule inaccessible to phytase and may explain the apparent inactivity of phytase supplementation in high Ca diets (Wise, 1983). Therefore, the higher concentration of phytase is needed to reverse the negative effect of phytate in diets that contain unbalanced Ca:P ratios. Previous research suggests that when the requirement for P is met, greater P availability will not necessarily result in greater performance due to a possible limit of phosphate uptake from the lumen of the bird (Yan et al., 2003). Therefore, a higher dose of phytase in broiler diets that already meets the P requirement, suggests an extra-phosphoric effect of phytase.

Addition of phytase allows for the utilization and absorption of previously-bound nutrients, but there are many factors that play a role in optimal phytase efficacy. Phytase inclusion, Ca and P concentration, and Ca:P ratio can all affect phytase efficacy and can influence the response of phytase (Lei et al., 1994; Kornegay and Yi, 1996b). Supplementing microbial phytase to poultry diets can remove the anti-nutrient factors, which allows for a decrease in overall P inclusion. Therefore, phytase improves the bioavailability of nutrients,

positively influences poultry digestion and excretion, as well as beneficial cost and environmental enhancements.

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CHAPTER 3: INFLUENCE OF DIETARY CALCIUM REQUIREMENTS AND CALCIUM AND NON-PHYTATE PHOSPHORUS RATIOS IN BROILERS

ABSTRACT

Two experiments were conducted to determine the influence of dietary Ca concentrations (Experiment 1) and a combination of dietary Ca and non-phytate phosphorus (**NPP**) to create distinct Ca-to-NPP ratios (Experiment 2) in corn-soybean meal diets fed to broiler chickens from 2 to 23 d of age. In Experiment 1, dietary treatments consisted of 7 concentrations of Ca (0.4, 0.6, 0.8, 1.0, 1.2, 1.4, or 1.6% of the diet; 7 treatments total) and NPP concentrations were maintained at 0.3%. Birds that were fed diets supplemented with 0.6% Ca (i.e., 2:1 Ca-to-NPP ratio) exhibited increased ($P < 0.01$) BW gain, feed intake and bone ash, whereas Ca concentrations greater than 0.6% elicited reductions ($P < 0.01$) in these measurements. Dietary treatment effects ($P < 0.01$) were also observed for apparent retention of P and Ca, which decreased ($P < 0.05$) linearly or quadratically for birds receiving dietary treatments with Ca concentrations greater than 0.6%. In Experiment 2, diets were formulated to contain 3 concentrations of Ca (0.4, 1.0, or 1.6% of the diet) with NPP concentrations either constant at 0.45% or adjusted to maintain a dietary Ca-to-NPP ratio of 2:1 (6 treatments total). Growth performance was not influenced by Ca concentration or the Ca-to-NPP ratio. Tibia break force was lower ($P < 0.01$) in birds fed diets containing 0.4% Ca, regardless of the NPP concentration. Tibia ash increased ($P < 0.01$) as the dietary Ca concentration increased. Nitrogen retention was only increased ($P < 0.05$) at the highest Ca concentration. Upon maintaining a constant 2:1 Ca-to-NPP ratio, P and Ca retention decreased ($P < 0.01$) at the highest Ca concentration. In conclusion, imbalanced Ca and NPP adversely influenced growth performance and nutrient retention of broilers, indicating the concentrations of Ca and NPP required to maximize bone structure and function may be higher than those required for performance.

INTRODUCTION

Dietary Ca and P serve a variety of integral functions in metabolism and skeletal integrity, which highlights the important economic implications of these minerals in production animal agriculture. Supplying a diet that is inadequate in Ca and P can lead to skeletal abnormalities, which negatively affect growth performance (Applegate et al., 2003). Excess dietary Ca can reduce the energy value of the diet and interfere with the availability of other minerals, including P, due to the ability of Ca to chelate molecules and make them unavailable for absorption (Qian et al., 1997; Sebastian et al., 1997). Due to the importance of P inclusion in poultry diets, it was previously a common practice to over-supplement this mineral in the diet (Driver et al., 2005). However, the inclusion of P in poultry diets contributes to increased feed cost, and over-supplementation resulted in large portions of dietary P not being utilized by the bird, which can have a negative environmental impact due to excess P excretion. Since dietary needs for Ca and P are interdependent, it is critical to formulate poultry diets to correct Ca to non-phytate phosphorus (**NPP**) ratios (Qian et al., 1997; Applegate et al., 2003; Yan et al., 2005).

Bird performance and bone development can have a multitude of responses depending on the concentration of Ca and NPP, even when a constant Ca-to-NPP ratio is maintained (Edwards and Veltmann, 1983). At low concentrations of NPP, dietary Ca concentrations (i.e., 0.8 to 1.0%) can impact bird performance and bone ash percentage in broilers (Davis, 1959; Waldroup et al., 1963a; Kondos and McClymont, 1967; Driver et al., 2005). Conversely, when dietary Ca concentrations are reduced, bird growth and P absorption tend to increase and bone abnormalities subside, reiterating the concept that a balanced ratio between Ca and NPP is essential when formulating poultry diets.

It appears that increased concentrations of Ca in broiler diets increase bone ash content, but such concentrations may also reduce bird growth performance and interfere with mineral

absorption (Simpson and Wise, 1990; Sebastian et al., 1996; Selle et al., 2009). A high ratio of Ca-to-NPP in the diet antagonizes digestibility and absorption of P due to increased precipitation of insoluble mineral complexes (Yan et al., 2005). Dietary Ca and NPP concentrations, as well as their ratio, can largely affect the absorption and utilization of nutrients. Minerals supplemented beyond the physiological threshold needed for maximum mineral retention are eliminated through the renal system, which leads to environmental and economic concerns (Manangi and Coon, 2007). Therefore, re-examining Ca and NPP concentrations can lead to a more accurate formulation of broiler diets. The objective of these experiments was to determine the influence of dietary Ca concentrations and the Ca-to-NPP ratio over a wide range of Ca and NPP concentrations in corn-soybean meal diets fed to birds from 2 to 23 d of age. Growth performance, mineral retention, and bone characteristics such as reference force indentation, break force, and bone ash were all determined in the same bone, and these measurements were examined to determine the differences between experimental diets.

MATERIALS AND METHODS

All animal care procedures used in this experiment were approved by the University of Illinois Urbana-Champaign Institutional Animal Care and Use Committee.

General Bird Husbandry

In two separate experiments, broiler chicks were obtained from a commercial hatchery (Hoover's Hatchery, Rudd, IA). The birds were housed at the University of Illinois Poultry Farm from 2 to 23 d of age in thermostatically-controlled brooder battery cages with raised wire floors in an environmentally-controlled room with continuous lighting. Birds were provided free access to water throughout each trial, and a corn-soybean meal based diet that contained 23% CP and titanium dioxide was included in all diets at 0.4% as an indigestible marker. On d 2 post-hatch,

birds were individually weighed, wing-banded, and allotted such that all treatment groups were similar in average initial body weight. Individual bird and feeder weights were recorded throughout each experiment for calculation of BW gain, feed intake (**FI**), and feed efficiency (BW gain/FI, **G:F**) for measurements of growth performance. All culled birds were weighed individually, and feed intake and feed conversion were adjusted according to number of bird days. All experimental diets were provided in mash form and chicks received these diets in a single feeding phase from 2 to 23 d of age.

Experiment 1

Three hundred and fifty male Ross 308 commercial broiler chicks were assigned to 7 dietary treatments from 2 to 23 d post-hatch, with 5 chicks per cage and 10 replicate cages per treatment. Dietary treatments consisted of a corn-soybean meal based diet that was formulated to contain 0.3% NPP and graded concentrations of Ca, ranging from 0.4 to 1.6% total Ca (Table 3.1). All diets were formulated to contain 3,093 kcal/kg nitrogen-corrected apparent metabolizable energy (**AME_n**) and only differed in dietary Ca, apart from Ca all nutrients met or exceeded recommendations for this age bird (NRC, 1994).

Experiment 2

One hundred and eighty male Ross 308 commercial broiler chicks were assigned to 6 dietary treatments from 2 to 23 d post-hatch, with 5 chicks per cage and 6 replicate cages per treatment. Dietary treatments consisted of a corn-soybean meal based diet that was formulated to contain 3 concentrations of Ca, with NPP concentrations either constant at 0.45% or adjusted to create a dietary Ca-to-NPP ratio of 2:1 (Table 3.2). Diets were formulated to contain 3,031 kcal/kg **AME_n** and all other nutrients met or exceeded the recommended NRC (1994) requirements.

Collection and Analyses

At the end of the 21-d feeding period, birds were euthanized by CO₂ inhalation and the right tibia was collected from all birds by cutting above the tibia, samples were stored at -20°C until processing. Processing of tibias involved removal of the skin and periosteum with a scalpel and scissors. Cheese cloth was used to clean any additional residue before submerging the bone in distilled water in a 4-ml sealable bag (Whirl-Pak; Nasco, Fort Atkinson, WI, USA). Extracted tibias (one bird/cage) were then randomly chosen and measured for total length as well as height and width at half length. That randomly chosen tibia was then used for each of the measurements described below.

Tibias were subsequently evaluated using reference point indentation methodology (BioDent; ActiveLife Scientific, Santa Barbara, CA), which consisted of 5 independent measurements per bone with 20 indentation cycles per measurement. Bone measurements were taken using a BP2 probe, which has a spherical tip and a blunted point. Holding the bone with condyles down, thus allowing the probe to contact the anterior surface of the bone, width was defined as the horizontal distance, lateral to medial, and height was defined as the vertical distance, anterior to posterior. The bones' resistance to fracture was measured by the indentation distance increase (**IDI**) between the first and last indentation cycles and total indentation distance (**TID**) quantified the total depth reached by the test probe.

Subsequent analytical analyses were used to evaluate ash composition after bones had been dried and defatted. Fat was extracted from the BioDent tibias using a cold extraction procedure with pure ethyl ether. Fat-extracted tibias were subsequently dried for 24 hours at 105°C and then ashed at 600°C for 24 h. Fat was extracted from the tibias following AOAC (method 932.16, 1990) with differences in ether used.

For determination of nutrient retention outcomes, excreta was collected from the trays beneath each cage and frozen (-20°C) for later analysis, along with samples of diets collected at the time of manufacture. Frozen excreta samples were lyophilized and ground using an electric coffee grinder to provide an evenly ground sample while avoiding significant loss. All subsequent nutrient analyses were completed in duplicate for each diet and excreta sample. Samples were weighed into sample boats (~0.2 g) and total nitrogen content was determined by the Dumas combustion method (TruMac[®] Series; LECO Corporation, St. Joseph, MI). To determine mineral content, all samples were weighed into crucibles (~0.5 g, dry wt.), dried for 24 hours at 105°C, ashed at 500°C for 24 h, and the ash was dissolved in 20% hydrochloric acid (method 968.08, AOAC, 2002). Total phosphorus content of diet and excreta samples were determined colorimetrically by the molybdo-vanadate method and Ca concentrations were determined by flame absorption spectrophotometry (method 965.17, AOAC, 1975).

Titanium dioxide concentrations of diet and excreta samples were determined following the procedures of Short et al. (1996). Samples were weighed into crucibles (~0.1 g, dry wt.), dried for 24 h at 105°C, ashed at 580°C for 24 h, and the ash was dissolved in 7.4 M sulfuric acid. Hydrogen peroxide (30% vol/vol.) was subsequently added, resulting in a yellow color with an intensity dependent upon the sample titanium dioxide concentration. Samples were aliquoted in duplicates and analyzed using a UV spectrophotometer at an absorbance of 410 nm.

The following equation was used to calculate apparent retention of excreta samples on a g/kg dry matter basis:

$$\text{Apparent Retention} = [1 - [(M_i / M_o) \times (X_o / X_i)],$$

where M_i = concentration of TiO_2 (marker) of the diet sample,

M_o = concentration of TiO_2 (marker) of the excreta sample,

X_o = nutrient concentration of the excreta sample,

X_i = nutrient concentration of the diet sample.

Statistical Analyses

A single cage of birds served as the experimental unit for growth performance and nutrient retention data, while individual bird was the experimental unit for all bone parameters. All statistical analyses were completed using procedures appropriate for a completely randomized design. Data are presented as least squares means per dietary treatment groups. All data were analyzed by a 1-way ANOVA for each experiment using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). Linear and quadratic effects were evaluated in Experiment 1 using orthogonal polynomial contrasts to test the response to increasing dietary Ca inclusion. Treatment means were separated using a Tukey's multiple comparison test in Experiment 2. Statistical significance was considered at $P < 0.05$ in all cases.

RESULTS

Experiment 1

Broiler BW gain and FI from 2 to 23 d of age were numerically highest in the dietary treatment that contained 0.6% Ca concentration and statistically different ($P < 0.01$) to those birds fed diets with higher Ca concentrations (Table 3.3). Dietary reduction of Ca improved ($P < 0.01$) G:F of birds relative to that of birds fed increased Ca inclusion concentrations. Tibia size was also influenced ($P < 0.01$) by dietary treatment. Tibia height, length, and width were overall larger in birds fed diets that contained a 0.4% or 0.6% Ca inclusion when compared with birds fed increased Ca inclusions (Table 3.4). Tibia break force and tibia ash content increased ($P < 0.01$) with 0.6% Ca inclusion compared with birds fed diets containing 1.6% Ca. Reference force

indentation characteristics exhibited quadratic ($P < 0.05$) responses as Ca inclusion increased from 0.4 to 1.6%. Dietary treatment effects were observed for apparent retention of dry matter, nitrogen, P, and Ca (Table 3.5). Dry matter and nitrogen apparent retention were reduced ($P < 0.01$) for birds fed diets that contained 0.4% Ca compared with those fed 1.6% Ca inclusion diets. Birds fed diets that contained 0.6% Ca (i.e., 2:1 Ca-to-NPP ratio) exhibited increased ($P < 0.01$) P retention when compared with birds fed the 0.4% Ca inclusion diets. Birds fed diets that contained 0.4% Ca had the greatest ($P < 0.01$) Ca apparent retention when compared with other dietary treatments.

Experiment 2

Neither growth performance (Table 3.6) nor tibia dimensions (Table 3.7) of broilers were influenced ($P > 0.05$) by dietary treatment. Tibia break force and ash were reduced ($P < 0.01$) at the lowest inclusion of Ca and NPP concentrations. Tibia break force and tibia ash did not differ ($P > 0.05$) when the Ca inclusion increased from 1.0% to 1.6%. By varying Ca and NPP concentrations to maintain a constant 2:1 ratio, apparent retention of dry matter decreased ($P < 0.05$) for birds fed dietary treatments that contained the 1.0% Ca inclusion when compared with birds fed the 1.0% Ca inclusion and the constant 0.45% NPP inclusion (Table 3.8). Nitrogen apparent retention was not influenced ($P > 0.05$) by dietary treatments. Varying Ca and NPP concentrations to maintain a constant 2:1 ratio, apparent retention of P decreased ($P < 0.05$) for birds fed 0.8% NPP and P retention increased for birds fed 0.2% NPP when compared with other dietary treatments. Increased Ca concentrations resulted in a decrease in Ca apparent retention ($P < 0.05$) when compared with birds fed 0.4% Ca, regardless of the NPP concentration.

DISCUSSION

Results of previous research on the interaction of Ca and NPP provide clear evidence that insufficient supply of one mineral interferes with homeostasis of the other, therefore the ratio of Ca-to-NPP may be more influential than individual mineral concentrations when formulating poultry diets (Shafey et al., 1990; Bradbury et al., 2014). In Experiment 1, dietary treatments that contained 0.6% Ca inclusion were able to support maximal growth performance and nutrient retention when compared with birds receiving more than 0.6% Ca. The additive and interactive effects of Ca inclusion beyond bird requirements negatively influences broiler growth and nutrient retention due to the ability of Ca to chelate with phytate, thus forming insoluble complexes (Selle et al., 2009). Additionally, both Ca and NPP concentrations, as well as the dietary Ca-to-NPP ratio, influences bird performance due to the antagonism of dietary Ca and the formation of phytate complexes (Plumstead et al., 2008). Increasing dietary Ca while maintaining NPP constant, thus further widening the Ca-to-NPP ratio, may have contributed to the overall reduction of broiler growth performance in Experiment 1. Conversely, growth performance was not influenced by Ca inclusion or Ca-to-NPP ratio in Experiment 2, however Ca concentration did influence bone mineralization in this study. Based on the evidence of broiler growth performance and nutrient retention from our studies, it appears that the Ca-to-NPP ratio seems to be more important than either of the dietary concentrations of Ca or NPP.

In Experiment 1, the NPP concentration was maintained at 0.3% and responses in growth performance were greatest in birds that received diets containing 0.6% Ca inclusion (i.e., a 2:1 Ca-to-NPP ratio). However, birds supplemented with Ca inclusions greater than 0.6% exhibited decreases in both BW gain and FI. When Ca inclusion was greater than 0.6%, we observed decreases in both apparent retention of Ca and P, as well as bone mineralization. This coincides

with data from Driver et al. (2005) who conducted an experiment with 6 Ca concentrations (0.325, 0.4, 0.475, 0.55, 0.625, and 0.9%) and reported that only the highest dietary Ca concentration decreased BW gain, FI, and P retention. Furthermore, data from Rama Rao et al. (2006) reported tibia ash content was maximal and P excretion was minimal when the dietary Ca-to-NPP ratio was maintained at 2:1. Excess dietary Ca can reduce the availability of other minerals, reduce digesta transit time, and impair absorption (Shafey and McDonald, 1991a). Increasing dietary Ca while holding dietary NPP steady (i.e., further widening the Ca-to-NPP ratio) reduced nutrient retention possibly due to the formation of insoluble complexes in the intestinal lumen (Hunziker et al., 1982). As suggested previously, a 2:1 Ca-to-NPP ratio is recommended for broilers while a wider Ca-to-NPP ratio can decrease nutrient retention, suggesting these minerals are not readily available at disproportionate Ca-to-NPP ratios in the diet (Sebastian et al., 1996; Rama Rao et al., 2006). In the current experiment, the use of a Ca-to-NPP ratio at 2:1 led to a reduction of dietary Ca inclusion without compromising growth performance, tibia characteristics, and apparent retention of nutrients.

While the 2:1 Ca-to-NPP ratio elicited beneficial responses in Experiment 1, data from Experiment 2 highlights the importance of controlling both the absolute concentrations of dietary Ca and NPP as well as their relative ratio. As such, broiler growth performance was not influenced by the dietary treatments in this experiment, however bone mineralization was influenced by dietary treatments. It has been reported that BW gain and bone mineralization do not respond in the same way to dietary manipulations, likely because 80% of total body P resides in the skeleton of the bird (Yi et al., 1996; Waldroup et al., 2000). Therefore, bone mineralization depends to a greater extent on dietary concentrations of NPP and Ca compared with BW gain as an outcome (Adedokun et al., 2004; Olukosi and Fru-Niji, 2014). In our study, as the inclusion of

Ca and NPP increased, this elicited increases in tibia break force and ash. This agrees with the results by Onyango et al. (2003), who reported the percentage of tibia ash increased linearly as the concentration of Ca increased from 0.45 to 0.91%, and further exemplified by evidence from Brugalli et al. (1999) who estimated the Ca and P requirements from hatch to 21 d of age as 1.00% and 0.45%, respectively. Presumably, the increased intake of Ca and NPP was used for the synthesis and deposition of hydroxyapatite in bone, which therefore resulted in increased bone mineralization. These findings are in agreement with data that indicate BW gain is not as sensitive of a measurement when quantifying Ca and NPP requirements (Waldroup et al., 2000; Dhandu and Angel, 2003). Therefore, the concentrations of Ca and NPP required to maximize bone mineralization are likely higher than the concentrations required to maximize growth performance.

The antagonism of dietary Ca on digestibility and absorption of P has been well established and shown to be dependent on both Ca and P concentrations, as well as the ratio of Ca to NPP in the diet (Hurwitz and Bar, 1971; van der Klis and Versteegh, 1996). Al-Masri (1995) reported that the dietary Ca inclusion, and its ratio with NPP, may affect P retention with lower P retention values with higher dietary Ca concentrations. Similar results were observed in the current study, where Ca and P retention decreased as dietary concentrations of Ca and NPP increased. Dietary treatments that contained 0.4% Ca exhibited an increase in Ca retention and Ca retention was reduced as the Ca inclusion increased, suggesting Ca retention is heavily dependent on the dietary Ca concentration. Browning et al. (2012) provided evidence that reduced dietary Ca was associated with increased efficiency of Ca retention compared with diets that contained higher concentrations of Ca, indicating a physiological response by the bird to overcome Ca deficiency by increasing absorption and utilization. The adaptive response to

increased absorption is partly mediated through feedback initiation of parathyroid hormone production in the parathyroid glands. When Ca concentrations become too low, the parathyroid gland produces parathyroid hormone and retains Ca by increasing renal Ca reabsorption and stimulating renal release of P (Frandsen and Spurgeon, 1992). When Ca intake exceeds physiological requirements, the parathyroid gland produces calcitonin to inhibit renal Ca reabsorption and therefore increases Ca excretion via the urine (Frandsen and Spurgeon, 1992). High Ca concentrations, or a wide dietary Ca-to-NPP ratio, antagonizes digestibility and absorption of inorganic soluble forms of P due to increased precipitation of insoluble Ca-P complexes (Hurwitz and Bar, 1971; Wise, 1983). In our study, dietary treatments that maintained a narrow Ca-to-NPP ratio were probably less likely to develop insoluble phytate complexes in the gut, which increase the amount of Ca and P available for absorption.

Producing a 2:1 Ca-to-NPP ratio is adequate for growing broilers, however maintaining a balanced 2:1 ratio with lower concentrations of Ca and NPP resulted in broiler performance superior to that of broilers fed a 2:1 ratio with higher concentrations of Ca and NPP. However, reduction of these minerals should be done in a balanced way to obtain maximal performance and mineral retention results. In conclusion, results of these experiments indicate that imbalanced Ca-to-NPP ratios negatively affected growth performance of broilers and resulted in lower utilization of NPP with high dietary Ca concentrations. While maintaining a 2:1 Ca-to-NPP ratio appears beneficial in broilers during the starter period, the individual dietary concentrations of Ca and NPP in broiler diets is also important.

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TABLES

Table 3.1 Composition and calculated analysis of experimental diets fed to broilers in Experiment 1¹

<i>Treatment</i>	1	2	3	4	5	6	7
<i>Ca-to-NPP</i>	1.33:1	2.00:1	2.67:1	3.33:1	4.00:1	4.67:1	5.33:1
<i>Ca, %</i>	0.4	0.6	0.8	1.0	1.2	1.4	1.6
Ingredient, % as-fed	<i>NPP, %</i>	0.3	0.3	0.3	0.3	0.3	0.3
Corn	49.98	49.95	50.03	50.00	49.95	49.94	49.92
Soybean meal	39.50	39.50	39.50	39.50	39.50	39.50	39.50
Limestone	0.72	1.25	1.78	2.30	2.85	3.36	3.88
Soy oil	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Salt	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Silica sand	1.55	1.30	1.00	0.75	0.50	0.25	0.00
Cellulose	1.55	1.30	1.00	0.75	0.50	0.25	0.00
Phosphoric acid ²	0.55	0.55	0.55	0.55	0.55	0.55	0.55
Vitamin premix ³	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Mineral premix ⁴	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Titanium dioxide ⁵	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Calculated analysis, as-fed							
AME _n , kcal/kg	3,093	3,092	3,095	3,094	3,092	3,092	3,091
Crude protein, %	23.00	23.00	23.00	23.00	23.00	23.00	23.00
Ca, %	0.40	0.60	0.80	1.00	1.20	1.40	1.60
Total P, %	0.56	0.56	0.56	0.56	0.56	0.56	0.56
NPP, %	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Analyzed values							
Ca, %	0.61	0.81	0.98	1.05	1.40	1.57	2.09
Total P, %	0.54	0.53	0.52	0.54	0.55	0.54	0.54

¹Abbreviations: AME_n = nitrogen-corrected apparent metabolizable energy.

²Included as a crystalline powder, 99% purity; Sigma-Aldrich, St. Louis, MO.

³Provided per kilogram of complete diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 µg; dl- α -tocopheryl acetate, 11 IU; vitamin B₁₂, 0.01 mg; riboflavin, 4.41 mg; d-Ca-pantothenate, 10 mg; niacin, 22 mg; and menadione sodium bisulfite complex, 2.33 mg.

⁴Provided per kilogram of complete diet: Mn, 75 mg from MnO; Fe, 75 mg from FeSO₄ · 7H₂O; Zn, 75 mg from ZnO; Cu, 5 mg from CuSO₄ · 5H₂O; I, 0.75 mg from ethylene diamine dihydroiodid; and Se, 0.1 mg from Na₂SeO₃.

⁵Included as a homogenous premix and used as an indigestible marker.

Table 3.2 Composition and calculated analysis of experimental diets fed to broilers in Experiment 2¹

<i>Treatment</i>	1	2	3	4	5	6	
<i>Ca-to-NPP</i>	0.9:1	2.2:1	3.9:1	2.0:1	2.0:1	2.0:1	
<i>Ca, %</i>	0.40	1.00	1.60	0.40	1.00	1.60	
Ingredient, % as-fed	NPP, %	0.45	0.45	0.45	0.20	0.50	0.80
Corn	47.81	47.81	47.81	47.81	47.81	47.81	
Soybean meal	40.00	40.00	40.00	40.00	40.00	40.00	
Limestone	0.72	2.30	3.90	0.72	2.30	3.90	
Soy oil	5.00	5.00	5.00	5.00	5.00	5.00	
Salt	0.40	0.40	0.40	0.40	0.40	0.40	
Silica sand	2.15	1.36	0.56	2.55	1.28	0.00	
Cellulose	2.15	1.36	0.56	2.55	1.28	0.00	
Phosphoric acid ²	1.02	1.02	1.02	0.22	1.18	2.14	
Vitamin premix ³	0.20	0.20	0.20	0.20	0.20	0.20	
Mineral premix ⁴	0.15	0.15	0.15	0.15	0.15	0.15	
Titanium dioxide ⁵	0.40	0.40	0.40	0.40	0.40	0.40	
Calculated analysis, as-fed							
AME _n , kcal/kg	3,031	3,031	3,031	3,031	3,031	3,031	
Crude Protein, %	23.00	23.00	23.00	23.00	23.00	23.00	
Ca, %	0.40	1.00	1.60	0.40	1.00	1.60	
Total P, %	0.70	0.70	0.70	0.45	0.75	1.06	
NPP, %	0.45	0.45	0.45	0.20	0.50	0.80	
Analyzed values							
Ca, %	0.56	1.20	1.80	0.51	1.04	1.93	
Total P, %	0.58	0.59	0.61	0.41	0.63	0.72	

¹Abbreviations: AME_n = nitrogen-corrected apparent metabolizable energy.

²Included as a crystalline powder, 99% purity; Sigma-Aldrich, St. Louis, MO.

³Provided per kilogram of complete diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 µg; dl- α -tocopheryl acetate, 11 IU; vitamin B₁₂, 0.01 mg; riboflavin, 4.41 mg; d-Ca-pantothenate, 10 mg; niacin, 22 mg; and menadione sodium bisulfite complex, 2.33 mg.

⁴Provided per kilogram of complete diet: Mn, 75 mg from MnO; Fe, 75 mg from FeSO₄ · 7H₂O; Zn, 75 mg from ZnO; Cu, 5 mg from CuSO₄ · 5H₂O; I, 0.75 mg from ethylene diamine dihydroiodide; and Se, 0.1 mg from Na₂SeO₃.

⁵Included as a homogenous premix and used as an indigestible marker.

Table 3.3 Growth performance of broiler chicks receiving diets varying in Ca concentrations in Experiment 1¹

Item	<i>Treatment</i>	1	2	3	4	5	6	7	SEM	<i>P-value</i>		
	<i>Ca-to-NPP</i>	1.33:1	2.00:1	2.67:1	3.33:1	4.00:1	4.67:1	5.33:1		Model	Lin ²	Quad ³
	<i>Ca, %</i>	0.4	0.6	0.8	1.0	1.2	1.4	1.6				
	<i>NPP, %</i>	0.3	0.3	0.3	0.3	0.3	0.3	0.3				
BW gain, g/chick												
d 1-7		84.9	91.2	86.4	84.7	88.0	79.3	79.9	2.81	0.006	0.003	0.077
d 7-14		233.6	241.8	209.6	194.2	216.7	196.9	192.7	7.50	0.001	0.001	0.240
d 14-21		285.9	297.8	250.4	225.2	242.9	227.7	226.6	8.22	0.001	0.001	0.015
d 1-21		551.3	575.7	497.2	451.0	493.0	455.0	451.1	14.80	0.001	0.001	0.080
Feed intake, g/chick												
d 1-7		116.7	124.6	127.9	123.2	128.2	115.2	115.9	4.17	0.044	0.289	0.005
d 7-14		144.7	148.8	159.1	160.5	167.9	170.8	159.8	8.49	0.310	0.034	0.203
d 14-21		431.6	456.4	394.2	371.1	412.8	389.4	386.6	16.81	0.005	0.004	0.200
d 1-21		781.8	822.7	731.7	688.5	757.7	701.5	695.1	24.34	0.001	0.001	0.430
Gain:feed, g/kg												
d 1-7		729.4	729.9	678.8	692.6	691.1	692.0	695.2	21.36	0.505	0.145	0.216
d 7-14		754.2	767.2	750.4	715.6	739.4	751.3	744.7	14.89	0.320	0.360	0.229
d 14-21		664.3	656.1	640.6	613.7	593.1	587.4	609.4	26.76	0.104	0.005	0.302
d 1-21		705.0	701.0	681.0	657.4	652.1	650.4	658.4	16.44	0.023	0.001	0.153

¹Data are means of 10 cages of 5 chicks fed experimental diets from 2-to-23 d post-hatch, average initial weight was 35.6 g.

²Lin = linear contrast.

³Quad = quadratic contrast.

Table 3.4 Tibia measurements in broiler chicks receiving diets varying in Ca concentrations in Experiment 1¹

Item	Treatment	1	2	3	4	5	6	7	SEM	Model	P-value	
	Ca-to-NPP	1.33:1	2.00:1	2.67:1	3.33:1	4.00:1	4.67:1	5.33:1			Lin ²	Quad ³
	Ca, %	0.4	0.6	0.8	1.0	1.2	1.4	1.6				
	NPP, %	0.3	0.3	0.3	0.3	0.3	0.3	0.3				
Tibia dimensions, mm												
Height		5.7	5.5	5.0	5.3	5.5	5.1	5.1	0.18	0.035	0.016	0.297
Length		72.4	70.8	66.1	66.6	68.4	67.0	65.7	1.20	0.001	0.001	0.052
Width		5.8	5.9	5.1	5.5	5.6	5.3	5.3	0.15	0.001	0.001	0.325
RFI ⁴ , μ m												
TID ⁵		67.0	63.1	58.9	59.7	56.7	70.6	86.9	7.53	0.071	0.065	0.007
IDI ⁶		9.6	9.3	8.7	9.6	8.9	10.8	13.9	1.30	0.065	0.019	0.022
Bone breaking force, N		11,285	13,061	7,540	9,591	9,938	8,461	7,978	1,108.7	0.006	0.003	0.551
Tibia ash ⁷ , %		37.4	40.8	39.1	36.6	35.5	37.0	33.9	1.15	0.001	0.001	0.074

¹Data are means of 10 cages of 5 chicks fed experimental diets from 2-to-23 d post-hatch. At 23 d of age, 1 bird/cage was randomly chosen for collection of the right tibia.

²Lin = linear contrast.

³Quad = quadratic contrast.

⁴RFI = Reference force indentation.

⁵TID = Total indentation distance.

⁶IDI = Indentation distance increase.

⁷Expressed as a proportion of dried, fat-free bone.

Table 3.5 Apparent dry matter and nutrient retention (%) in broiler chicks receiving diets varying in Ca concentrations in Experiment 1¹

<i>Treatment</i>	1	2	3	4	5	6	7		<i>P</i> -value		
<i>Ca-to-NPP</i>	1.33:1	2.00:1	2.67:1	3.33:1	4.00:1	4.67:1	5.33:1		Model	Lin²	Quad³
<i>Ca, %</i>	0.4	0.6	0.8	1.0	1.2	1.4	1.6				
<i>NPP, %</i>	0.3	SEM									
Dry Matter	64.6	69.9	69.1	68.3	67.6	71.5	71.5	0.76	0.001	0.001	0.688
Nitrogen	51.7	55.5	55.5	52.8	56.3	63.2	62.8	1.68	0.001	0.001	0.098
Phosphorus	47.4	60.3	56.3	48.5	44.4	46.4	44.4	1.27	0.001	0.001	0.002
Calcium	76.8	66.2	49.1	16.4	21.2	17.6	36.3	2.10	0.001	0.001	0.001

¹Data are means of 10 cages of 5 chicks fed experimental diets from 2 to 23 d post-hatch.

²Lin = linear contrast.

³Quad = quadratic contrast.

Table 3.6 Growth performance of broiler chicks receiving diets varying in Ca and NPP concentrations in Experiment 2¹

<i>Treatment</i>	1	2	3	4	5	6			
<i>Ca-to-NPP</i>	0.9:1	2.2:1	3.9:1	2.0:1	2.0:1	2.0:1			
<i>Ca, %</i>	0.40	1.00	1.60	0.40	1.00	1.60			
<i>NPP, %</i>	0.45	0.45	0.45	0.20	0.50	0.80			
Item							SEM		<i>Model P-value</i>
BW gain, g/chick									
d1-7	94.6	108.0	99.0	102.3	99.2	101.2	4.36		0.415
d7-14	214.4	233.7	211.1	215.0	219.6	205.8	7.08		0.139
d14-21	333.9	355.0	344.4	327.4	373.3	363.9	11.24		0.055
d1-21	643.3	696.7	654.5	643.4	694.4	670.9	19.09		0.190
Feed intake, g/chick									
d1-7	125.7	136.9	132.4	131.5	133.9	133.2	5.52		0.811
d7-14	322.3	329.7	313.2	312.6	319.3	307.4	9.77		0.651
d14 -21	497.5	535.4	516.9	500.5	553.5	537.1	15.89		0.114
d1-21	945.6	1,001.9	962.5	944.6	1,006.7	977.7	27.24		0.441
Gain:feed, g/kg									
d1-7	748.8	790.2	747.8	777.5	745.4	773.9	31.14		0.863
d7-14	664.8	708.1	674.7	687.2	687.9	674.2	15.45		0.458
d14-21	671.2	662.3	667.0	654.2	675.1	678.3	9.48		0.512
d1-21	679.3	695.0	680.0	681.0	691.2	688.2	8.18		0.687

¹Data are means of 6 cages of 5 chicks fed experimental diets from 2-to-23 d post-hatch, average initial weight was 42.7 g.

Table 3.7 Tibia characteristics in broiler chicks receiving diets varying in Ca and NPP concentrations in Experiment 2¹

<i>Treatment</i>	1	2	3	4	5	6			
<i>Ca-to-NPP</i>	0.9:1	2.2:1	3.9:1	2.0:1	2.0:1	2.0:1			
<i>Ca, %</i>	0.40	1.00	1.60	0.40	1.00	1.60		<i>Model</i>	
<i>NPP, %</i>	0.45	0.45	0.45	0.20	0.50	0.80	SEM	<i>P-value</i>	
Tibia dimensions, mm									
Height	5.6	5.6	6.0	5.7	5.7	5.9	0.16	0.611	
Length	74.7	73.8	72.7	73.1	74.8	73.7	0.87	0.489	
Width	6.0	6.1	6.2	5.9	6.2	6.5	0.17	0.275	
Bone breaking force, N	12,805 ^b	18,761 ^a	18,171 ^a	13,620 ^b	19,556 ^a	21,240 ^a	1,084.80	0.001	
Tibia ash ² , %	36.0 ^b	41.9 ^a	42.4 ^a	36.2 ^b	42.2 ^a	43.5 ^a	0.64	0.001	

^{a-b}Means within a row that do not share a common superscript are different ($P < 0.05$).

¹Data are means of 6 cages of 5 chicks fed experimental diets from 2 to 23 d post-hatch. On d 23, 1 bird/cage was randomly chosen for tibia analysis.

²Expressed as a proportion of dried, fat-free bone.

Table 3.8 Apparent dry matter and nutrient retention (%) in broiler chicks receiving diets varying in Ca and NPP concentrations in Experiment 2¹

<i>Treatment</i>	1	2	3	4	5	6			
<i>Ca-to-NPP</i>	0.9:1	2.2:1	3.9:1	2.0:1	2.0:1	2.0:1			
<i>Ca, %</i>	0.40	1.00	1.60	0.40	1.00	1.60		<i>Model</i>	
Nutrient	<i>NPP, %</i>	0.45	0.45	0.45	0.20	0.50	0.80	SEM	<i>P</i>-value
Dry Matter		66.0 ^b	69.5 ^a	68.2 ^{ab}	67.7 ^{ab}	66.0 ^b	69.0 ^{ab}	0.80	0.015
Nitrogen		51.3	58.2	59.5	54.3	49.7	56.0	2.54	0.072
Phosphorus		41.0 ^b	43.3 ^b	39.8 ^b	61.7 ^a	35.8 ^b	10.5 ^c	2.06	0.001
Calcium		73.5 ^a	44.3 ^b	21.3 ^c	73.8 ^a	33.3 ^{bc}	30.2 ^c	2.80	0.001

^{a-c}Means within a row that do not share a common superscript are different ($P < 0.05$).

¹Data are means of 6 cages of 5 chicks fed experimental diets from 2 to 23 d post-hatch.

CHAPTER 4: EFFECTS OF A HIGH LEVEL OF PHYTASE ON BROILER PERFORMANCE, BONE ASH, AND PHOSPHORUS UTILIZATION

ABSTRACT

An experiment was conducted to evaluate how the addition of microbial phytase influenced growth performance, bone mineralization, tissue P content, apparent digestibility and retention, and inositol phosphate concentrations in broilers fed diets with varying mineral matrices from 2 to 23 d of age. At 2 d of age, chicks were randomly allotted to receive 1 of 6 experimental diets arranged as a 3×2 factorial of mineral matrix [control diet with 1.0% Ca and 0.5% non-phytase P (**NPP**); mineral matrix 1 with 0.84% Ca and 0.35% NPP; and mineral matrix 2 with 0.77% Ca and 0.29% NPP] and phytase supplementation (0 or 1,500 FTU/kg). Feed intake was influenced (quadratic, $P = 0.012$) by the mineral matrix, but no interaction or main effect of phytase were observed. Phytase increased ($P = 0.011$) BW gain regardless of the mineral matrix applied. Feed efficiency (**gain:feed**) was not influenced ($P > 0.05$) by mineral matrix, phytase, or their interaction. Phytase increased bone ash content differentially across matrices (interaction, $P < 0.01$), and tibia P content was lowest in birds fed matrix 2 and highest in the control (linear, $P < 0.05$). Concentrations of P in muscle, spleen, and liver were not affected by treatment. An interactive effect ($P < 0.01$) was observed for apparent ileal digestibility (**AID**) of P, where phytase increased AID in matrix 1. An interactive effect ($P < 0.01$) was observed for apparent retention of P and Ca, where phytase reduced P and Ca retention in the control diet. A main effect ($P < 0.01$) of mineral matrix was observed for AID of Ca, with birds fed matrix 1 having the lowest AID of Ca compared with control and matrix 2 treatments. Phytase influenced ($P < 0.05$) inositol phosphate concentrations differently across matrices. Overall, phytase and the mineral matrix, either as main effects or in an interactive manner,

influenced growth performance, apparent nutrient digestibility and retention, bone and inositol phosphate concentration responses in broiler chicks.

INTRODUCTION

Poultry diets are primarily composed of plant-based ingredients and more than half of the total P in plants is found as part of phytate, which is poorly utilized by poultry (Nelson et al., 1971; Waldroup et al., 2000). Therefore, inorganic P is typically added to meet physiological P requirements of the bird. Phosphorus is an essential mineral for poultry because it is an important component in metabolic and structural processes, and as such, is an essential mineral to attain maximal potential in growth performance. However, endogenous plant phytate binds to minerals and other nutrients to severely decrease nutrient availability and negatively affect digestive and absorptive processes. To alleviate this problem, phytase, an exogenous enzyme, is commonly used as a feed additive to release phytate-bound P. Dietary supplementation with exogenous phytase is an effective method of improving P digestibility (Wu et al., 2003; Selle and Ravindran, 2007; Adeola and Cowieson, 2011).

Phytate is the main storage form of both P and inositol, and phytate can directly bind carbohydrates, proteins, and lipids within the intestinal lumen, which may reduce nutrient solubility and intestinal absorption (Cowieson et al., 2011). The inclusion of phytase in poultry diets allows for the dephosphorylation of nutrient binding phytate, myo-inositol hexaphosphate (IP6), which thereby increases absorption of previously-bound nutrients. The addition of phytase has recently shifted from P release and the reduced anti-nutritive effects of phytate to now focusing on the role of inositol in broilers in response to applying high concentrations of phytase. Implementing high doses of phytase may allow for the degradation of phytate ester IP6, as well

as lower esters, such as IP3 and IP2. The IP1 ester serves as a substrate for endogenous alkaline phosphatases and broilers are able to remove the last P from IP1 to produce the nutrient inositol (Cowieson et al., 2011). Achieving maximum degradation of phytate is essential for the extra-phosphoric effect of phytase. Importantly, complete degradation of phytic acid to produce inositol has been shown to improve growth performance in broilers (Zyla et al., 2004). The benefits of supplementing a high dose of phytase has been shown in broilers through inositol provision and phytate destruction, which allows for the improvement of Ca and P digestibility as well as an overall improvement in growth performance (Cowieson et al., 2011). Nutrient matrix values affect phytase efficacy in broiler diets (Cowieson et al., 2011). Therefore, a study was conducted to vary the Ca and NPP concentrations in plant-based diets and examine the effects on bird performance, bone mineralization, tissue P concentrations, and nutrient digestibility and retention.

MATERIALS AND METHODS

All animal care procedures used in this experiment were approved by the University of Illinois Urbana-Champaign Institutional Animal Care and Use Committee before initiation of the experiment.

Animals and Management Practices

An experiment was conducted using 240 male Ross 308 commercial broiler chicks (Hoover's Hatchery, Rudd, IA). The experiment included 6 dietary treatments with 5 chicks per cage and 8 replicate cages per treatment. Birds were housed at the University of Illinois Poultry Farm in thermostatically-controlled brooder battery cages with raised-wire floors located in an environmentally-controlled room with continuous lighting. Temperature in battery cages was

maintained at 29 to 31°C for the first week of the study, and the temperature was decreased as the birds aged, reaching a final temperature of 27 to 28°C. Access to feed and water were provided *ad libitum* throughout the 21-d feeding period. Feed was fed in mash form via a feed trough and water was provided via a water trough located at the end of each cage.

Experimental Diets

Broiler chicks were fed 1 of 6 dietary treatments that consisted of 3 concentrations each of dietary Ca and non-phytate P (**NPP**) and 2 concentrations of phytase in a 3 × 2 factorial arrangement (Table 4.1). The mineral matrices consisted of control (1.0% Ca, 0.5% NPP), matrix 1 (0.84% Ca, 0.35% NPP), and matrix 2 (0.77% Ca, 0.29% NPP), and each matrix was further supplemented with microbial phytase (Quantum Blue, AB Vista, Marlborough, Wiltshire, UK) at either 0 or 1,500 FTU/kg of diet. These corn-soybean meal based diets contained NPP and Ca ratios that were achieved by varying the inclusion of limestone and dicalcium phosphate to produce reduced mineral matrices. Matrix 1 had reductions of 0.15% NPP and 0.16% Ca and matrix 2 had reductions of 0.21% NPP and 0.23% Ca reduction relative to the control. All diets were formulated to contain 3,060 kcal/kg nitrogen-corrected apparent metabolizable energy (**AME_n**) and were identical in amino acid composition. All other nutrients met or exceeded recommendations for broilers (NRC, 1994). Titanium dioxide was included in all diets at 0.4% inclusion as an indigestible marker to permit calculation of nutrient digestibility and retention. Experimental diets were fed in mash form and chicks received these diets from 2-to-23 d of age.

Measurements

Chicks were weighed, wing-banded, and randomly allotted such that average initial group weights and weight distributions were similar across dietary treatments on d 2 post-hatch; birds

were monitored daily for morbidity and mortality throughout the study. All culled birds were weighed individually and feed intake and feed efficiency outcomes were adjusted for mortality according to the number of bird days. At the end of the 21 d feeding period, all birds and feeders were weighed to determine BW gain, feed intake (**FI**), and feed efficiency (gain:feed, **G:F**) to assess growth performance. Overall, mortality was low and was not associated with a particular dietary treatment.

Collection and Analyses

At the end of the 21 d feeding period, birds were euthanized by CO₂ inhalation to permit collection of tissue samples. The entire liver and spleen were collected from each bird, as well as a section of the pectoral muscle from the right breast of each bird. For determination of digestibility, ileal digesta were collected by gently flushing the terminal ileum (4-to-30 cm proximal to the ileo-cecal junction) using deionized water. For determination of retention, excreta was collected from trays beneath each cage. All ileal digesta and excreta samples were pooled within cage and frozen (-20°C) for later analysis. Frozen ileal digesta and excreta samples were lyophilized and ground using an electric coffee grinder prior to analysis.

The right tibia was collected from every bird and pooled within cage at the end of the 21 d feeding period to quantify bone mineral content. Tibias were autoclaved for 45 minutes at 121°C under 18 PSI before being cleaned of all adhering material. The endcaps were removed from each tibia and each tibia was cut in half using wire cutters to ensure no part of the tibia was lost during the cutting process. Fat was extracted from the tibias using a cold extraction procedure with pure ethyl ether. Fat-extracted tibias were then dried for 24 h at 105°C and subsequently ashed at 600°C for 24 h. Fat was extracted from the tibias following AOAC (method 932.16, 1990) with differences in ether used.

All diet, muscle, liver, spleen, tibia, and ileal digesta and excreta samples were dried at 105°C for 24 h and subsequently ashed at 500°C for 24 h. The ashed samples were then dissolved in 20% hydrochloric acid (method 968.08, AOAC, 2002) before quantifying total P content using a colorimetric procedure based on the molybdo-vanadate method (method 965.17, AOAC, 1975). Calcium concentrations were also quantified in diet, ileal digesta, and excreta samples using flame absorption spectrophotometry.

Quantification of inositol phosphates in ileal and excreta samples were determined using a modification of the method from Kwanyuen and Burton (2005) using high-performance liquid chromatography (HPLC). Freeze dried samples were extracted with 10 mL of 0.5 M HCl for 1 h at 20°C by ultrasonication. The extracts were then centrifuged for 10 minutes at 2,200 × g, and 5 mL of the supernatant was evaporated to dryness in a vacuum centrifuge. The samples were then re-dissolved in 1 mL of distilled, deionized water by ultrasonication for 1 h at 20°C and centrifuged for 15 minutes at 18,000 × g. The resulting supernatant was filtered through a 13 mm syringe filter with a 0.45 μm membrane (GH Polypro Acrodisc[®], Pall Corporation, Ann Arbor, MI) and placed in a 30 kDa centrifugal filter (Microcon[®] Ultracel YM-30, Millipore Corporation, Bedford, MA) and finally centrifuged for 30 minutes at 9,000 × g. The samples were then analyzed for inositol phosphate (**IP**) moieties (IP₂–IP₆) using a standard HPLC analytical column (4 × 250 mm CarboPac PA1 column, Thermo Scientific, Sunnyvale, CA). Phytic acid dodecasodium salt hydrate (Sigma-Aldrich, St. Louis, MO) was used as the standard for both IP₆ and the lower IP to calculate the ratio between peak area and P amount of IP in the isolated fractions.

Titanium dioxide concentrations of diet, ileal digesta, and excreta samples were determined following the procedures of Short et al. (1996). Duplicate samples were weighed

into crucibles, dried at 105°C for 24 h, and subsequently ashed at 550°C for 24 h. The ashed samples were then dissolved in 7.4 M sulfuric acid. Hydrogen peroxide (30% vol./vol.) was subsequently added to produce a yellow color with an intensity proportional to the titanium dioxide concentration in each sample. Duplicate aliquots of these sample solutions were analyzed using a UV spectrophotometer by measuring the absorbance at 410 nm.

The following equation was used to calculate apparent nutrient digestibility of ileal samples and apparent nutrient retention on a g/kg dry matter basis:

$$\text{Apparent nutrient digestibility and retention} = [1 - [(M_i / M_o) \times (X_o / X_i)],$$

where M_i = concentration of TiO_2 (marker) of the diet sample,

M_o = concentration of TiO_2 (marker) of the ileal or excreta sample,

X_o = nutrient concentration of the ileal or excreta sample,

X_i = nutrient concentration of the diet sample.

Statistical Analyses

An individual cage of birds served as the experimental unit for all outcomes with 8 replicate cages for each of 6 dietary treatments arranged in a complete randomized design. Data were corrected for mortality and presented as least square means per treatment group. All data were analyzed by a 2-way ANOVA, with the model including mineral matrix, phytase addition, and their interaction using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). Treatment means were separated using a Tukey's multiple comparison test for outcomes when a significant interactive effect was observed. Statistical significance was considered when $P < 0.05$.

RESULTS

Growth Performance

Broiler growth performance data during the starter period of d 2 to 23 post-hatch are presented in Table 4.2. While no interactive effects were noted for any growth performance outcome, BW gain exhibited main effects ($P < 0.05$) of both Ca-to-NPP ratio and phytase addition. As such, addition of phytase improved BW gain regardless of mineral matrix, while increasing the Ca-to-NPP ratio from 2.00:1 to 2.65:1 via reductions in dietary mineral concentrations caused an overall linear reduction in BW gain and reductions were minimal with the addition of phytase. A main effect of mineral matrix was also observed for FI ($P < 0.01$), whereby a quadratic response was detected, where birds fed matrix 1 exhibited increased FI, while birds fed matrix 2 concomitantly exhibited a decrease in FI, relative to the control. No significant effects of either mineral matrix or phytase addition were observed for G:F.

Tibia Ash and Phosphorus Content

A phytase dose by matrix interaction (quadratic, $P < 0.01$) was observed in tibia ash (Figure 4.1), where without the addition of phytase, tibia ash values were lower for matrix 1 and 2 when compared with the control dietary treatment. However, when supplemented with a high dose of phytase, reductions were minimal for matrix 1 and 2 ($P < 0.01$). Therefore, the tibia ash content of birds supplemented with high concentrations of phytase resulted in minimal tibia ash reductions. Specifically looking at the P concentration of dried, defatted tibias (Table 4.3), it was solely influenced by matrix ($P < 0.05$), where reductions in dietary mineral concentrations caused a linear reduction in P concentration ($P < 0.05$). Therefore, birds fed the control dietary treatment exhibited the greatest tibia P concentration. Total P concentrations of muscle and liver were not influenced by phytase dose, matrix, or their interaction ($P > 0.05$). However, the

addition of phytase increased ($P < 0.05$) P concentrations in the spleen from 11.6 to 12.1 g/kg, regardless of the mineral matrix applied.

Calcium and Phosphorus Digestibility and Retention

Interactive effects were observed for apparent ileal digestibility (**AID**) and apparent retention (Table 4.4). Birds fed matrix 2 had a reduction ($P < 0.05$) in AID of dry matter with the addition of phytase. Additionally, birds fed the control dietary treatment had reductions ($P < 0.01$) in AID and apparent retention of dry matter when supplemented with phytase. An interactive effect was observed for matrix 1, whereby AID of P increased with the addition of phytase ($P < 0.01$) when compared with birds fed matrix 1 and no phytase supplementation. No interactive effects were noted for AID of Ca, however a main effect of mineral matrix was observed. Birds fed matrix 1 had the lowest ($P < 0.01$) AID of Ca compared with birds fed the control or matrix 2 dietary treatments, regardless of phytase addition. A phytase dose by matrix interaction was observed for apparent retention of P and Ca, whereby birds fed the control dietary treatment had reductions ($P < 0.01$) in apparent retention of P and Ca when supplemented with phytase when compared with birds fed the control dietary treatment and no phytase supplementation.

Inositol Phosphate Profiles

Interactive effects were observed for inositol phosphates (**IP**) in both ileal and excreta content (Table 4.5), where inositol hexaphosphate (**IP₆**) concentrations were reduced ($P < 0.01$) when birds were supplemented with phytase, but effects differed by mineral matrix. A main effect of phytase and mineral matrix were observed, where the addition of phytase reduced ($P < 0.01$) ileal and excreta inositol heptaphosphate (**IP₅**) concentrations in birds fed the control dietary treatment. An interactive effect ($P < 0.01$) was observed where only birds fed the control

dietary treatment exhibited ileal inositol tetrakisphosphate (**IP₄**) and inositol triphosphate (**IP₃**) concentrations that increased with the addition of phytase. Similarly, interactive effects ($P < 0.01$) were observed in excreta samples where birds fed the control and matrix 1 dietary treatments had increased **IP₄** and **IP₃** concentrations when supplemented with phytase, but the same did not occur for birds fed the matrix 2 dietary treatments. The addition of phytase increased ($P < 0.05$) ileal inositol diphosphate (**IP₂**) concentrations, regardless of dietary treatment. Additionally, phytase supplementation increased ($P < 0.05$) the amount of inositol present in ileal digesta regardless of the mineral matrix, however **IP₂** and inositol were not influenced ($P > 0.05$) by dose, matrix, or their interaction in excreta samples.

DISCUSSION

Microbial phytase has been supplemented in poultry diets for many years and the beneficial effects of phytase depends on both concentrations of NPP and Ca in the diet (Wu et al., 2003; Selle and Ravindran, 2007; Adeola and Cowieson, 2011). Our study evaluated responses to microbial phytase and varying mineral matrix on growth performance, bone mineralization, tissue P content, and apparent nutrient digestibility and retention in broilers fed diets differing in their mineral matrix and phytase addition. As expected, growth performance responses in the control dietary treatment exceeded those of either reduced mineral matrix dietary treatments in the absence of added phytase, and this was expected as the Ca and NPP concentrations of the reduced mineral matrix dietary treatments were included below NRC (1994) recommendations. However, phytase supplementation increased BW gain of birds, regardless of dietary mineral concentration, due to the ability of this exogenous phosphatase to liberate P and other nutrients as part of the phytate complex. Feed intake was influenced by the matrix applied to the diet, where matrix 2 generally caused a decrease in FI, indicating birds fed

matrix 2 were deficient in P. This coincides with finding of Scott et al. (1982) who reported a deficiency of P in poultry is characterized by a decrease in feed consumption and a failure in growth. Gain-to-feed of broilers was not influenced by the addition of phytase, which agrees with previous literature (Schoner et al., 1991; Vogt, 1992; Denbow et al., 1995; Onyango et al. 2005; Bahadoran et al. 2011). In contrast, Dos Santos (2013) reported improved G:F with the addition of phytase. The contradicting G:F response may be due to multiple different responses such as bird genetics, housing and environmental conditions, phytase sources, ingredients, and or processing.

As a response to dietary supplementation, microbial phytase increased tibia ash content of broilers in agreement with results of previous studies (Sebastian et al., 1996; Dilger et al., 2004; Olukosi et al., 2013). The improvement in tibia ash percentage indicates bone mineralization was likely increased due to availability of minerals liberated from the phytate mineral complex when supplemental phytase was added to the diets. Benefits of supplementing microbial phytase to poultry diets that contain low P due to the increased utilization of P from phytate have been reported (Sebastian et al., 1996). Therefore, the addition of phytase can reduce supplementation of inorganic P and still allow birds to maintain normal bone development and growth (Sebastian et al., 1996; Viveros et al., 2002; Wu et al., 2003).

Regarding P status of broilers, dietary P requirements differ depending on the outcome evaluated. Phosphorus is quantifiably more important in bone mineralization than for soft tissue growth, because P is a major component of the bird's skeleton. Broilers may become more vulnerable to mineral imbalances as Ca and NPP concentrations are reduced, because bone mineralization requirements are met before growth requirements when P nutrition is the focus. Therefore, tibia ash content is a more sensitive response measurement than growth response

(Zyla et al., 2004). Collectively, broiler growth performance and tibia bone ash data confirm that addition of phytase was effective in increasing utilization of phytate P when dietary Ca and NPP concentrations were reduced.

The addition of phytase in the reduced mineral matrix dietary treatments increased AID and apparent retention of P, which coincides with data from previous experiments (Shirley and Edwards, 2003; Selle and Ravindran, 2007; Adeola and Cowieson, 2011). Selle and Ravindran (2007) suggested when phytate is degraded by phytase, it allows more P to be released in the small intestine, indicative of an improvement in the release of phytate-phosphorus. Silversides et al. (2004) also reported increased P digestibility, with the addition of phytase, when NPP was decreased from 0.40% to 0.23% in broiler diets. Supplementing phytase to low NPP diets increased P digestibility which reduced P content in excreta. Therefore, supplementing phytase and feeding lower Ca and NPP concentrations may confer benefits to reducing excretion of excess P into the environment (Simons et al., 1990; Perney et al., 1993; Waldroup et al., 2000; Dilger et al., 2004). The use of supplemental phytase in conjunction with reduced dietary NPP concentrations are well known as an effective method of improving P utilization and decreasing P excretion.

Similarly, the addition of phytase increased apparent retention of Ca in reduced mineral matrix diets. Supplementation of phytase at lower Ca concentrations suggest fewer Ca-phytate complexes were formed and therefore more phytate molecules were exposed to phytase hydrolysis (Selle et al., 2000). Silversides et al. (2004) also reported increases in Ca digestibility when phytase was added to diets that contained reduced NPP and Ca concentrations. This may explain why a lower Ca-to-NPP ratio is recommended in poultry diets that are supplemented with phytase compared with diets that contain no phytase supplementation (Lei et al., 1994). On

the contrary, birds fed the control dietary treatment exhibited decreases in both Ca and P digestibility and retention when supplemented with phytase. This is an indication that excess concentrations of NPP and Ca in the diet can actually have a negative impact on the digestion of nutrients. This could be due to a greater amount of P and Ca in the intestine than can be utilized by the bird, which leads to a reduction in Ca and P digestibility and increased mineral excretion. Liao et al. (2007) reported that as the dietary content of Ca increased there was a smaller increase in the apparent retention of Ca in response to phytase supplementation, which could be due to high Ca concentrations in the diet that can lead to the formation of insoluble Ca-phytate complexes within the digestive tract. Managi and Coon (2008) reported 5,000 FTU/kg of phytase are necessary to make almost all of the phytate phosphorus in corn-soybean meal diets available for utilization by young broiler chicks.

As a novel aspect of this experiment, analysis of the inositol phosphate profiles in ileal digesta and excreta samples suggested the greatest proportion of IP₆ concentrations, and the addition of phytase clearly hydrolyzed this form into more IP₅ than other lower esters. This suggests phytase targets the higher molecular weight esters, which coincides with data from previous studies (Wyss et al., 1990; Cowieson et al., 2011). As discussed above, supplementing phytase decreased the amount of P in excreta, presumably due to the hydrolysis of IP₆, which is further supported by the data obtained from our experiment. It has been reported that the effects of supplementing phytase on IP₄ and IP₃ are primarily seen in the crop and gizzard, which is expected due to an optimal pH for phytase activity (Schlemmer et al., 2001; Elkhilil et al., 2011). In our study, IP₄ and IP₃ increased with the addition of phytase. This could be due to the pancreatic duct secreting zinc into the small intestine, which causes an increase in digesta pH and may reduce the activity of phytase (Pontoppidan et al., 2012). Additionally, Carre (2004)

reported an increase in IP₄ and IP₃ due to the addition of phytase may have resulted from an unequal transit rate, where smaller feed particles traveled through the gizzard faster than what could be retained for digestion. The addition of phytase improved inositol concentrations in the ileal digesta, which coincides with data from previous experiments (Cowieson et al., 2014; Vieira et al., 2015). Increased inositol concentrations suggest 1,500 FTU of phytase per kg of diet was able to degrade dietary phytate to produce lower IP esters and pure inositol, which may have beneficial effects in broiler production.

In conclusion, birds fed dietary treatments that had reductions in the amount of dietary NPP and Ca exhibited a decrease in performance. However, there was a main effect of phytase, which resulted in an increase in BW gain. Without phytase supplementation both matrices exhibited a lower tibia ash value when compared with the control, however tibia values had less of a reduction when supplemented with a high dose of phytase, which led to a phytase by matrix interaction. As expected, tibia ash content was more sensitive in response to the amount of NPP and Ca in the diet. Supplementing a high dose of microbial phytase resulted in increased concentrations of P in the spleen, while tibia P, apparent digestibility, and retention responses depended on the mineral matrix that was applied to the diet. Supplementing phytase improved IP₆ degradation and increased the ileal inositol concentration of the bird.

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TABLES AND FIGURES

Table 4.1 Composition and calculated analysis of experimental diets fed to broilers¹

Ingredient, % as-fed	Treatment Ca-to-NPP Ca, % NPP, %	Control 2.00:1 1.00 0.50	Matrix 1 2.40:1 0.84 0.35	Matrix 2 2.65:1 0.77 0.29
Corn		58.61	60.07	60.69
Soybean meal		33.65	33.40	33.30
Limestone		1.03	1.12	1.14
Dicalcium phosphate		2.17	1.34	1.01
Soy oil		2.54	2.05	1.85
Salt		0.30	0.30	0.30
Sodium bicarbonate		0.23	0.23	0.23
DL-Methionine		0.29	0.29	0.29
Lysine HCl		0.21	0.22	0.22
L-Threonine		0.03	0.03	0.03
Choline chloride (60%)		0.20	0.20	0.20
Vitamin premix ²		0.20	0.20	0.20
Mineral premix ³		0.15	0.15	0.15
Titanium dioxide ⁴		0.40	0.40	0.40
Calculated analysis, as-fed				
AME _n , kcal/kg		3,060	3,060	3,060
Crude protein, %		21.50	21.50	21.50
Ca, %		1.00	0.84	0.77
Total P, %		0.82	0.65	0.59
NPP, %		0.50	0.35	0.29
Analyzed values				
Ca, %		2.67	1.91	2.22
Total P, %		0.59	0.46	0.47

¹Abbreviations: AMEn = nitrogen-corrected apparent metabolizable energy.

²Provided per kilogram of complete diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 µg; dl-α-tocopheryl acetate, 11 IU; vitamin B₁₂, 0.01 mg; riboflavin, 4.41 mg; d-Ca-pantothenate, 10 mg; niacin, 22 mg; and menadione sodium bisulfite complex, 2.33 mg.

³Provided per kilogram of complete diet: Mn, 75 mg from MnO; Fe, 75 mg from FeSO₄ · 7H₂O; Zn, 75 mg from ZnO; Cu, 5 mg from CuSO₄ · 5H₂O; I, 0.75 mg from ethylene diamine dihydroiodid; and Se, 0.1 mg from Na₂SeO₃.

⁴Included as a homogenous premix and used as an indigestible marker.

Table 4.2 Growth performance of broiler chicks receiving diets varying in Ca, non-phytate phosphorus (NPP), and phytase concentrations¹

<i>Treatment</i>	Control		Matrix 1		Matrix 2		SEM	P-value			
	Ca-to-NPP		2.40:1		2.65:1			Matrix	Phytase	Interaction	
Item	<i>Ca, %</i>	1.00	1.00	0.84	0.84	0.77	0.77				
	<i>NPP, %</i>	0.50	0.50	0.35	0.35	0.29	0.29				
	<i>Phytase</i> ²	-	+	-	+	-	+				
BW gain, g/chick		759.1	763.1	701.3	766.5	667.0	717.5	18.43	0.002	0.011	0.234
Feed intake, g/chick		970.3	987.0	1000.2	994.3	896.3	953.0	20.04	0.002	0.176	0.297
Gain:feed, g/kg		798.2	766.1	701.9	774.4	737.2	735.0	19.02	0.076	0.130	0.074

¹Data are means of 8 cages of 5 chicks fed experimental diets from 2 to 23 d post-hatch; average initial weight was 43.3 g.

²Microbial phytase added at either 0 or 1,500 FTU/kg of diet.

Table 4.3 Tibia and organ P concentrations in broiler chicks receiving diets varying in Ca, non-phytate phosphorus (NPP), and phytase concentrations¹

<i>Treatment</i>	Control		Matrix 1		Matrix 2		SEM	<i>P-value</i>		
	2.00:1		2.40:1		2.65:1			Matrix	Phytase	Interaction
<i>Ca-to-NPP</i>										
<i>Ca, %</i>	1.00	1.00	0.84	0.84	0.77	0.77				
<i>NPP, %</i>	0.50	0.50	0.35	0.35	0.29	0.29				
<i>Phytase</i> ²	-		+		-		+			
Item										
Tibia P, g/kg ³	168.5	167.1	163.2	164.4	163.0	164.1	1.85	0.031	0.847	0.700
Muscle P, g/kg	9.2	9.3	9.3	9.4	9.4	9.4	0.12	0.517	0.198	0.899
Liver P, g/kg	10.5	10.4	10.1	9.9	10.4	10.4	0.31	0.242	0.744	0.942
Spleen P, g/kg	11.6	12.0	11.6	11.9	11.7	12.3	0.26	0.498	0.020	0.831

¹Data are expressed on dry matter basis and are means of 8 cages of 5 chicks fed experimental diets from 2 to 23 d post-hatch; average initial weight was 43.3 g.

²Microbial phytase added at either 0 (-) or 1,500 (+) FTU/kg of diet.

³Expressed as a proportion of dried, fat-free bone.

Table 4.4 Apparent dry matter, nutrient digestibility, and retention (%) in broiler chicks receiving diets varying in Ca, non-phytate phosphorus (NPP), and phytase concentrations¹

<i>Treatment</i>	Control		Matrix 1		Matrix 2		SEM	P-value		
	2.00:1		2.40:1		2.65:1			Matrix	Phytase	Interaction
<i>Ca, %</i>	1.00	1.00	0.84	0.84	0.77	0.77				
<i>NPP, %</i>	0.50	0.50	0.35	0.35	0.29	0.29				
<i>Nutrient Phytase</i> ²	–	+	–	+	–	+				
AID ³										
Dry Matter	78.8 ^a	75.1 ^b	73.1 ^b	74.6 ^b	75.7 ^b	73.3 ^b	0.68	0.001	0.011	0.001
Phosphorus	71.9 ^a	63.5 ^{ab}	55.0 ^b	72.5 ^a	69.4 ^a	75.7 ^a	3.36	0.040	0.069	0.002
Calcium	58.0	53.2	42.1	46.8	64.7	58.9	2.57	0.001	0.362	0.094
Apparent Retention										
Dry Matter	83.0 ^a	77.0 ^b	77.1 ^b	77.1 ^b	78.6 ^b	77.0 ^b	0.96	0.012	0.002	0.010
Phosphorus	55.5 ^{ab}	32.2 ^d	44.0 ^c	48.7 ^{bc}	56.2 ^{ab}	62.6 ^a	2.30	0.001	0.036	0.001
Calcium	56.3 ^a	46.4 ^{abc}	36.9 ^c	40.8 ^{bc}	47.8 ^{ab}	54.7 ^a	2.50	0.001	0.887	0.004

^{a-d}Means within a row that do not share a common superscript are different, as interactive effects between mineral matrix and phytase concentration were observed ($P < 0.05$).

¹Data are means of 8 cages of 5 chicks fed experimental diets from 2 to 23 d post-hatch; average initial weight was 43.3 g.

²Microbial phytase added at either 0 (–) or 1,500 (+) FTU/kg of diet.

³AID = apparent ileal digestibility.

Table 4.5 Inositol phosphate concentrations (nmol/g DM) in broiler chicks receiving diets varying in Ca, non-phytate phosphorus (NPP), and phytase concentrations¹

<i>Treatment</i>	Control		Matrix 1		Matrix 2		SEM	P-value		
	2.00:1		2.40:1		2.65:1			Matrix	Phytase	Interaction
<i>Ca-to-NPP</i>	1.00	1.00	0.84	0.84	0.77	0.77				
<i>Ca, %</i>	1.00	1.00	0.84	0.84	0.77	0.77				
<i>NPP, %</i>	0.50	0.50	0.35	0.35	0.29	0.29				
<i>Phytase²</i>	-	+	-	+	-	+				
Item³										
Ileal digesta										
IP ₆	45,680 ^a	6,199 ^c	31,008 ^b	6,657 ^c	33,247 ^b	7,710 ^c	2,063.2	0.004	0.001	0.001
IP ₅	2,975	1,483	1,891	1,335	1,508	1,044	232.3	0.001	0.001	0.061
IP ₄	361 ^b	4,011 ^a	677 ^b	1,645 ^b	121 ^b	693 ^b	460.0	0.002	0.001	0.003
IP ₃	687 ^b	2,230 ^a	1,111 ^b	1,328 ^b	593 ^b	756 ^b	198.9	0.001	0.001	0.001
IP ₂	2,085	3,111	2,292	2,220	2,143	2,590	253.4	0.395	0.029	0.107
Inositol	2,108	8,957	5,726	12,431	6,578	12,998	1,003.3	0.001	0.001	0.974
Total IP	53,895 ^a	25,992 ^c	42,705 ^b	25,615 ^c	43,368 ^b	25,791 ^c	1,956.3	0.008	0.001	0.013
Excreta										
IP ₆	54,822 ^a	9,405 ^c	38,680 ^b	8,824 ^c	38,883 ^b	9,379 ^c	1,394.8	0.001	0.001	0.001
IP ₅	4,251	2,649	2,919	2,181	2,466	1,734	247.9	0.001	0.001	0.113
IP ₄	810 ^c	5,952 ^a	737 ^c	3,136 ^b	619 ^c	1,662 ^c	301.2	0.001	0.001	0.001
IP ₃	1,127 ^c	3,926 ^a	1,281 ^c	2,393 ^b	1,112 ^c	1,458 ^c	157.7	0.001	0.001	0.001
IP ₂	2,980	2,736	2,349	2,937	2,399	3,343	377.9	0.774	0.149	0.246
Inositol	99	852	1,087	1,035	615	880	291.3	0.120	0.165	0.356
Total IP	64,088 ^a	25,413 ^c	47,053 ^b	20,507 ^{cd}	46,092 ^b	17,605 ^d	1,566.4	0.001	0.001	0.001

^{a-d}Means within a row that do not share a common superscript are different, as interactive effects between mineral matrix and phytase concentration were observed ($P < 0.05$).

¹Data are means of 8 cages of 5 chicks fed experimental diets from 2 to 23 d post-hatch; average initial weight was 43.3 g.

²Microbial phytase added at either 0 (-) or 1,500 (+) FTU/kg of diet.

³Subscript number denotes the number of phosphate groups attached to the inositol ring.

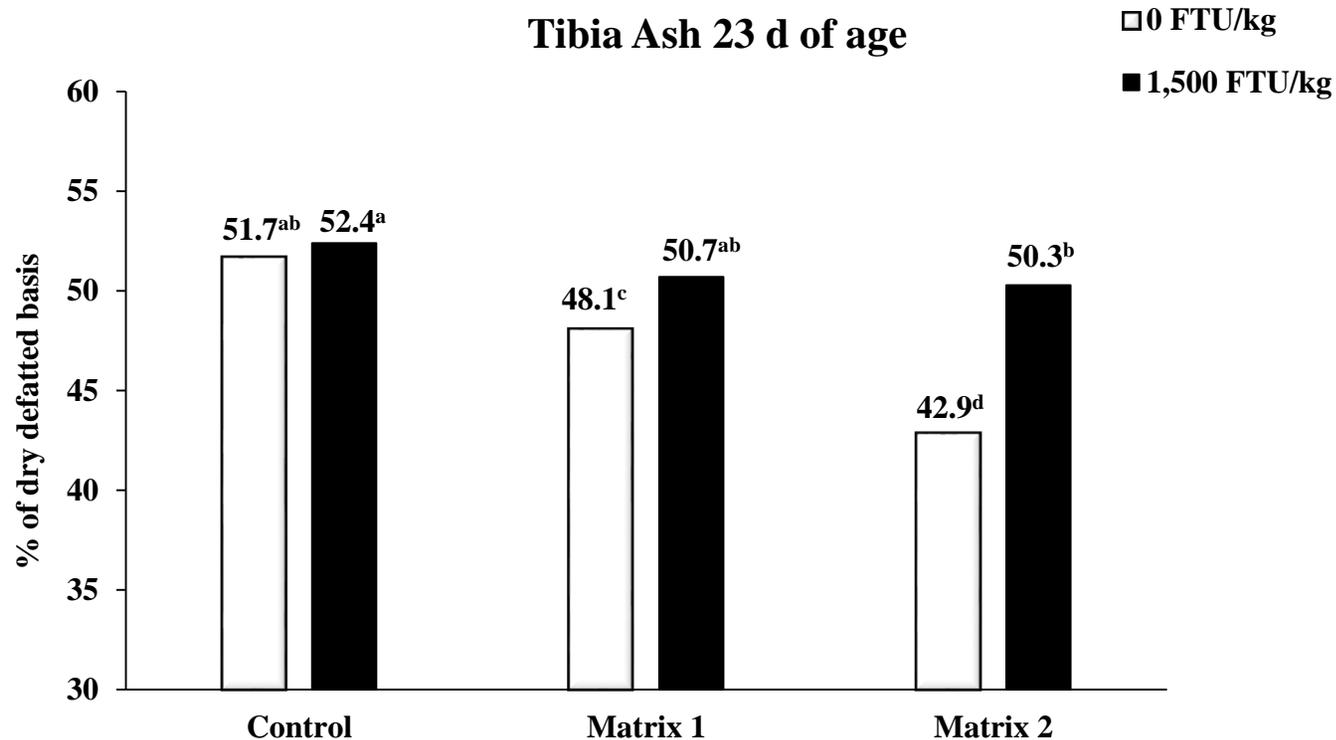


Figure 4.1. ^{a-b}Means that do not share a common superscript are different, as interactive effects between mineral matrix and phytase concentration were observed ($P < 0.05$). Effect of dietary mineral matrix and the addition of phytase on dried defatted tibia ash of broiler chicks at 23 d post hatch. Birds were fed 1 of 6 experimental diets; control dietary treatment with 1.0% Ca and 0.5% non-phytase P (NPP); mineral matrix 1 with reductions of 0.15% NPP and 0.16% Ca compared with control; mineral matrix 2 with reductions of 0.21% NPP and 0.23% Ca compared with control, and microbial phytase was added at 0 or 1,500 FTU/kg of diet. Values represent least square means of 5 chicks/cage from 8 replicate cages.

CHAPTER 6: GENERAL CONCLUSIONS

The overall focus of these studies was to determine the effects of dietary Ca-to-NPP ratio and the effects of phytase supplementation on growth performance, digestibility, retention, and bone characteristics in broiler chickens fed corn-soybean meal diets. In Experiment 1, the NPP concentration was maintained at 0.3% and growth performance responses were greatest in birds that received diets containing 0.6% Ca inclusion (i.e., a 2:1 Ca-to-NPP ratio). Reductions in BW gain, feed intake (**FI**), apparent retention, and bone mineralization were observed in broilers fed diets containing Ca inclusions greater than 0.6%. Therefore, use of a Ca-to-NPP ratio of 2:1 led to a reduction of dietary Ca inclusion without compromising growth performance, tibia characteristics, or apparent retention of nutrients. Experiment 2 indicated the importance for controlling both the absolute concentrations of dietary Ca and NPP as well as their relative ratio. There was no impact of Ca concentration or the Ca-to-NPP ratio on broiler growth performance, however the Ca-to-NPP ratio did influence bone mineralization. This is important to note, as BW gain and bone mineralization do not respond in the same way to dietary manipulations. Reductions in Ca and P retention were observed as dietary concentrations of Ca and NPP increased. Imbalanced Ca-to-NPP ratios appeared to exacerbate the effect on apparent retention of Ca and P in broilers, and dietary treatments that maintained a narrow Ca-to-NPP ratio positively influenced the amount of Ca and P available for absorption. Regarding Experiment 3, the addition of phytase (1,500 FTU/kg) increased broiler BW gain, while FI and feed efficiency were not influenced by phytase supplementation. A phytase dose by matrix interaction was observed in tibia ash, where in the absence of added phytase, tibia ash values were lower for matrix 1 and 2 and reductions were minimal when supplemented with phytase. Reductions in dietary mineral concentrations adversely impacted tibia P concentrations and varying effects of

phytase dose by matrix interaction were observed for nutrient digestibility, retention, and inositol phosphate in broilers. Collectively, data from these experiments indicate a 2:1 Ca-to-NPP ratio appears beneficial in broilers during the starter period, however individual dietary concentrations of Ca and NPP to produce the 2:1 ratio is highly important. Therefore, dietary phytase supplementation may be particularly advantageous for broilers fed reduced mineral matrices.