# THE IMPACT OF DIETARY PHYTASE SUPPLEMENTATION ON SKELETAL BIOMECHANICS AND CHARACTERISTICS IN GROWING SWINE AND MICE

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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August 2009

## ABSTRACT

The misconception that bone is a static tissue underestimates its importance in human and animal health, as well as the many factors that influence its development and maintenance. Genetic, hormonal, mechanical and nutritional factors all influence bone strength and mass, particularly in early life. Musculoskeletal diseases, such as osteochondrosis and osteoporosis, are debilitating to humans and animals and create a substantial financial burden on the country's health care system and food animal production industries. Efforts to discover strategies for the prevention and management of musculoskeletal diseases have become a major area of research.

Our research examined the impact of nutritional supplementation on bone strength and mass as a means to improve bone integrity early in life and reduce the susceptibility of the skeleton to musculoskeletal diseases. Phytase, an acid phosphatase, was the primary dietary supplement examined because it improves bone strength in swine, broiler chicks and rats fed a phosphorus deficient diet. We were interested in the ability of phytase to improve bone strength in young animals through a non-phosphorus mechanism. Therefore, we conducted a series of feeding trials in swine (Yorkshire x Hampshire x Laundrace) and mice (129/SVJ×C57BL/6 line) fed phosphorus adequate diets. In addition, two other bone-impacting dietary supplements, inulin and strontium, were examined as well. These two additional supplements were examined with phytase because studies have suggested that they might have an additive effect and further improve bone strength and mass when supplemented with phytase.

In swine, phytase supplementation for 5 weeks in a nutrient adequate diet increased ($p < 0.05$) femur bending rigidity and mineral content. When phytase was supplemented in the diet with inulin or strontium, no significant impact on skeletal tissue was observed. Dietary inulin supplementation alone for 5 weeks altered ($p < 0.05$) the ellipticity index of femurs and dietary strontium supplementation alone for 10 weeks increased ($p < 0.05$) femur mineral density.

In the mouse, we did not observe an impact of phytase supplementation on skeletal functional parameter, such as femur breaking strength or bending rigidity, when its substrate, phytate, was absent from the nutrient adequate diet. Superoxide dismutase knockout mice (SOD1 -/-; Cu, Zn-SOD; EC 1.15.1.1) provided an animal model with a compromised skeletal system. Phytase supplementation was not effective in improving breaking strength or bending rigidity in this model.

Our results suggest that phytase supplementation has some beneficial effects on bone breaking strength early in development when supplemented in a diet with its native substrate. Phytase supplemented with either inulin or strontium did not provide any additional benefit on bone parameters examined in these studies.

## BIOGRAPHICAL SKETCH

Catherine A. Faber attended high school at Niles North High School in Skokie, Illinois. After graduation, she enrolled as an undergraduate at the University of Iowa and received her Bachelor of Science in Biology in 2004. She then received an Intramural Research Training Award from the National Institutes of Health and was a post-baccalaureate research from 2005 to 2007. During this time she worked in the Institute of Mental Health in the Laboratory of Genetics. Eager to continue her research career she pursued graduate research at Cornell University in the Department of Animal Science with Dr. Xin Gen Lei. Catherine began her Master of Science in August 2007. Following the completion of her degree in August 2009, Catherine began a Doctorate of Veterinary Medicine at Purdue University.

## ACKNOWLEDGMENTS

I wish to acknowledge the following individuals, for with out their support and expertise this research would not have been possible; Dr. Xin Gen Lei, Dr. Cornelia Farnum, Dr. Marjolein van der Meulen, Dr. Jay Cao, Karl Roneker, Carol Roneker, Mike Rutzke, Jeremy Weaver, Theresa Gallie, Joanne Krzyspiak, Florence Hsu, Courtney Mills, Shikui Wang, Koji Yasuda, and Xi Yan.

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# CHAPTER ONE
# INTRODUCTION

## 1.1 The Skeleton

### *1.1.1 Composition*

Skeletal tissue is a specialized form of connective tissue that serves two main functions. Bone provides internal structural support and acts as a mineral reservoir, particularly for essential elements such as calcium and phosphorus (1,2). Bone consists of bone cells, vessels, and a matrix of collagen and minerals (1). Collagen gives bone flexibility and also provides a surface for the nucleation of bone mineral crystals. Hydroxyapatite crystals, $Ca_{10}(PO_4)_6(OH)_2$, are the predominant type of mineral in bone but these crystals have a tendency to absorb and substitute other minerals into their lattice structure, such as fluoride or strontium (3).

Bone is composed of two tissue structures, cortical and cancellous, that are classified by their porosity, or volume fraction of bone tissue (3). Cancellous, or trabecular, bone has a porosity of approximately 75-95%. The cancellous bone matrix consists of plates or structs, known as trabeculae, each approximately 200 μm thick. The cavities between these trabeculae are filled with bone marrow. On the other hand, cortical, or compact bone, is quite dense with a porosity of 5-10% and is located in long bones, forming a shell around cancellous bone (4). Cortical bone is intercalated with Haversian canals and Volkmann's canals. The former contains capillaries and nerves

aligned with the long axis of the bone, while the latter are transverse and connect Haversian canals to each other and to the outside surface of the bone. Volkmann's canals are shorter than Haversian canals, but also contain blood vessels and nerves (Fig. 1.1) (3).

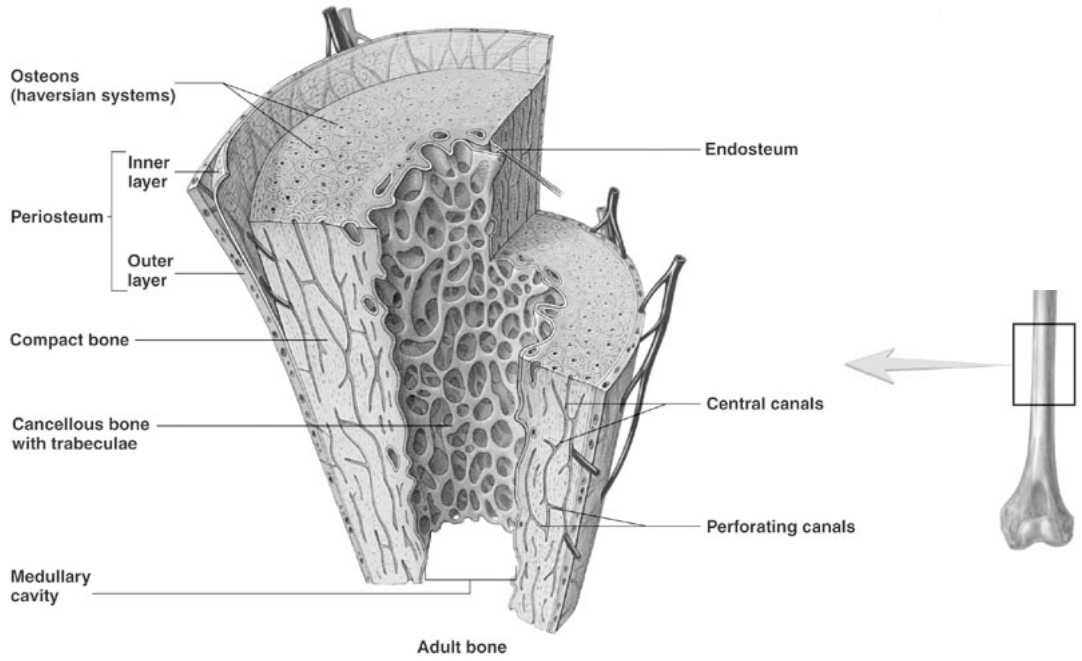



**Figure 1.1** A schematic demonstration of a cross and longitudinal section of the femur diaphysis. Modified from Seeley, R. R., Stephens, T. D. & Tate, P. (Eds). (2007). *Anatomy and Physiology*. McGraw-Hill Science.

### *1.1.2 Bone Remodeling*

Bone is not a static structure and is continuously undergoing a remodeling process to repair microfractures and modify skeletal areas in response to biomechanical and hormonal signals (1). Remodeling involves the destruction

and absorption of mineralized bone followed by the deposition of new bone, thereby preserving the structure and function of the skeleton (5). Two distinct types of bone cells are responsible for the remodeling process of the mineralized extracellular matrix: osteoclasts and osteoblasts (2). Osteoclasts catabolize bone by acidification and proteolysis of the bone matrix. The resulting cavity is filled by recruited osteoblasts, which deposit the new bone matrix in two steps. First, osteoblasts produce osteoid, an unmineralized organic matrix of bone comprised of collagen and noncollagenous proteins, proteoglycans, and water (5). After deposition of the osteoid, osteoblasts direct mineralization of the osteoid and mineral is deposited within and between the collagen fibers. During the remodeling process, osteoclasts and osteoblast are coupled together and work as a team called the basic multicelluar unit (BMU) (3). As described above, the BMU remodeling sequence follows a characteristic order initiated with activation (precursors cells signaled to differentiate), resorption and formation, or the A-R-F sequence.

### *1.1.3 Endochondral Ossification*

The longitudinal growth of bones is accomplished by endochondral ossification, the replacement of cartilage by bone tissue during skeletal maturation (3). The prenatal skeleton is composed of cartilaginous structures, which act as a template for ossification (Fig 1.2) (6). Prior to partition, ossification occurs at the center of the bone shaft, or diaphysis, forming the

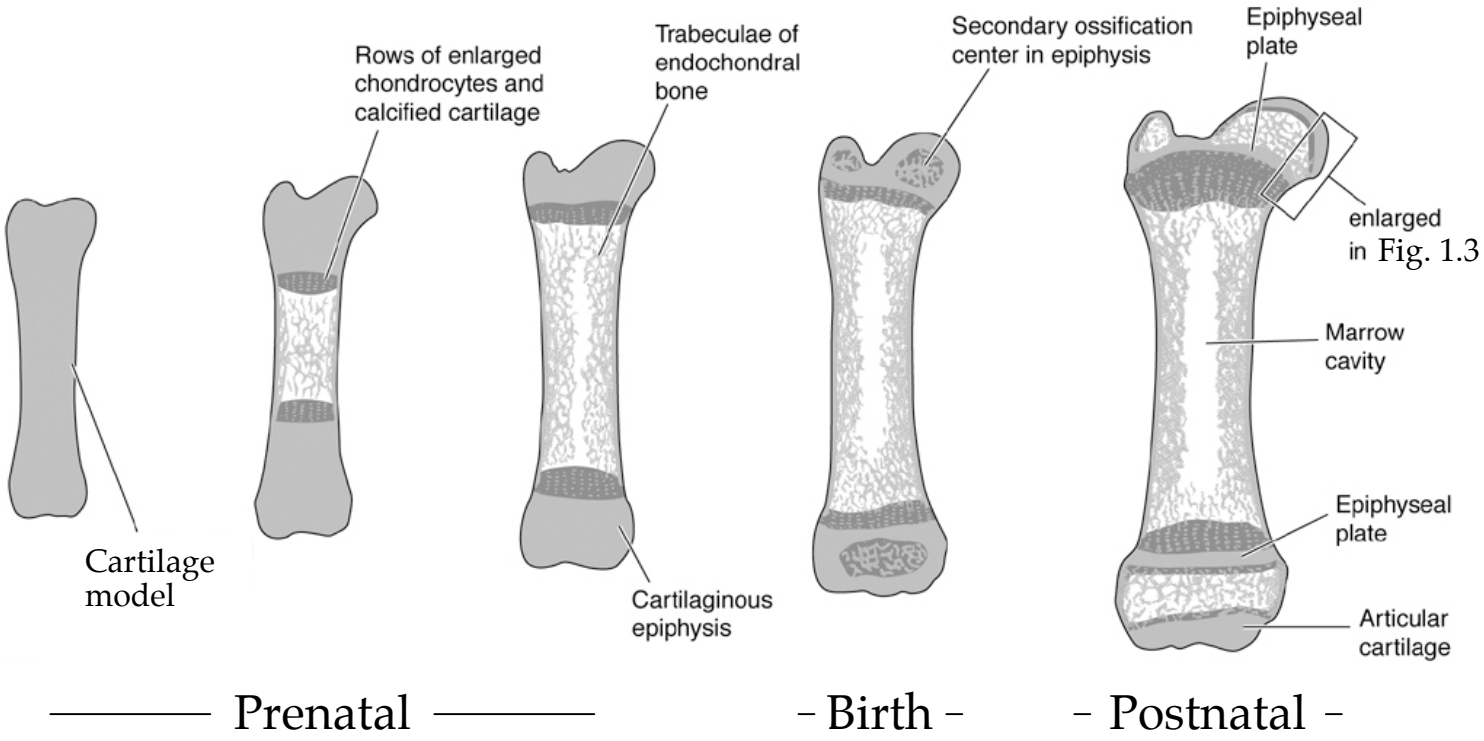



**Figure 1.2** Scheme of endochondral ossification in a long bone. An approximate time-line in shown below the images to represent when the changes bone structure are occurring in the developing animal. Modified from Buckwalter, J. A., Thomas A., M. D. E. & Simon, S. R. (2000). *Skeletal Function and Form: Mechanobiology of Skeletal Development, Aging and Regeneration.* Cambridge University Press.

primary ossification center (POC). The initiation of the POC results from morphological changes of cartilages cells, or chondrocytes in this area (6)

Chondrocytes begin to enlarge and end their secretion of collagen andproteoglycans, components of the extracellular matrix (7). Thus, instead of contributing to the ECM, chrondrocytes initiate the calcification of the matrix via secretion of alkaline phosphatase. In addition, chrondrocytes secrete vascular endothelial cell growth factor (VEGF), a growth factor involved in recruiting blood vessel (7). Blood vessels supply the POC with nutrients as well as provide a route for the migration of other cell types, such as osteoblasts. Chondroblasts, osteoclast-like cells, are responsible for catabolizing the calcified cartilage, while osteoblast subsequently lay down new bone (3). Remodeling activity begins to convert calcified cartilage to bone.

At about the time of partition, a secondary ossification center originates in the center of the epiphysis, which partially divides the epiphyseal growth cartilage into the epiphyseal plate and articular cartilage (6). Endochondral ossification occurs at both of these sites and involves the proliferation, degeneration, and apoptosis of the chondrocytes followed by the calcification and ossification of the matrix to increase bone length and size (7,8). The epiphyseal plate is divided into zones representing the life cycle of chrondrocytes and includes the resting, proliferative, hypertrophic, and calcification zones (Fig. 1.3) (3). These zones are similar to the morphological transformations of chrondroytes described in the POC, but are arranged in a vertical pattern in the epiphyseal cartilage that allows the bone to grow in

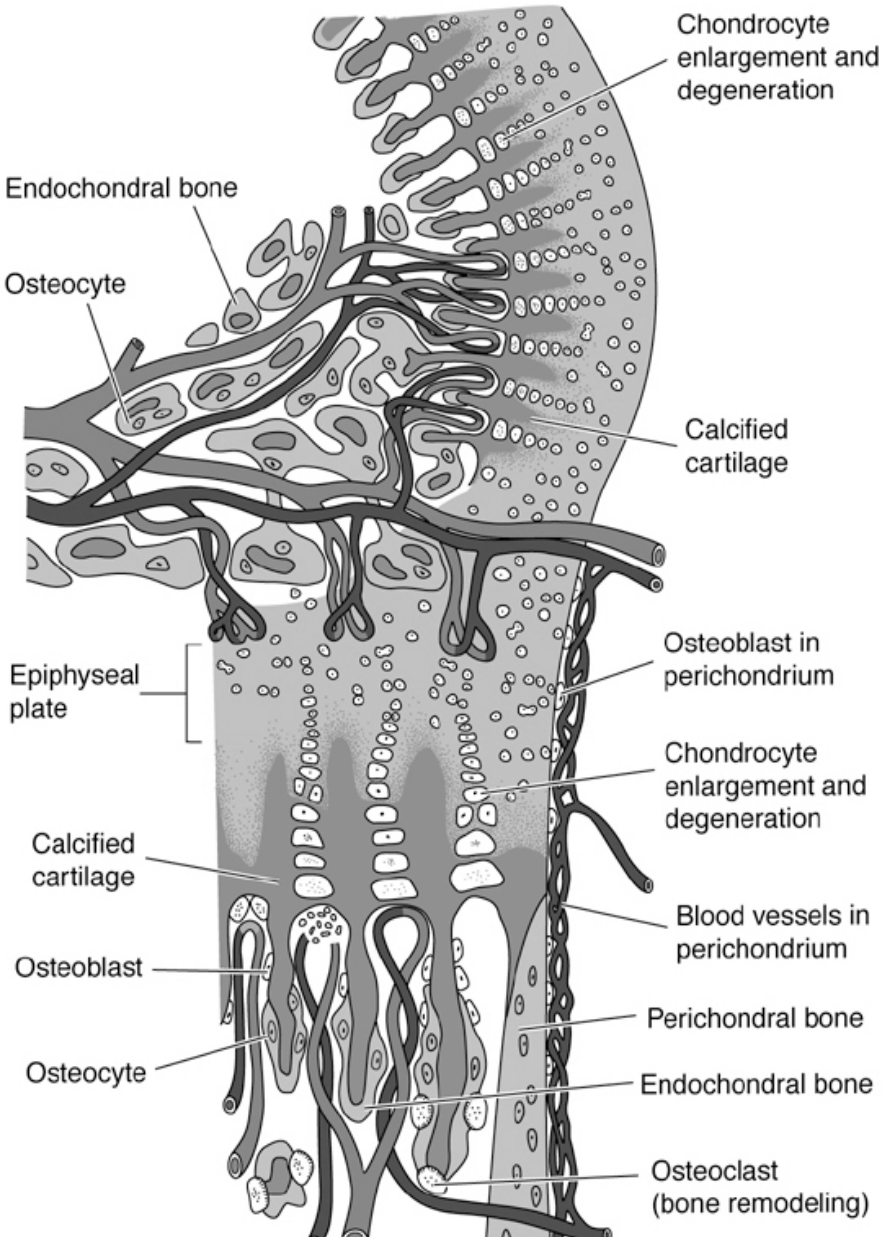



**Figure 1.3** Enlargement of epiphyseal plate and articular cartilage of a long bone. At both sites, chondrocytes undergo a series of morphological changes, which leads to the calcification of cartilage. Osteoclast and osteoblast cells work together to replace the calcified cartilage with bone. Modified from Liem, K., Bemis, W., Walker, W. F. & Grande, L. (2003). *Functional Anatomy of the Vertebrates: An Evolutionary Perspective*. Brooks Cole.

length. The resting zone is nearest the epiphyseal side of the epiphyseal plate and contains stems cells that differentiate into chondrocytes. In the proliferative zone, chondrocytes divide rapidly and produce molecules that build the surrounding ECM. Mature chondrocytes no longer proliferate, but increase in size (9). In the hypertropic zone, mature chondrocytes stop contributing to the ECM and begin to calcify the matrix. Mature chondrocytes in the zone of calcification degenerate and die as a result of apoptosis (3). A calcified matrix remains and is replaced by bone through the action of chondroclasts and osteoblasts, and further remodeling activity (3). Endochondral ossification also occurs in the articular cartilage, or the articular-epiphyseal cartilage (AECC), to increase bone size and achieve the adult form (7,8,10). When skeletal maturation is complete and bones are of adult length, the epiphyseal plate is no longer needed and is ossified. Ossification of the epiphyseal plate connects the epiphysis to the metaphysis and also unites the blood circulatory system (3).

### 1.2 Musculoskeletal diseases

Musculoskeletal diseases are diseases of the muscles, associated ligaments, bones and cartilage. Alterations in normal bone development and maintenance can interfere with skeletal function, leading to musculoskeletal disease and abnormalities. A number of factors impact bone health, such as genetics, hormones, nutrition, and mechanical loading and are particularly influential during skeletal maturation (11). Therefore, creating an optimal environment for the skeletally maturating animal is a strategy for maximizing

bone health and possibly reducing the risk of musculoskeletal diseases. Osteochondrosis and osteoporosis are two musculoskeletal diseases that are particularly debilitating to a number of species and create a financial burden on society.

### *1.2.1 Osteochondrosis*

Osteochondrosis is a developmental orthopedic disease (DOD), a musculoskeletal disease that occurs in the growing animal (12). Osteochondrosis (OC) occurs in a number of species including humans, but mostly commonly affects domesticated animals, such as pigs, lambs, cattle, horses, and large dogs (7,13-16). These rapidly growing animals display clinical signs of osteochondrosis in adolescence with the disease becoming more detrimental as the animal ages. Clinical signs include leg weakness syndrome, or abnormal ambulation, joint stiffness, and degrees of lameness are usually observed with osteochondrosis, while limb deformities are possible but less frequent (17).

Osteochondrosis is a focal disturbance in endochondral ossification of long bones at either the epiphyseal plate or the immature articular-epiphyseal cartilage (18). An area of growth cartilage fails to differentiate and calcify, delaying endochondral ossification, a hallmark of OC. As a consequence, cartilage accumulates at the expense of bone and the deepest layers of cartilage are sequestered from nutrients (19). Cartilage relies on the diffusion

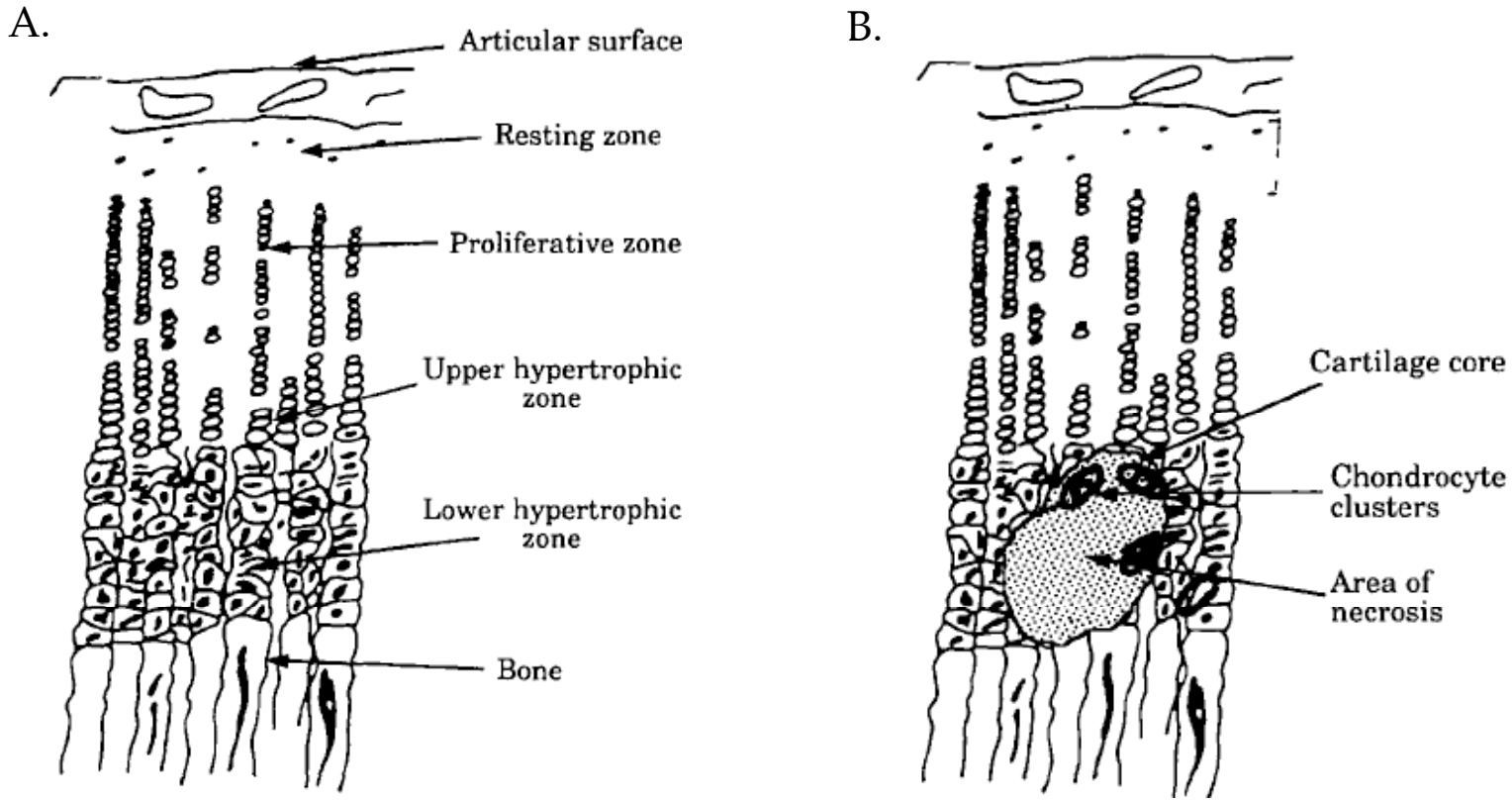



**Figure 1.4** (A) Zonal nature of chondrocytes in the growth cartilage at the articular-epiphyseal complex. (B) Representation of an osteochondrotic lesion formed in the growth cartilage. Modified from Bonjour et al., 2009.

of nutrients from synovial fluid in the joint cavity because blood vessels are absent from the uncalcified cartilage matrix (12). The thickened cartilage reduces cartilage nutrition and creates localized ischemic, which lead to the necrosis of the cartilage. Endochondral ossification fails when the ossification front reaches the necrotic area and remodeling activity is unable to convert uncalcified cartilage to bone (Fig. 1.4) (10).

A lesion in immature cartilage is a clinical sign of OC and is formed from necrotic cartilage and debris (10). Lesions at the articular-epiphyseal cartilage disrupt normal joint movement while those at the epiphyseal plate disrupt longitudinal growth and may alter the entire shape of the bone. Lesions weaken the bone area leading to fissures in the cartilage (19). At the articular surface, cartilage that forms the lesion can fragment allowing debris to enter into the joint space, creating painful swollen joints. Furthermore, mechanical loading can exacerbate the size of fissures, creating cartilage flaps, perpetuating joint inflammation (19). The cartilage flaps may completely separate from the epiphysis and form loose bodies, or joint mice, in the joint cavity, creating a painful peddle-in-shoe effect (10).

The prevalence of osteochondrosis in a variety of domesticated animals has become an animal welfare concern. Furthermore, this disease is responsible for major economic losses in both the equine and livestock industry due to the culling of breeding animals and carcass condemnations of affected animals (16,17). Therefore, OC prevention is essential to human and animal well-being and it is necessary to mitigate economic losses in food animal production and

equine industries. However, the etiology of osteochondrosis is multifactorial and includes rapid growth rate, anatomical characteristics, genetics, nutrition and weak cartilage and bone structure (13). In food animal production, as well as in the equine industry, animals are selected and bred for rapid growth to maximize economic efficiency and production. Rapid weight gain in young growing animals is an additional challenge for the maturing skeleton and may increase the risk of osteochondrosis (13). For example, an increase in growth rate in dogs coincides with skeletal diseases, like osteochondrosis (20). Larger dogs were shown to have a less dense epiphyseal bone below the articular cartilage. The lower density of bone in large dog suggests that the skeleton is less strong and may increase the risk for osteochondrosis (20). Therefore, strategies to optimize bone characteristics, such as mass and strength, in the developing animal may aid in the prevention of osteochondrosis.

### *1.2.2 Osteoporosis*

Osteoporosis is a musculoskeletal disease that is characterized by compromised bone strength, which leads to an increase risk in fracture (21). More than 10 million people in the United States suffer from this disease, resulting in approximately 300,000 hip fractures, 700,000 spinal fractures, 250,000 wrist fractures, and 300,000 fractures at other sites annually (22). Osteoporosis is prevalent among the elderly population and is the result of the overall weakening of the skeleton with age. Bone loss is a normal part of the remodeling cycle, however it plays a more dominant role in the aging individual. The shift in the remodeling cycle to favor bone loss along with a

number of factors, such as genetic, hormonal, gender, and nutritional contribute to the onset of osteoporosis (21).

Osteoporosis also affects a number of domesticated animals, such as cattle, pigs, and poultry (17,23,24). Breeding production animals are most susceptible to the disease during lactation when calcium is in high demand for milk production and is mobilized from bones. Sows in late gestation and during lactation have particularly weak bones that are vulnerable to fractures (17). Furthermore, sows have little time to recover skeletal mass between breeding cycles which progressively deteriorates the skeleton, causing a considerable numbers of sows to be culled due to fractures and lameness (25). In addition, osteoporotic fractures are a major cause of mortality in high-production poultry flocks (24). A progressive loss of structural bone in laying hens is due to shift in the remodeling cycle, but exacerbated by the relative inactivity of-caged bird. Such osteoporotic fractures are costly to the swine and poultry industry and are an animal welfare concern.

In both humans and animals, osteoporosis is a common musculoskeletal disease that is debilitating to the affected individual and creates a financial burden on the health care system and food animal production industries. Fortunately, research has suggested that this disease is largely preventable by optimizing bone strength and mass early in life and maintaining it throughout the lifespan of the organism (26). Growth in bone size and strength occurs early in life, but the accumulation of bone mass is not complete until the third decade of life in humans and around eighteen months in pigs (25,26).

Currently, there is no accurate noninvasive measurement for overall bone strength so bone mineral density (BMD) is frequently used as a proxy measure, and accounts for approximately 70 percent of bone strength (26). Individuals with high BMD, suggesting high bone mass and strength, after adolescence have the greatest protective advantage when the inexorable decline in bone density associated with increasing age, illness, and diminished sex-steroid production take their toll (11,27). Bone strength and mass attained early are perhaps the most important determinant of life-long skeletal health (26).

### 1.3 Potential Dietary Supplements to Improve Whole Bone Strength

A number of factors influence bone strength in children and young animals such as, genetics, mechanical loading, hormones, and nutrition (Fig. 1.5) (11). Nutrition status directly affects bone health and is essential for proper skeletal development and maintenance (26). Because nutrition is a modifiable factor that affects bone, it is one approach for increasing bone strength and mass early in life. Our research investigated three potential dietary supplements for increasing bone strength and mass in young animals as a means to decrease the risk of musculoskeletal diseases, like osteochondrosis and osteoporosis.. The dietary supplements investigated were phytase, inulin, and strontium, which have all been shown to have potential benefits in improving bone integrity and strength when supplemented in the diet in a variety of species (26,28,29).

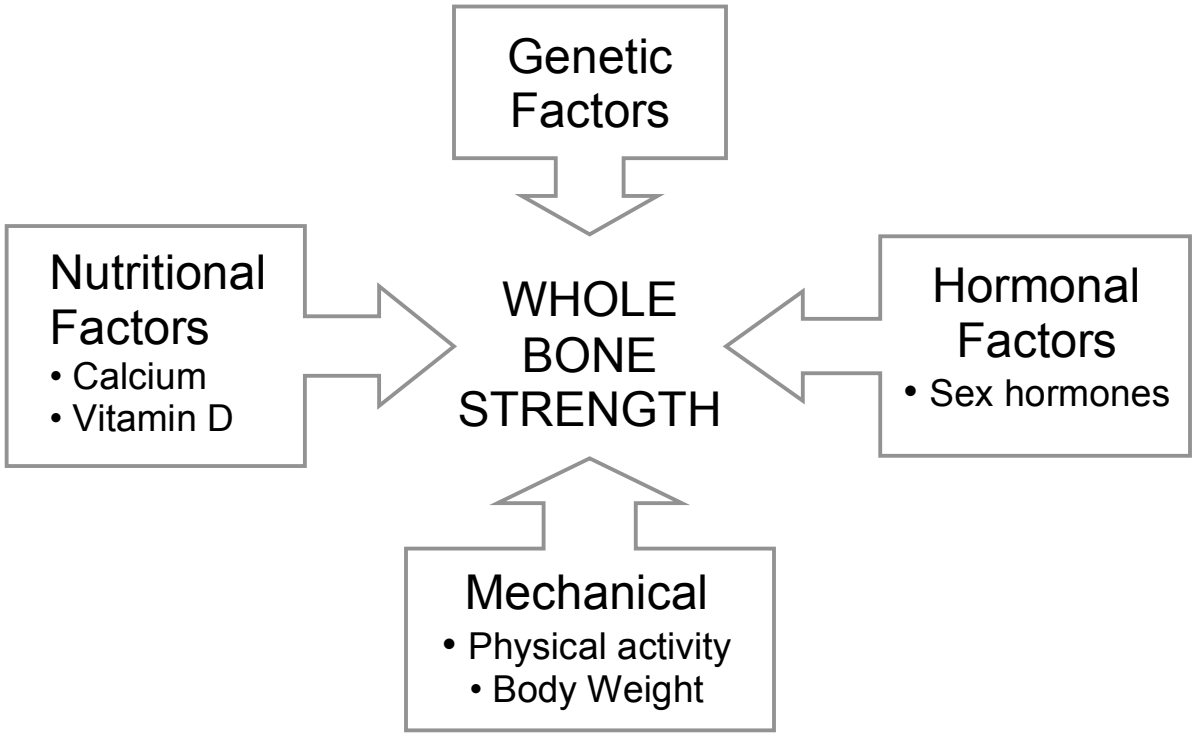



**Figure 1.5** Factors that influence whole bone strength. Modified from Bonjour et al., 2009.

### *1.3.1 Phytase*

Phytic acid, or *myo*-inositol hexa*kis*phosphate, is an essential compound for phosphorus and mineral storage in plants (30,31). Cereal grains, such as wheat, corn, rice and soybean meal, and legume plants, particularly grasses, are major sources of phytic acid (31). Phytic acid contains negatively charged phosphate groups, which spontaneously bind to multivalent cations, particularly elements like calcium, iron, and zinc (30,32). As a mixed cation salt, the phytic acid compound is referred to as phytate, or more specifically phytin when bound to calcium and magnesium ions (31,33). Phytate is degraded by phytase, a phosphohydrolase present in plants and bacteria. Phytase catalyzes the removal of orthophosphates and minerals from the phytate (Figure 1.6) (34).

#### *1.3.1.1 Phytase in Agriculture*

Livestock feed is primarily composed of corn and soybean-meal and as a result 60 to 75 percent of the phosphorus is organically bound in the form of phytate (30). Ruminants possess bacteria in their gastrointestinal tract that produce sufficient quantities of phytase to utilize the phosphorus and mineral contained in phytate (35). Other livestock animals, such as pigs, poultry and fish, do not possess phytase in sufficient amounts and are unable to break down phytate in the plant-based diet (36). In nature, simple-stomached animals are able to obtain phosphorus from primary sources that contain

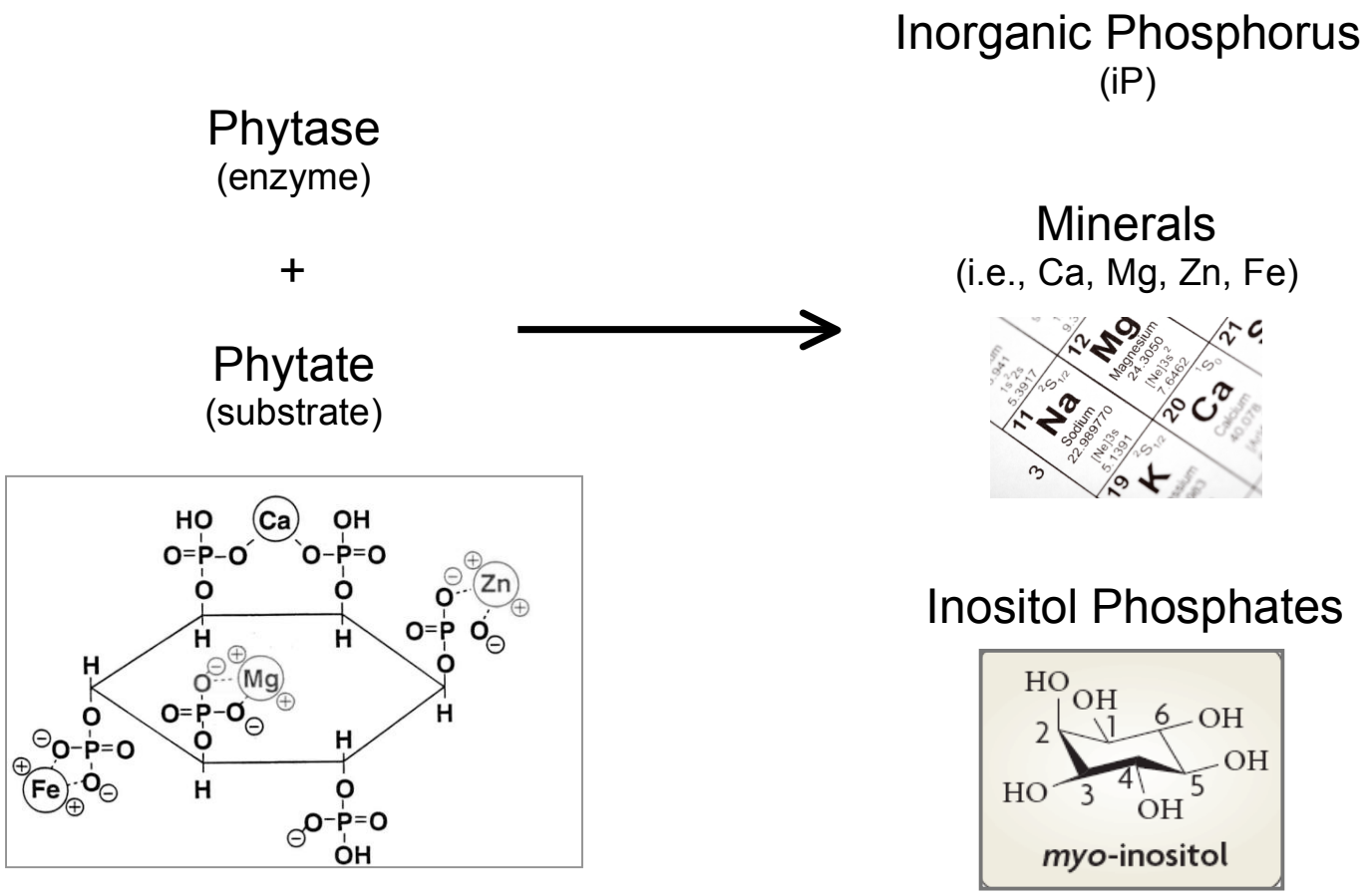



**Figure 1.6** Degradation of phytate by phytase. Phytase catabolizes phytate yielding inorganic phosphate, mineral cations, and inositol phosphates.

endogenous phytase, however plant-based feeds destined for agricultural use are highly heat-treated during processing and plant phytases may be inactivated. Thus, the plant-based feed used in agricultural practices contains phosphorus in phytate form with limited endogenous phytase, leading to substantial phosphorus and minerals being unavailable to the monogastric animals.

*1.3.1.2 Nutritional and Ecological Effects*

The inability of monogastric organisms to utilize the naturally occurring phytate in feed is of agricultural and ecological concern. First, the diets of monogastic animals must be supplemented with a non-phytate form of phosphorus, like inorganic phosphorus (iP), in order for the animal to receive sufficient levels of phosphorus and achieve proper growth (34,35,37). Inorganic phosphorus is expensive and a limitied resource (35). Secondly, the feces of monogastic animals contain large amounts of non-digested phytate and unabsorbed phosphorus. The unabsorped phosphorus is due to the presence of a phytase-producing microbes in the colon of monogastric animals. However, P and cations liberated from phytae by microbal phytases are poorly absorbed in the colon and the majority is excreted. Large amounts of phosphorus are released into the environment and can lead to the damaging natural ecosystems such as eutrophication in aquatic habitats (32,35). Therefore, interest in supplementing the diets of monogastric livestock with phytases engineered to have high activity early in the gastrointestinal tract as a potential means for increasing essential mineral bioavailability in

livestock diet and eliminating phosphorus waste in the environment is of great interest.

#### *1.3.1.3 Genetically Modified Phytase*

Phytases investigated in animal feeding trials are of microbial origin and are genetically modified to have enhanced thermostability and kinetic activity at pH 2.5 - 3.5 - the pH of the stomach (38). Optimal phytase activity in the stomach allows phytate to be degraded early in the intestinal tract. As a consequence, P and minerals cleaved from phytate pass through the entire gastrointestinal tract, contacting the intestinal epithelium more frequently, which increases opportunities for absorption.

#### *1.3.1.4 Supplementation*

Phytase research has primarily focused on determining its optimal use as a supplement in livestock feed. Animal feeding trials with phytase supplementation examine skeletal characteristics as evidence of phytase efficiency to increase phosphorus availability. Approximately 85% of phosphorus in the body is retained in the skeleton and skeletal characteristics and biomechanics are sensitive indicators of P status. Animals on a restricted P diet display symptoms of hypophosphatemia, such as rickets or osteomalacia (39,40). In swine, broiler chicks and rats, microbial phytase supplementation in the diet has been shown to improve bone properties, such

as breaking strength, percent ash analysis (a measure of bone mineralization), and bone geometry in animals fed a phosphorus or mineral deficient diet (41-47). Phytase (*Escherichia coli*) supplementation at 1,000U/kg is able to replace approximately 0.20% inorganic phosphorus in the diet and rescue the bones of pigs receiving inadequate levels of inorganic phosphorus (48). In some situations, this would eliminate the need for the addition of iP in pig and poultry nutrition (48). Furthermore, in several feeding trials, improvements in bone characteristics and biomechanics were greater than those of the nutrient adequate control. (44-46,49,50). Phytase supplementation in swine fed a P-adequate diet demonstrated an increase in bone mineral content in femur and metacarpal bones by 14-15% as well as a 9-11% increase in bone mineral density (29). These results suggest that in addition to an increase in P and mineral bioavailability, supplemental phytase may provide additional benefits to skeletal tissue, such as increase in bone strength and bone mass, in young animals. Therefore, dietary phytase supplementation showed potential to improve bone integrity and may be a strategy to reduce the risk of acquiring musculoskeletal disease that manifest in animals with compromised skeletons.

### *1.3.2 Strontium*

Early research with stable strontium isotopes ($Sr^{2+}$) focused on evaluating its toxicity in animals. These studies demonstrated a high tolerance in animals for strontium and symptoms of strontium toxicity, such as strontium rickets, where usually observed only when calcium was insufficient (51,52). For example, swine can tolerate strontium supplemented at 0.2 percent in diet

even though usual strontium consumption is approximately 0.007 percent for swine in a normal plant-based diet (51). These results rectified the initial confusion between stable strontium and radioactive strontium. Biological interest in strontium rose, especially when its ability to replace calcium in vivo was observed. Strontium and calcium are earth minerals and divalent cations (51,53). Their similar origin and chemistry may be allow them to share the same mechanism for absorption from the gastrointestinal tract and accumulating in bone (51). Strontium has a heterogeneous distribution in skeletal tissue. Bone turnover is greater in cancellous bone and thus cancellous bone accumulates more Sr compared to cortical bone (53).

Strontium has a high affinity for bone and is incorporated into it by two mechanisms: surface exchange and ionic substitution (53). Surface exchange is the incorporation or absorption of Sr at the surface of the mature hydroxyapatite crystal. Alternatively, ionic substitution involves the replacement of calcium with strontium during the formation of the crystal (one strontium atom out of a 10 calcium can be substituted by strontium) (53). However, as the animal matures mechanisms develop which discriminate against strontium substitution for calcium (51). Studies with rats, monkeys, and humans have shown that strontium reaches a plateau in bone during oral administration with strontium ranelate or strontium chloride. In addition, once strontium treatment is stopped, it is rapidly eliminated form the bone suggesting that the mineral is only superficially incorporated into the bone (53).

*1.3.2.1 Beneficial Effects on Bone*

Strontium supplementation not only makes strontium more available for its physical incorporation into skeletal tissue but also seems to impact bone remodeling cells in a manner that favors bone formation. Particularly, strontium ranelate has shown its ability to stimulate osteoblasts, but decrease osteoclasts differentiation and activity, thereby altering bone remolding to favor bone formation (26,54,55). In non-human primates, strontium supplementation inhibited bone resorption as indicated by a decrease in osteoclast number, but not bone formation (56). In rats, strontium supplementation increased bone mass, as measured by an increase in trabecula number and thickness, as well as an increase in cortical density (26).

The mechanism of strontium on bone cells is still unclear, but it is thought to act through calcium-sensing receptors (CsR) on bone cells to stimulate differentiation or apoptosis (57). Overall, strontium supplementation increases strontium atoms on the bone surface matrix and stimulates bone cells to favor bone formation (55). These interactions lead to positive changes in microarchitecture and function in skeletal tissue, such as increased bone mass and breaking strength.

We investigated dietary strontium supplementation in combination with phytase because previous studies showed an increase in strontium concentrations in the femur and metatarsal of young pigs fed supplemental phytase (29). Therefore, we were interested in if the increase in strontium concentration in bones by phytase was responsible for improvements in bone

paramaters and if additional strontium in the presence of phytase can further increase strontium accumulation in bones, and thus increase bone strength.

### *1.3.3 Inulin*

Inulin, a linear fructan with a terminal alpha-D-glucose (β2-1 linkage), is present in plants, such as wheat and chicory root (28). Inulin is not digestible by human gastrointestinal enzymes and is instead fermented by bacteria in the colon (58). Studies with dietary inulin supplementation have shown the supplement to possess prebiotic properties as it increases beneficial bacteria populations, such as *Lactobacillus* and *Bifidobacterium*, in the gastrointestinal tract in animals (59). In addition, supplemental inulin has been shown to increase mineral absorption in the gastrointestinal tract (58). Colonic fermentation of inulin yields short chain fatty acids, such as acetate and butyrate, which lowers the pH of the large intestinal lumen. The decrease in pH seems to increase solubility of minerals, such as calcium, magnesium and zinc, which allows for their passive absorption by gut mucosal cells as well as diffusion paracellularly (60). The enhanced mineral absorption due to dietary inulun may also be due to changes in cecum morphology. By-products from bacterial fermentation of inulin, particularly butyrate, have been noted to alter cecum morphology, particularly an increase in mass and villus height (28). These changes in the intestinal epithelium most likely increase absorptive surfaces and thus enhance mineral absorption. Furthermore, increases in calcium binding proteins have been observed with dietary inulin supplementation and may better facilitate active calcium transport (61).

Inulin supplementation seems to improve iron bioavailability in a dose dependent manner when added to a corn and soybean meal diet fed to young pigs (62). The mechanism responsible for the increase in iron with inulin is unclear. It was hypothesized that inulin might reduce or remove iron chelators, such as phytate, in the diet but a reduction in phytase activity was not observed. Dietary inulin might trigger an up regulation of genes encoding for Fe transporters in the enterocyte (59).

#### *1.3.3.1 Beneficial Effects on Bone*

Supplemental inulin increases mineral bioavailability and absorption due to its prebiotic properties in the gastrointestinal tract (61). Alterations in gut microbial populations leads to changes in lumen pH, cecum morphology and mineral transport proteins, which ultimately allow an organism to absorb and utilize minerals previously unavailable (28,58,60,61). In addition to the increase in mineral absorption, feeding trials with supplemental inulin in humans and animals have also shown beneficial effects on skeletal tissue (28,58,63). In rats fed inulin-type fructans, bone mineralization was improved as well as tibia breaking strength at the mid-diaphysis (28). Furthermore, dietary inulin increased whole BMC and BMD and Ca absorption in human adolescents (63,64). These studies are particularly supportive of dietary inulin's potential to increase bone integrity. Therefore, we investigated inulin supplementation with phytase to determine if the addition of inulin could further improve bone strength and mass beyond that of phytase supplementation alone.

## 1.4 Research Objectives

Our objective was to investigate whether phytase supplementation in a high phosphorus diet can increase bone strength and mass in young pigs and mice. We hypothesize that the non-phosphorus by-products, minerals and inositol derivatives, from the enzymatic reaction of phytase with phytate are responsible for possible improvements in bone parameters examined in young animals. Furthermore, we examined two other known bone-impacting supplements, strontium and inulin, to determine if either had synergistic activity on bone properties when supplemented in the diet with phytase. In mice, we examined if phytase could improve bone properties when phytate, the intrinsic substrate of phytase, was absent from the diet. This experiment was designed to demonstrate if the phytase enzyme alone is able to improve bone properties.

Overall, these experiments will better elucidate whether these dietary supplements (phytase, strontium, and inulin) can positively impact skeletal tissue, particularly by increasing bone strength and mass, when supplemented in the diet of young animals. The mouse experiment will also provide insight into the mechanism of phytase in improving bone characteristics. These studies will lay groundwork for future nutritional experiments that explore strategies to optimize bone health early in life as a means to reduce the risk of musculoskeletal diseases.

# CHAPTER TWO
# THE IMPACT OF DIETARY PHYTASE AND INULIN SUPPLEMENTATION ON BONE BIOMECHANICS IN YOUNG PIGS

## 2.1 Abstract

Increasing bone strength and mass early in life may result in a skeleton that is less susceptible to diseases. We investigated improving bone health via nutritional means and examined two dietary supplements; phytase and inulin. Previous studies have demonstrated potential benefits of phytase and inulin supplementation on skeletal tissue in humans, rats, and pigs. We conducted an experiment to compare the effects of microbial phytase (3,500U/kg, OptiPhos, JBS United, Sheridan, IN) and inulin (4%, Synergy Orafti, Tienen Belgium) supplemented alone or together on bone characteristics in young pigs. Thirty-five pigs (body weight: 8.5 ± 1.6 kg) were fed a corn-soybean-meal basal diet with 0.35% inorganic phosphorus (BD), BD + phytase, BD + inulin, or BD + phytase + inulin. At the end of 6 weeks, five pigs were selected from each diet group and one femur was analyzed from each animal using the three-point bending test. Supplemental phytase increased bending rigidity ($p < 0.05$), the moment of inertia medial-lateral, $I_{ml}$ ($p = 0.08$), and the moment of inertia anterior-posterior, $I_{ap}$ ($p = 0.06$). Inulin supplementation altered ($p < 0.05$) the ellipticity index of femurs and enhanced ($p = 0.06$) femoral height. Femoral mineral analysis showed that phytase supplementation increased Mg ($p < 0.05$), Sr ($p < 0.001$), and Zn ($p < 0.001$) concentrations and inulin supplementation increased ($p < 0.05$) Ca, K, Mg, Na, P, and Sr concentrations in cortical bone. In cancellous bone, phytase supplementation enhanced ($p < 0.05$) S, Sr, and Zn concentration and inulin supplementation increased ($p <$

0.05) Cr concentration. Based on our analysis, phytase supplementation impacted bending rigidity and both supplements had an effect on femoral mineral composition when supplemented in the diet alone. It may be important to extend the length of this study beyond 6 weeks to better determine if these supplements can influence bone properties in older animals.

**2.2 Introduction**

In this study, we examined bone strength in young pigs fed supplemental phytase, and acid phosphatase, and inulin, a carbohydrate. These supplements have shown potential for improving bone health in a variety of species (28,29,48,58). We conducted this feeding trial to better understand the extent and nature of these skeletal improvements over a course of 6 weeks. A high phosphorus (P) diet was used to eliminate any possible beneficial effects on skeletal tissue from the increase in phosphorus from the degradation phytate by phytase. Therefore, improvements in bone parameters examined would most likely be due to phytase or the non-phosphorus by-products (minerals and inositol phosphates) of the enzymatic reaction. In addition to breaking strength, bone geometry and material properties were examined to better understand how phytase and inulin might be influencing bone biomechanics. Overall, this study of supplemental phytase and inulin will better guide the use of these dietary supplements and define their role for combating musculoskeletal disease.

## 2.3 Methods

### *2.3.1 Experimental diet*

The four experimental diets were composed from a corn-soybean-meal basal diet (BD) containing 0.35% inorganic phosphorus (P) concentration. The microbial phytase used was *Escherichia coli* AppA2 (OptiPhos, JBS United, Sheridan, IN) at 3500 U/kg After the phytase activity was analyzed, the phytase enzyme was added to the diets at feed mixing (65). Phytase activity in the experimental diets was further verified by laboratory analysis. Inulin (Synergy, Orafti, Tienen Belgium) was supplemented at 4% of the diet.

### *2.3.2 Experimental animals and design*

Thirty-five mixed gender weanling pigs (Yorkshire x Hampshire x Laundrace, 8.5 ± 1.0 kg of body weight) from the Cornell University Swine Farm were weaned at 4 weeks of age and assigned to one of four dietary treatment groups based on body weight, litter and sex. This study was designed as a 2 x 2 factorial arrangement of diet treatments (2 levels of phytase, 0 and 3500 U/kg) and 2 levels of inulin (0 and 4%) in a high phosphorus diet (0.35% inorganic phosphorus, iP) and was conducted for 6 weeks. The treatments groups were BD, BD + phytase, BD + inulin, and BD + phytase + inulin. Pigs were penned in an environmentally controlled barn (20-25$^{o}$C; 12 h light: 12 h dark cycle) and were fed ad libitum. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Cornell University.

### *2.3.3 Growth performance and sample collection*

The body weights of individual pigs were measured initially (wk 0) and then biweekly. Blood samples of individual pigs were collected initially and biweekly from the cranial vena cava into heparinized tubes after an overnight fast (8 h). The collected whole blood samples were chilled on ice and centrifuged at 3000 x g for 10 min at 4°C (GS-6KR centrifuge, Beckman Instruments). Plasma samples were stored at -20°C until inorganic phosphorus concentration (PIP) and alkaline phosphatase (AKP) activity assays were performed. At the end of the experiment, five pigs were selected from each group for biomechanical and bone composition analysis and were killed by electrical stunning and exsanguinations. The right rear limb of each animal was amputated and stored at -20°C until the femur could be isolated for the 3-point mechanical bending test.

### *2.3.4 Plasma biochemical assays*

Plasma samples were thawed and then deproteinated with 12.5% tricholoacetic acid. Plasma samples were assayed for inorganic P concentrations using Elon (p-methylaminophenol sulfate) solution (66). The hydrolysis of *p*-nitrophenol phosphate to *p*-nitrophenol was used to measure plasma alkaline phosphatase activity (67). The enzyme unit was defined as 1μmol of *p*-nitrophenol released per minute at 30°C.

*2.3.5 Mineral composition analysis*

After biomechanical testing, cortical and cancellous samples were collected from the femur for mineral analysis. A bone saw was used to collect a cortical sample at the mid diaphysis. A stainless steel scalpel was used to further isolate the cortical bone from any remaining tissue. Cancellous bone was collected at the ephiphysis using a stainless trephine (Michele Trephine 6 1/4" shaft, 5/16" O.D., graduated 1/4 to 1 1/4") and chisel. Specimens were defatted in a chloroform-methanol solution (2:1) for three days with a change in solution every 24 h. The samples were air-dried for 24 h at room temperature and then for 24 h at 105ºC to obtain a dry weight. Ash weight was determined by ashing bone samples in a box furnace (Lindberg Moldatherm 1100°C box furnace; model BF51748A) for 72 h at 600ºC. Femoral percent ash was calculated as 100 x (ash weight/dry weight).

To detect ash mineral composition, ash samples (0.1 g) were digested in 1 ml of concentrated nitric and perchloric acid (1:1, v:v) and the mixture was heated at 220ºC until dry. The ash was dissolved in 20 ml of 5% nitric acid and mineral composition was determined using an inductively coupled argon plasma spectrophotometer (ICAP 61E Trace Analyzer, Thermo Jarell Ash Corporation). Standard reference materials (1573a, tomato leaves, and 1577b, bovine liver; National Institute of Standards and Technology) were used to validate the analytical procedures (68). The transfer optics had been modified using a short depth of field transfer optics to reduce matrix effects (Ruzke MA, 2002; US Patent, 2002). The same procedure was used to analyze the mineral composition of diet and plasma. For diet, 0.5 g were dissolved in 30.25 ml of

5% nitric acid while 0.5 ml of plasma was dissolved in 10.25 ml of 5% nitric acid. Mineral concentration values were an average of three replicates.

### *2.3.6 Femur geometry and whole bone strength*

The right femur was prepared for mechanical testing by removing all surrounding skin, muscle, and soft tissue. The femur was wrapped in gauze soaked in normal saline to maintain the normal physiological environment. Bones were weighed wet and length was measured. Femurs were allowed to thaw at room temperature prior to mechanical testing (model 4500; Instron Corp, Canton, MA). All specimens were loaded in the anterior-posterior direction at a crosshead speed of 50 mm/min and the sample rate was 10 points/s. Specimen supports were 50 mm apart. A load-deformation curve was recorded for each sample for data analysis. Post-failure femurs were cut with a bone saw at the load site to expose a cross section. The medial-lateral axis, anterior-posterior axis, and cortical thickness at the medial and lateral position were measured with digital calipers and used to calculate the moment of inertia. Mechanical properties of bones were calculated using the following formulas (Fig. 2.1):

Moment (M) = PL/4

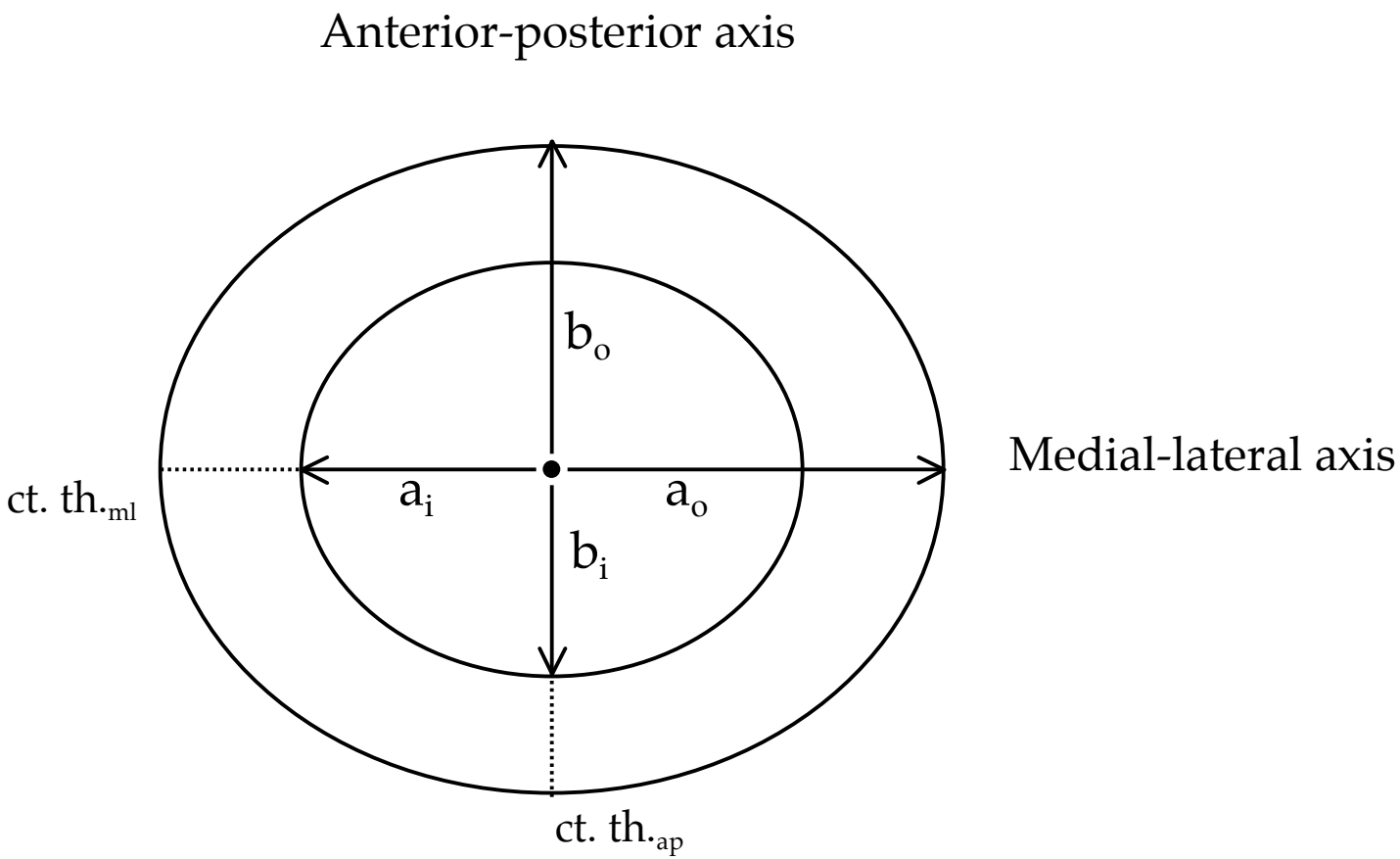
Bending stiffness (EI) = $mL^3/48$

Moment of inertia approximated using the hollow ellipse formula:

$I_{ml} = \pi/4\ (b_o a_o^3 - b_i a_i^3)$

$I_{ap} = \pi/4\ (a_o b_0^3 - a_i b_i^3)$

**Figure 2.1** Schematic illustration of cross section of bone through the diaphysis. Letters indicate where measurements were taken. Cortical thickness medial-lateral (ct. $th._{ml}$), cortical thickness anterior-posterior (ct. $th._{ap}$), outer radius medial-lateral ($a_o$), inner radius medial-lateral ($a_i$), outer radius anterior-posterior ($b_o$), and inner radius medial-lateral ($b_i$).


Anterior-posterior axis
$b_o$
Medial-lateral axis
ct. $th._{ml}$
$a_i$
$a_o$
$b_i$
ct. $th._{ap}$

P = maximal load at failure

L = span

m = slope of the load-displacement curve

$I_{ml}$ = moment of inertia of the medial-lateral axis

$I_{ap}$ = moment of inertia of the anterior-posterior axis

$a_o$ = outer radius of the medial-lateral axis

$a_o = a/2$

a = width (diameter) of the medial-lateral axis

$a_i$ = interior radius of the medial-lateral axis

$a_i = (a_o/2)$ - ct. th.

ct. th. = cortical thickness (average of medial and lateral)

$b_o$ = outer radius of the anterior-posterior axis

$b_o = b/2$

b = height (diameter) of the anterior-posterior axis

$b_i$ = interior radius of the anterior-posterior axis

$b_i = (a_o/b_o)a_i$

### 2.3.7 *Statistical analysis*

Data were analyzed using SigmaPlot 9.0 (2004 SYSTAT Software Inc.). Main effects of dietary phytase and inulin supplementation were analyzed using two-way ANOVA as a 2 x 2 factorial arrangement of diet treatments with significance level set at $p \leq 0.05$. Holm-Sidak method was used for post-hoc analysis with overall significance level equal to 0.05.

## 2.4 Results

### *2.4.1 Growth performance and plasma biochemical assays*

Body weight, average daily gain, and gain: feed ratio were not affected by phytase or inulin supplementation (Table 2.1). At 6 weeks, dietary phytase supplementation decreased ($p = 0.05$) plasma inorganic phosphorus concentration in pigs (Table 2.2). There was no observed effect of treatments on plasma alkaline phosphatase activity.

### *2.4.2 Plasma mineral analysis*

Phytase supplementation had a marginal effect ($p = 0.09$) on plasma Fe concentration and increased plasma Fe concentration approximately 58% in pigs fed BD + phytase compared to pigs fed BD at 5 weeks (Table 2.3 and 2.4). Inulin supplementation had a marginal increase ($p = 0.05$) on plasma S concentration. The BD + phytase + inulin diet had a significant impact ($p < 0.05$) on Ca, Mg, S, and Sr and had a marginal impact Na ($p = 0.06$), P ($p = 0.08$), and K ($p = 0.05$). For the most part, the individual supplements increased plasma mineral concentration; however the combined supplement diet returned plasma minerals concentration values to approximately those of the control, or BD.

**Table 2.1** Growth performance of swine during the 6-week feeding trial[1]

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | Main effect, p-value | | |
| Inulin, % | 0 | 0 | 4 | 4 | SEM | Phytase | Inulin | Phy x Inu[2] |
| Body Weight, kg | | | | | | | | |
| Wk 0 | 8.3 | 8.4 | 8.7 | 8.4 | 1.0 | 0.8 | 0.8 | 0.7 |
| Wk 5 | 30.9 | 31.1 | 30.2 | 29.6 | 2.2 | 0.9 | 0.3 | 0.8 |
| Average Daily Gain, kg | | | | | | | | |
| Wk 0 | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.6 | 0.6 |
| Wk 5 | 0.8 | 0.8 | 0.7 | 0.7 | 0.5 | 0.6 | 0.09 | 0.6 |
| Gain: Feed | | | | | | | | |
| Wk 0 | 0.6 | 0.9 | 0.7 | 0.8 | 0.09 | 0.2 | 0.8 | 0.4 |
| Wk 5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.07 | 0.9 | 0.9 | 0.8 |

[1] Values are means, n = 5
[2] Phy, phytase; Inu, inulin

**Table 2.2** Plasma inorganic phosphorus (PIP) and alkaline phosphatase activity (AKP) of swine at wk 0 and wk 6 of the feeding trial[1]

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | Main effect, p-value | | |
| Inulin, % | 0 | 0 | 4 | 4 | SEM | Phytase | Inulin | Phy x Inu[3] |
| Plasma Inorganic Phosphorus, mg/dl[3] | | | | | | | | |
| Wk 0 | 8.0 | 7.8 | 7.6 | 7.4 | 0.8 | 0.6 | 0.3 | 1.0 |
| Wk 5 | 11.6 | 9.01 | 10.4 | 9.7 | 1.3 | 0.05 | 0.8 | 0.3 |
| Alkaline Phosphatase Activity, U/ml | | | | | | | | |
| Wk 0 | 90.1 | 86.3 | 110.8 | 95.9 | 22.2 | 0.4 | 0.2 | 0.6 |
| Wk 5 | 80.8 | 91.6 | 89.2 | 83.3 | 21.7 | 0.8 | 1.0 | 0.5 |

[1] Values are means, n = 5
[2] Phy, phytase; Inu, inulin
[3] To convert mg/dl to mmol/L multiply by 0.323.

**Table 2.3** Plasma macromineral concentration[3] (mg/L) during the feeding trial[1]

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | Main effect, p-value | | |
| Inulin, % | 0 | 0 | 4 | 4 | SEM | Phytase | Inulin | Phy x Inu[2] |
| **Na** (0.0435) | | | | | | | | |
| Wk 0 | 1891.8 | 1877.8 | 1869.5 | 1818.7 | 14.4 | 0.3 | 0.2 | 0.5 |
| Wk 5 | 1742.6 | 1875.7 | 2024.9 | 1584.2 | 74.5 | 0.3 | 1.0 | 0.06 |
| **S** (0.0312) | | | | | | | | |
| Wk 0 | 448.1 | 411.6 | 464.7 | 424.2 | 20.1 | 0.4 | 0.7 | 1.0 |
| Wk 5 | 453.2 | 532.6 | 508.1 | 285.4 | 30.4 | 0.1 | 0.05 | 0.004 |
| **K** (39.09)[4] | | | | | | | | |
| Wk 0 | 295.9 | 280.4 | 308.5 | 274.8 | 29.7 | 0.1 | 0.8 | 0.6 |
| Wk 5 | 251.2 | 314.1 | 338.6 | 242.7 | 73.3 | 0.7 | 0.8 | 0.05 |
| **P** (0.0323) | | | | | | | | |
| Wk 0 | 112.8 | 110.6 | 116.3 | 116.2 | 3.2 | 0.9 | 0.5 | 0.9 |
| Wk 5 | 122.2 | 132.9 | 145.8 | 105.3 | 7.1 | 0.3 | 0.9 | 0.08 |
| **Ca** (0.0249) | | | | | | | | |
| Wk 0 | 93.8 | 93.7 | 92.1 | 89.7 | 1.3 | 0.7 | 0.3 | 0.7 |
| Wk 5 | 90.9 | 105.5 | 109.7 | 85.2 | 4.3 | 0.5 | 0.9 | 0.03 |
| **Mg** (0.0412) | | | | | | | | |
| Wk 0 | 18.3 | 18.3 | 17.2 | 17.2 | 0.3 | 1.0 | 0.1 | 1.0 |
| Wk 5 | 15.0 | 17.7 | 18.1 | 14.6 | 0.7 | 0.8 | 1.0 | 0.04 |

[1] Values are means, n = 5
[2] Phy, phytase; Inu, inulin
[3] To convert mg/L to mmol/L multiply values by the factor in parentheses next to each element, except for K.
[4] To convert mg/L to mmol/L (mEq/L) divide values by the factor in parentheses.

**Table 2.4** Plasma micromineral concentration[3] (μg/L) in swine during the feeding trial[1]

| | Treatment | | | | | Main effect, p-value | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | | | |
| Inulin, % | 0 | 0 | 4 | 4 | SEM | Phytase | Inulin | Phy x Inu[2] |
| **Fe** (0.018) | | | | | | | | |
| Wk 0 | 2326.5 | 1514.4 | 1217.2 | 1643.7 | 933.6 | 0.7 | 0.3 | 0.2 |
| Wk 5 | 1958.4 | 3015.6 | 2358.4 | 3467.8 | 1341.4 | 0.1 | 0.5 | 1.0 |
| **Cu** (0.016) | | | | | | | | |
| Wk 0 | 1654.2 | 1679.3 | 1785.4 | 1841.6 | 187.7 | 0.7 | 0.1 | 0.9 |
| Wk 5 | 1487.7 | 1617.7 | 1869.0 | 1438.3 | 345.2 | 0.4 | 0.6 | 0.1 |
| **Zn** (0.015) | | | | | | | | |
| Wk 0 | 533.7 | 518.9 | 511.4 | 483.0 | 14.9 | 0.5 | 0.4 | 0.8 |
| Wk 5 | 944.4 | 1169.9 | 1173.7 | 1040.5 | 60.8 | 0.7 | 0.7 | 0.2 |
| **Sr** (0.011) | | | | | | | | |
| Wk 0 | 31.3 | 29.2 | 26.3 | 26.9 | 1.9 | 0.9 | 0.4 | 0.7 |
| Wk 5 | 30.8 | 42.2 | 44.5 | 38.2 | 2.1 | 0.5 | 0.2 | 0.04 |

[1] Values are means, n = 5
[2] Phy, phytase; Inu, inulin
[3] To convert μg/L to μmol/L multiply values by the factor in parentheses next to each element.

### *2.4.3 Ash fraction and bone mineral analysis*

Dietary supplementation with phytase, inulin, or both did not produce any significant differences in cortical or cancellous ash fraction (Table 2.5). However, inulin supplementation increased ($p = 0.08$) percent ash in cancellous bone.

In cortical bone, phytase supplementation increased ($p < 0.05$) Mg, and Sr and Zn concentrations ($p < 0.001$) (Table 2.6). Inulin supplementation increased ($p < 0.05$) Ca, K, Mg, Na, P, and Sr concentrations. No differences in minerals analyzed were observed in animals fed BD + phytase + inulin in cortical bone. In cancellous bone, phytase supplementation enhanced ($p < 0.05$) S and Sr concentrations and greatly increased ($p < 0.001$) Zn concentration (Table 2.7).

Inulin supplementation increased ($p < 0.05$) Cr concentration in cancellous bone. Femurs from pigs fed BD + phytase + inulin had an increase ($p < 0.05$) in Mg and Cr concentrations as well as an increase ($p = 0.06$) in P concentration in cancellous bone.

### *2.4.4 Femur geometry and whole bone strength*

Dietary phytase supplementation increased ($p < 0.05$) bending rigidity in swine femora (Table 2.8). Additionally, supplemental phytase marginally increased the medial-lateral moment of inertia, $I_{ml}$, ($p = 0.08$) and anterior-posterior moment of inertia, $I_{ap}$ ($p = 0.06$). Inulin supplementation altered ($p < 0.05$) ellipticity index of femurs and marginally enhanced ($p = 0.06$) femoral

**Table 2.5** Femur percent ash at two skeletal sites[1]

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | Main effect, p-value | | |
| Inulin, % | 0 | 0 | 4 | 4 | SEM | Phytase | Inulin | Phy x Inu[2] |
| Cortical | 72.6 | 72.3 | 72.9 | 72.2 | 1.7 | 0.6 | 0.1 | 0.8 |
| Cancellous | 50.5 | 49.2 | 51.0 | 52.2 | 1.9 | 1.0 | 0.08 | 0.2 |

[1] Values are means, n = 5
[2] Phy, phytase; Inu, inulin

**Table 2.6** Mineral concentration of cortical femur of pigs at wk 6[1]

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | Main effect, p-value | | |
| Inulin, % | 0 | 0 | 4 | 4 | SEM | Phytase | Inulin | Phy x Inu[2] |
| Macrominerals[3], mg/kg | | | | | | | | |
| K[4] | 2902 | 2820 | 2911 | 2701 | 242.0 | 0.2 | 0.7 | 0.6 |
| S | 911.1 | 980.5 | 962.4 | 971.0 | 48.3 | 0.1 | 0.4 | 0.2 |
| Ca | 391.0 | 394.3 | 399.8 | 399.8 | 4.4 | 0.5 | 0.005 | 0.5 |
| P | 192.0 | 192.6 | 195.2 | 195.6 | 1.8 | 0.6 | 0.003 | 0.9 |
| Na | 8.7 | 8.9 | 9.0 | 9.1 | 0.2 | 0.1 | 0.008 | 0.3 |
| Mg | 7.3 | 7.7 | 7.7 | 7.8 | 0.2 | 0.04 | 0.02 | 0.3 |
| Microminerals[5], μg/kg | | | | | | | | |
| Zn | 224.7 | 272.8 | 242.7 | 289.3 | 20.8 | <0.001 | 0.1 | 1.0 |
| Sr | 91.7 | 108.4 | 101.8 | 114.8 | 7.0 | <0.001 | 0.03 | 0.6 |
| Fe | 25.9 | 49.6 | 43.9 | 55.5 | 58.1 | 0.6 | 0.7 | 0.8 |
| Cr[6] | 11.7 | 11.9 | 11.8 | 11.6 | 0.4 | 0.9 | 0.6 | 0.4 |

[1] Values are means, n = 5
[2] Phy, phytase; Inu, inulin
[3] To convert mg/kg to mmol/kg see footnote 3 in Table 2.3
[4] To convert mg/kg to mmol/kg see footnote 4 in Table 2.3
[5] To convert μg/kg to μmol/kg see footnote 3 in Table 2.4
[6] To convert μg/kg to μmol/kg multiply by 0.019

**Table 2.7** Mineral concentration in cancellous femur at wk 6[1]

| | Treatment | | | | | Main effect, p-value | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | | | |
| Inulin, % | 0 | 0 | 4 | 4 | SEM | Phytase | Inulin | Phy x Inu[2] |
| Macromineral[3], mg/kg | | | | | | | | |
| K[4] | 4119.1 | 3375.0 | 4624.5 | 5143.8 | 830.5 | 0.8 | 0.02 | 0.1 |
| Ca | 399.3 | 387.8 | 400.4 | 400.4 | 7.7 | 0.2 | 0.1 | 0.2 |
| P | 202.4 | 197.4 | 200.4 | 202.3 | 3.4 | 0.4 | 0.4 | 0.06 |
| Na | 13.2 | 12.8 | 13.5 | 12.1 | 1.0 | 0.09 | 0.7 | 0.3 |
| Mg | 8.0 | 7.8 | 8.0 | 8.2 | 0.2 | 0.8 | 0.1 | 0.03 |
| S | 3.0 | 2.7 | 3.3 | 2.8 | 0.3 | 0.03 | 0.2 | 0.4 |
| Microminerals[5], μg/kg | | | | | | | | |
| Fe | 354.0 | 368.3 | 391.7 | 377.6 | 62.9 | 1.0 | 0.5 | 0.7 |
| Zn | 299.0 | 371.5 | 302.6 | 382.6 | 23.9 | <0.001 | 0.5 | 0.8 |
| Sr | 98.3 | 108.4 | 98.5 | 115.3 | 7.5 | 0.002 | 0.3 | 0.4 |
| Cr[6] | 11.8 | 10.9 | 11.9 | 12.5 | 0.6 | 0.6 | 0.02 | 0.04 |

[1] Values are means, n = 5
[2] Phy, phytase; Inu, inulin
[3] To convert mg/kg to mmol/kg see footnote 3 in Table 2.3
[4] To convert mg/kg to mmol/kg see footnote 4 in Table 2.3
[5] To convert μg/kg to μmol/kg see footnote 3 in Table 2.4
[6] To convert μg/kg to μmol/kg multiply by 0.019

**Table 2.8** Geometric and biomechanical properties of swine femur after 6 weeks of dietary supplementations[1]

| | Treatment | | | | | Main effect, p-value | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | | | |
| Inulin, % | 0 | 0 | 4 | 4 | SEM | Phytase | Inulin | Phy x Inu[2] |
| **Geometry** | | | | | | | | |
| Weight, g | 134.6 | 135.7 | 132.9 | 133.9 | 8.7 | 0.8 | 0.7 | 1.0 |
| Length, mm | 129.8 | 130.6 | 127.7 | 128.2 | 3.3 | 0.7 | 0.2 | 0.9 |
| Height (b), mm | 18.2 | 19.4 | 19.6 | 19.9 | 0.9 | 0.1 | 0.06 | 0.3 |
| Width (a), mm | 18.3 | 18.8 | 18.3 | 18.5 | 0.7 | 0.3 | 0.7 | 0.7 |
| Cortical thickness, mm | 3.2 | 3.4 | 3.4 | 3.7 | 0.6 | 0.4 | 0.6 | 0.9 |
| **Moment of Inertia, mm$^4$** | | | | | | | | |
| Moment of inertia ($I_{ap}$) | 4343 | 5529 | 5482 | 5993 | 831.9 | 0.06 | 0.07 | 0.4 |
| Moment of inertia ($I_{ml}$) | 4348 | 5141 | 4790 | 5204 | 643.4 | 0.08 | 0.4 | 0.5 |
| Ellipticity index, ($I_{ap}$/ $I_{ml}$) | 1.0 | 1.07 | 1.15 | 1.15 | 0.09 | 0.3 | 0.01 | 0.4 |
| **Whole Bone Mechanics** | | | | | | | | |
| Maximum displacement, mm | 4.1 | 4.7 | 5.1 | 4.1 | 0.9 | 0.7 | 0.6 | 0.08 |
| Breaking energy, J | 4.0 | 6.0 | 7.1 | 5.9 | 2.8 | 0.7 | 0.3 | 0.3 |
| Bending Moment, N-m | 30.1 | 33.8 | 29.6 | 32.3 | 5.4 | 0.3 | 0.7 | 0.9 |
| Bending Rigidity, N-m$^2$ | 3.3 | 3.7 | 3.2 | 3.7 | 0.4 | 0.03 | 0.9 | 0.9 |

[1] Values are means, n = 5
[2] Phy, phytase; Inu, inulin

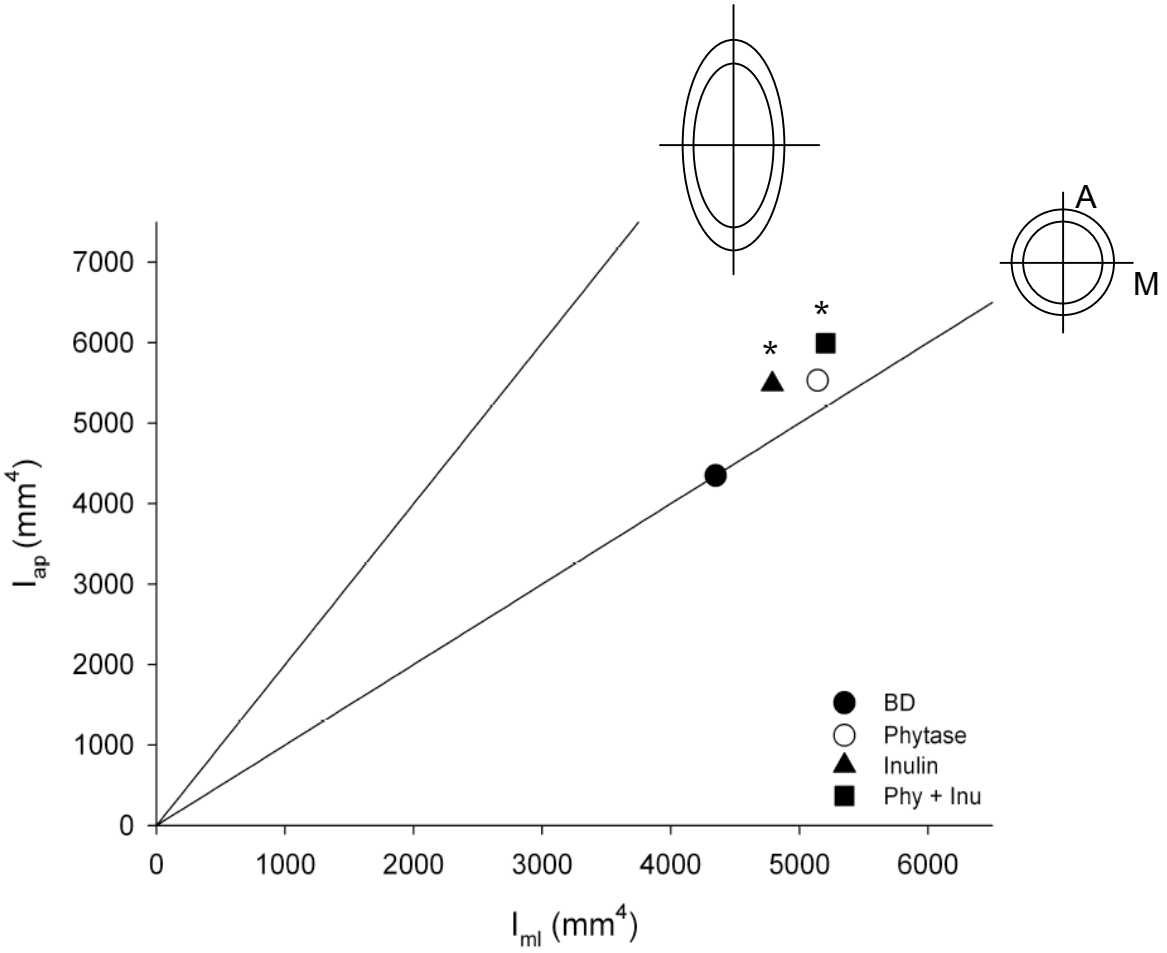
height (Figure 2.2). Femurs from swine fed BD + phytase + inulin displayed a marginal main effect ($p = 0.08$) with maximum displacement, resembling the value of the BD group.

## 2.5 Discussion

Phytase supplementation increased ($p < 0.05$) bending rigidity and bending moment by approximately 10%. Furthermore, phytase supplementation significantly altered the mineral composition of both cortical and cancellous bone. Most noticeably there were increases in Sr and Zn. The increase in Sr concentration in femur was approximately 15% in cortical bone and 10% in cancellous bone with phytase supplementation. Femur Zn concentration increased approximately 17% in cortical bone and 19% in cancellous bone with phytase supplementation compared to the BD treatment. Phytase supplementation had marginal effects on moment of interia, a measure of the distribution of the material of a beam around a cross section and an indicator of bone geometry, in the two axes measured, $I_{ap}$ and $I_{ml}$. These geometrical changes and mineral composition may be responsible for the observed significant increase in bending rigidity.

Similar alterations in bone mineral composition with phytase supplementation have been observed (29). The change in mineral composition in the skeletal tissue may be due to presence of more minerals. Phytate degradation by phytase releases P, mineral cations, and inositol intermediates from phytate. This enzymatic reaction increases the bioavailability of some minerals, such as Sr and Zn. The organism might be able to utilize minerals that were currently

**Figure 2.2** Mean anterior-posterior moment of inertia plotted against medial-lateral moment of inertia for each dietary treatment group to represent ellipticity index. The two reference lines represent an ellipticity index of 1 and 2 and a schematic representation of the cross-section of bone accompanies each.


A
M
7000
6000
5000
4000
3000
2000
1000
0
0
1000
2000
3000
4000
5000
6000
$I_{ap}$ (mm$^4$)
$I_{ml}$ (mm$^4$)
*
*
BD
Phytase
Inulin
Phy + Inu

unavailable and these minerals may have an affinity for skeletal tissue. Interestingly, an increase in P concentration in plasma or bone was not observed with phytase supplementation, suggesting that the P status of the pigs was not altered and the increase in P availability from the degradation of phytate did not influence bone biomechanics (29,47). Therefore, the enzymatic activity of phytase may increase the bioavailability of non-phosphorus bone relevant minerals in healthy growing pigs and allow for incorporation of these minerals into skeletal tissue, positively impacting biomechanical parameters. Additionally, inositol derivatives released from the degradation of phytate may have also influenced bone strength, or bending rigidity. Phytase cleaves orthophosphate groups from phytase, yielding a spread of inositol derivatives. Inositol derivatives are important cellular signaling molecules and their increased abundance from supplemental phytase activity may benefit skeletal tissue. The inositol-1-4-5 pathway is important for intracellular calcium release, a prerequisite for the expression of bone matrix proteins, such as collagen and fibronectin (69,70). The presence of inostiol derivates may further activate such an inositol pathway, improving the material composition of bone.

Inulin supplementation did not have any significant effect on the biomechanical parameters tested in the femur despite the significant impact ($p = 0.01$) on the ellipticity index. A 15% increase in ellipticity index was observed along the anterior-posterior axis and corresponds to the marginal increase ($p = 0.07$) in $I_{ap}$. Furthermore, in cortical bone inulin supplementation significantly increased Ca, Mg, Na, and Sr and there was a non-significant increase of approximately 20% in Fe concentration. These changes in bone

mineral content were not accompanied by changes in plasma mineral concentration.

All femurs were loaded in the anterior-posterior direction so it is interesting that the change in geometry with inulin supplementation did not result in a significant functional change. Other studies have demonstrated significant biomechanical changes using three-point bending in rats with 10% inulin supplementation (28). In this study, inulin was supplemented at a lower level, 4%, and perhaps three-point bending was able to detect functional changes in the femur due to geometric and material transformations of low-level inulin supplementation in swine.

Inulin supplementation studies have shown this supplement to be effective in increasing Fe, Ca and Mg bioavailability through increased absorption in the large intestine in animals and humans (58,59). The changes in femur mineral content observed in this study may be due to the increased mineral absorption with inulin supplementation. It has been suggested that this increase in mineral absorption, and thus bioavailability, allows these minerals to be incorporated into skeletal tissue (58). Furthermore, the geometric changes in femur may be due to this change in mineral composition. Distribution of these minerals made available by inulin, might favor incorporation along the anterior-posterior axis of skeletal tissue. However, percent ash analysis of cortical and cancellous bone suggests that mineral content did not increase, but only mineral composition. Perhaps, percent ash analysis at only a cortical and cancellous site bone is not representative of whole bone ash or mineral content. Performing DXA analysis on these femurs might resolve the issue of

whether the changes in mineral makeup is due to an increase in mineralization, of these minerals in bone.

The combined phytase and inulin, BD + phytase + inulin, treatment did not have any effect on bone biomechanics or geometry examined; nonetheless this treatment group did have some significant effects on non-phosphorus plasma parameters and cancellous mineral profile. The lack of interaction between phytase and inulin on bone biomechanics indicates that these two supplements probably are most effective on skeletal parameters when supplemented independently and no synergism is apparent.

It is unclear how increases and decreases of individual minerals in plasma and bone influence bone biomechanics. All minerals examined are shown in the tables, but it is unknown how each mineral specifically influences the material and functional properties of bone. Plasma mineral analysis was performed primarily to examine P and Ca status, and ensure that these were within normal physiological range. Bone mineral analysis was performed to collectively examine any change in mineral composition and better understand the mechanism of phytase and inulin.

In conclusion, the individual supplements, phytase and inulin, had an impact on the bone characteristics examined and both may benefit skeletal tissue through their ability to increase the bioavailability of bone-relevant minerals. Phytase supplementation had a significant impact on bending rigidity and femur mineral composition. Positive changes in bending moment or rigidity were not observed in the inulin treatment group despite both geometric and

mineral transformations in the femur. Further examination of phytase and inulin supplementation is necessary to determine their use in the prevention of musculoskeletal diseases. Additional supplementations studies should include DXA analysis to better interpret the observed changes is femur mineral composition. Evaluating the effects of these supplements for greater than 6 weeks is also important to determine if the positive effects observed in skeletal tissue are sustainable as the animal matures.

# CHAPTER THREE
# THE IMPACT OF DIETARY PHYTASE AND STRONTIUM SUPPLEMENTATION ON BONE BIOMECHANICS OF YOUNG PIGS

## 3.1 Abstract

Maximizing bone strength and mass early in life may provide protection against some musculoskeletal diseases, such as osteochondrosis and osteoporosis. The aim of this study was to examine if two dietary supplements, phytase and strontium, can enhance bone strength and mass when added to the diet of young animals. Fifty-two mixed gender weanling pigs (Yorkshire x Hampshire x Laundrace, 10.9 ± 0.95 kg of body weight) were fed a corn-soybean-meal basal diet with 0.35% inorganic phosphorus (BD), BD + phytase (3500U/kg, OptiPhos, JBS United, Sheridan, IN), BD + strontium (50 mg/kg strontium as $SrCO_3$, Alfa Aesar, Ward Hill, MA), or BD + phytase + strontium for 10 weeks. Femurs were collected for mechanical testing at 5 weeks (n = 13). Both supplements had marginal effects on bone biomechanics in young pigs. Phytase supplementation increased ($p = 0.09$) bending rigidity and strontium supplementation had a marginal effect ($p = 0.09$) on energy to break. Dual x-ray energy absorptiometry (DXA) analysis of femurs at 5 and 10 weeks showed that phytase supplementation increased ($p < 0.05$) bone mineral content in the femur at 5 weeks and strontium supplementation increased bone mineral density (BMD) at 10 weeks. In conclusion, independent supplementation with phytase and strontium appeared to positively impact skeletal tissue in a high phosphorus diet in young pigs.

## 3.2 Introduction

The results from the study described in Chapter 2 motivated us to conduct a follow-up study exploring the impact of dietary phytase supplementation on the skeleton of young pigs beyond 6 weeks. In this study, phytase supplementation was extended to 10-weeks. A total of 52 pigs were used in this study, allowing us to examine a subset of animals at 5-weeks for comparison. In addition, because we observed an increase in strontium content in swine femur in the previous study, we included supplemental strontium in this feeding trial to determine if an increase in strontium bioavailability can further improve bone strength, particularly when combined in the diet with phytase. Overall, the purpose of this study was to examine if there is an additive effect on bone strength in young pigs fed supplemental strontium and phytase together.

## 3.3 Methods

### *3.3.1 Experimental diet*

The four experimental diets were composed of a corn-soybean-meal basal diet (BD) containing 0.35% inorganic phosphorus (P) concentration. The microbial phytase used was *Escherichia coli* AppA2 (OptiPhos, JBS United, Sheridan, IN), at 3500 U/kg. After the phytase activity was analyzed, the phytase enzyme was added to the diets at feed mixing (65). Phytase activity in the experimental diets was further verified by laboratory analysis. Strontium

($SrCO_3$, Alfa Aesar, Ward Hill, MA) was supplemented in the diet at 50 mg/kg.

*3.3.2 Experimental animals and study design*

Fifty-two mixed gender weanling pigs (Yorkshire x Hampshire x Laundrace, 10.9 ± 0.95 kg of body weight) from the Cornell University Swine Farm were weaned at 4 weeks of age and assigned to one of four dietary treatment groups based body weight, litter and sex. This study was designed as a 2 x 2 factorial arrangement of diet treatments (2 levels of phytase, 0 and 3500 U/kg) and 2 levels of strontium (0 and 50 mg/kg) in a high phosphorus diet (0.35% inorganic phosphorus, an amount that exceeds the NRC requirement for inorganic phosphorus) (71,72). As in the previous study, swine were fed a high phosphorus diet to eliminate any phosphorus influence on skeletal tissue from the degradation of phytate (73). The treatments groups were BD, BD + phytase, BD + strontium, and BD + phytase + strontium. The experiment was conducted for 10 weeks. At 5 weeks, five pigs were selected for skeletal tissue collection. At 10 weeks, the remaining pigs (n = 8) were sacrificed for skeletal tissue collection. Pigs were penned in an environmentally controlled barn (20-25ºC; 12 h light: 12 h dark cycle) and fed ad libitum. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Cornell University.

*3.3.3 Growth performance and sample collection*

*3.3.4 Plasma biochemical assays*

*3.3.5 Mineral composition analysis*

*3.3.6 Femur geometry and whole bone strength*

*3.3.7 Statistical analysis*

Refer to Chapter 2.

*3.3.8 Dual energy x-ray absorptiometry (DXA)*

Bone mineral content (BMC) and bone mineral density (BMD) were measured a Hologic densitometry system (Discovery A, S/N 82243) at the Human Metabolic Research Unit (HMRU) of Cornell University. Femurs were scanned in the anterior-posterior position and supported by plexiglass to maintain this orientation during scanning. The lumbar spine program was used for analysis (version 12.4.2:3).

## 3.4 Results

*3.4.1 Growth performance*

During the 10-week feeding trial, there were no significant differences in body weight or gain: feed ratio due to phytase and/or strontium supplementation (Table 3.1). The pigs feed BD + phytase + strontium had an increase in body weight at week five ($p = 0.07$) and week 10 ($p = 0.09$). At 5 weeks, BD + phytase + strontium significantly ($p < 0.05$) increased average daily gain, but

**Table 3.1** Growth performance of swine during the 10 week feeding trial[1]

| | Treatment | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | Main effect, p-value | | |
| Strontium, mg/kg | 0 | 0 | 50 | 50 | SEM | Phytase | Strontium | Phy x Sr[2] |
| Body Weight, kg | | | | | | | | |
| Wk 0 | 10.9 | 10.8 | 10.9 | 11.1 | 0.1 | 0.8 | 0.6 | 0.5 |
| Wk 5 | 32.8 | 31.2 | 31.1 | 32.5 | 0.4 | 0.9 | 0.7 | 0.04 |
| Wk 10 | 65.3 | 61.8 | 63.9 | 65.5 | 0.7 | 0.5 | 0.4 | 0.09 |
| Average Daily Gain, kg | | | | | | | | |
| Wk 0 | 0.4 | 0.4 | 0.4 | 0.5 | 0.01 | 0.7 | 0.3 | 0.4 |
| Wk 5 | 0.9 | 0.8 | 0.8 | 0.8 | 0.01 | 0.5 | 0.1 | 0.02 |
| Wk 10 | 0.9 | 0.9 | 0.9 | 0.9 | 0.03 | 0.7 | 0.9 | 0.7 |
| Gain: Feed | | | | | | | | |
| Wk 0 | 0.6 | 0.6 | 0.6 | 0.6 | 0.01 | 0.9 | 0.9 | 0.6 |
| Wk 5 | 0.6 | 0.6 | 0.6 | 0.6 | 0.01 | 0.3 | 0.1 | 0.9 |
| Wk 10 | 0.3 | 0.4 | 0.4 | 0.4 | 0.01 | 0.3 | 0.1 | 0.3 |

[1] Values are means. Wk 0, n = 13 per treatment group; Wk 5, n = 13 per treatment group; Wk 10, n = 8 for BD and BD + phytase + strontium; n = 7 for BD + phytase and BD + strontium

[2] Phy, phytase; Sr, strontium

this effect was not observed at 10 weeks.

### *3.4.2 Plasma biochemical assays and mineral analysis*

Phytase supplementation increased ($p = 0.08$) plasma AKP at week 10 (Table 3.2). The combination supplementation treatment did not alter AKP values. Phytase supplementation had a marginal effect ($p = 0.08$) on plasma inorganic phosphorus concentration (PIP) at week 5. Neither BD + strontium nor BD + phytase + strontium treatment had an apparent effect on PIP at week 5 or 10.

Phytase supplementation increased ($p < 0.05$) plasma Ca and Zn concentration at week 10 and decreased ($p = 0.08$) plasma P concentration at week 5 (Table 3.3 and 3.4). Strontium supplementation had an increase ($p < 0.001$) in plasma Sr concentration at week 5 and 10. Pigs fed BD + phytase + strontium had an increase ($p < 0.05$) in plasma Zn concentrations at 5 and 10 weeks.

### *3.4.3 Dual energy x-ray absorptiometry (DXA)*

Phytase supplementation resulted in approximately a 5 percent increase ($p < 0.05$) in femur BMC at week 5 compared to the BD treatment (Figure 3.1). No effects were observed in femur from pigs fed BD + phytase or the BD + phytase + strontium supplement with DXA analysis. At week 10, strontium supplementation increased ($p < 0.05$) femur mineral density (Figure 3.2). DXA analysis showed no effect of BD + phytase or BD + phytase + strontium at week 10.

**Table 3.2** The change in plasma inorganic phosphorus (PIP) and alkaline phosphatase (AKP) activity in swine during the 10 wk feeding trial[1]

| | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | Main effect, p-value[3] | |
| Strontium, mg/kg | 0 | 0 | 50 | 50 | SEM | Phytase | Strontium | Phy x Sr[2] |
| Plasma Inorganic Phosphorus[4], mg/dL | | | | | | | | |
| Δ Wk 5 | 10.9 | 10.4 | 11.3 | 10.4 | 0.09 | 0.08 | 0.4 | 0.6 |
| Δ Wk 10 | 9.4 | 9.9 | 10.1 | 10.4 | 0.57 | 0.3 | 0.7 | 0.8 |
| Alkaline Phosphatase Activity, U/ml | | | | | | | | |
| Δ Wk 5 | 121.5 | 133.7 | 111.8 | 131.3 | 0.06 | 0.4 | 1.0 | 0.1 |
| Δ Wk 10 | 78.97 | 89.7 | 89.9 | 96.6 | 0.14 | 0.08 | 0.2 | 0.2 |

[1] Values are means. Wk 5, n = 13 and Wk 10, n = 8 for BD and BD + phytase + strontium; n =7 for BD + phytase and BD + strontium
[2] Phy, phytase; Sr, strontium
[3] Statistical analyses were performed on the change from baseline, wk 0.
[4] To convert mg/dl to mmol/L multiply by 0.323.

**Table 3.3** Plasma macromineral concentration[3] (mg/L) during the 10 week feeding trial[1]

| | Treatment | | | | | Main effect, p-value | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | | | |
| Strontium, mg/kg | 0 | 0 | 50 | 50 | SEM | Phytase | Strontium | Phy x Sr[2] |
| **Na** (0.0435) | | | | | | | | |
| Wk 0 | 1911 | 1893 | 1887 | 1883 | 42.1 | 0.6 | 0.4 | 0.7 |
| Wk 5 | 2713 | 2705 | 2690 | 2665 | 57.6 | 0.6 | 0.3 | 0.8 |
| Wk 10 | 3469 | 3489 | 3375 | 3570 | 148 | 0.1 | 0.9 | 0.2 |
| **S** (0.0312) | | | | | | | | |
| Wk 0 | 356.7 | 362.1 | 303.2 | 351.2 | 34.0 | 0.1 | 0.06 | 0.2 |
| Wk 5 | 679.9 | 651.6 | 669.7 | 671.4 | 26.5 | 0.3 | 0.7 | 0.3 |
| Wk 10 | 537.7 | 525.7 | 542.7 | 542.4 | 42.0 | 0.8 | 0.6 | 0.9 |
| **P** (0.0323) | | | | | | | | |
| Wk 0 | 110.7 | 107.8 | 103.31 | 109.5 | 7.4 | 0.7 | 0.4 | 0.2 |
| Wk 5 | 130.9 | 128.7 | 134.7 | 128.8 | 4.5 | 0.08 | 0.4 | 0.4 |
| Wk 10 | 128.7 | 125.7 | 125.9 | 129.3 | 8.4 | 1.0 | 0.9 | 0.4 |
| **Ca** (0.0249) | | | | | | | | |
| Wk 0 | 101.3 | 100.9 | 96.5 | 100.0 | 2.9 | 0.3 | 0.05 | 0.2 |
| Wk 5 | 112.2 | 110.4 | 109.4 | 110.1 | 2.7 | 0.7 | 0.3 | 0.4 |
| Wk 10 | 107.5 | 111.6 | 105.9 | 112.6 | 4.5 | 0.02 | 0.9 | 0.5 |

[1] Values are means. Wk 0, n = 13 per treatment group; wk 5, n = 13 per treatment group; wk 10, n = 8 for BD and BD + phytase + strontium; n = 7 for BD + phytase and BD + strontium.
[2] Phy, phytase; Sr, strontium
[3] To convert mg/L to mmol/L multiply values by the factor in parentheses next to each element, except for K.
[4] To convert mg/L to mmol/L (mEq/L) divide values by the factor in parentheses.

**Table 3.4** Plasma micromineral concentration[3] (μg/L) during the 10 week feeding trial

| | Treatment | | | | | Main effect, p-value | | |
|---|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | | | |
| Strontium, mg/kg | 0 | 0 | 50 | 50 | SEM | Phytase | Strontium | Phy x Sr[2] |
| **Fe** (0.0179) | | | | | | | | |
| Wk 0 | 1601 | 1542 | 1495 | 1600 | 100 | 0.6 | 0.6 | 0.8 |
| Wk 5 | 3621 | 2625 | 2857 | 2893 | 90.0 | 0.3 | 0.6 | 0.3 |
| Wk 10 | 3295 | 4286 | 4000 | 2953 | 190 | 1.0 | 0.7 | 0.2 |
| **Zn** (0.0153) | | | | | | | | |
| Wk 0 | 809 | 821 | 764 | 861 | 64.6 | 0.1 | 0.9 | 0.2 |
| Wk 5 | 1261 | 1235 | 1165 | 1404 | 109 | 0.6 | 0.5 | 0.02 |
| Wk 10 | 1062 | 1088 | 982 | 1238 | 107 | 0.02 | 0.5 | 0.04 |
| **Sr** (0.0114) | | | | | | | | |
| Wk 0 | 30.2 | 28.4 | 28.4 | 31.6 | 2.7 | 0.6 | 0.6 | 0.07 |
| Wk 5 | 45.6 | 42.3 | 192 | 194 | 9.1 | 0.9 | <0.001 | 0.6 |
| Wk 10 | 40.3 | 44.5 | 180 | 190 | 12.0 | 0.3 | <0.001 | 0.7 |

[1] Values are means. Wk 0, n = 13 per treatment group; wk 5, n = 13 per treatment group; wk 10, n = 8 for BD and BD + phytase + strontium; n = 7 for BD + phytase and BD + strontium.
[2] Phy, phytase; Sr, strontium
[3] To convert μg/L to μmol/L multiply values by the factor in parentheses next to each element.

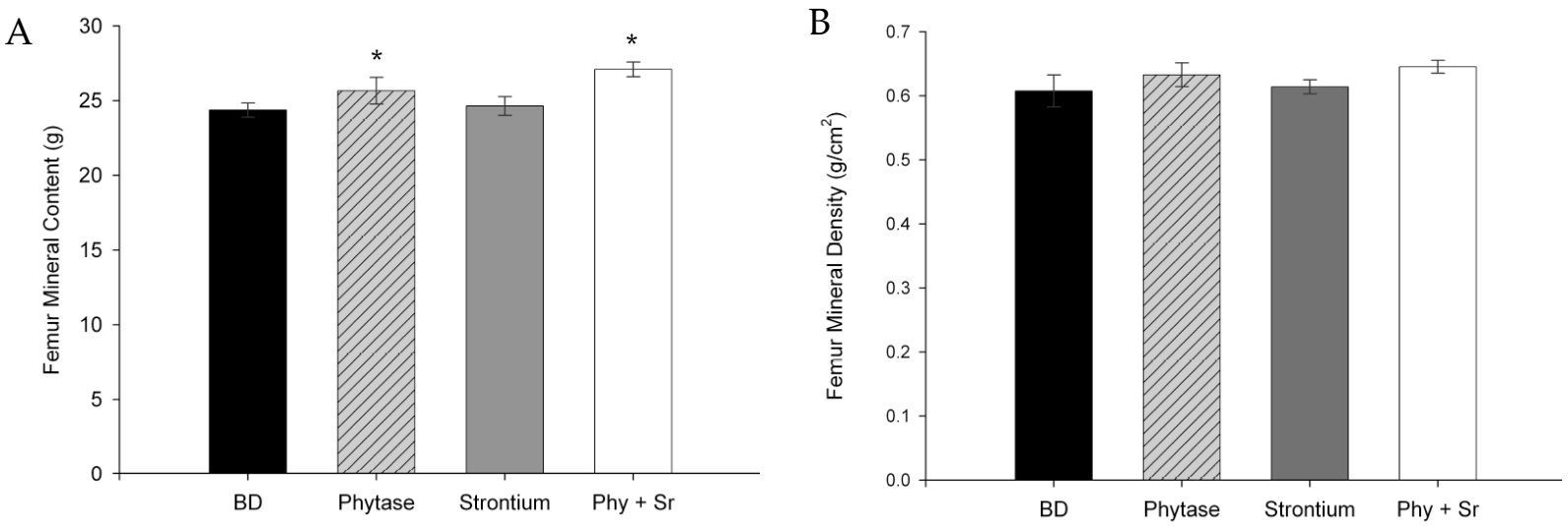



**Figure 3.1** Dual X-ray absorptiometry analysis of femurs at 5 weeks during the supplemental feeding trial. The mineral content (A) and mineral density (B) were obtained with DXA analysis. Bars represent mean values per treatment group and error bars are the standard error. The asterisks (A) represent the significant main effect ($p < 0.05$) observed with phytase supplementation on femur mineral content. For all treatment groups n = 8. Phy, phytase; Sr, strontium; BD, basal diet.

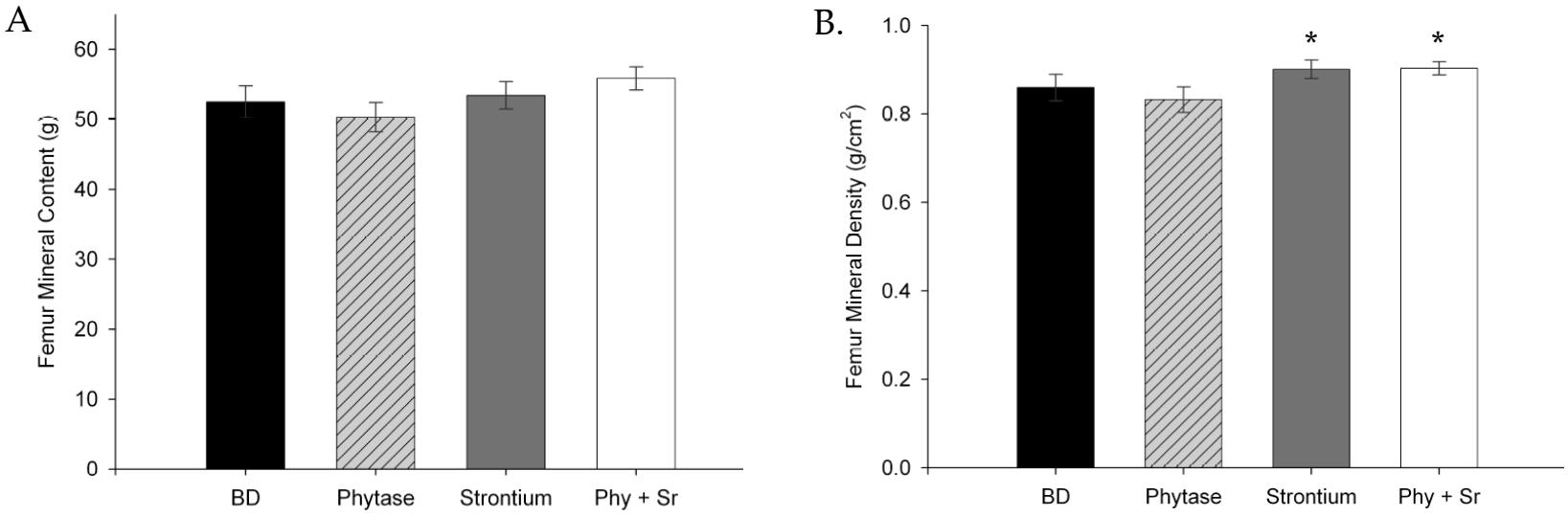



**Figure 3.2** Dual X-ray absorptiometry analysis of femurs after 10 weeks of the feeding trial. The femur mineral content (A) and mineral density (B) were obtained with DXA analysis. Bars represent mean values per treatment group and error bars are the standard error. The asterisks (B) represent the significant main effect ($p < 0.05$) observed with strontium supplementation on bone mineral density. For all treatment groups BD and BD + phytase + strontium, n = 8. For treatment groups BD + phytase and BD + strontium, n = 7. Phy, phytase; Sr, strontium; BD, basal diet.

### *3.4.4 Femur geometry and whole bone strength*

Weight, length, height (anterior-posterior axis), width (medial-lateral axis), and cortical thickness were not affected by supplementation with phytase, strontium, or both (Table 3.5). At week 5, 3-point bending test on femurs showed a decrease ($p = 0.04$) in displacement at maximum load and a marginal increase ($p = 0.09$) of approximately 10% in bending rigidity. Strontium supplementation had a marginal effect ($p = 0.09$) on energy to break.

## 3.5 Discussion

In this study we used three-point bending to determine whole bone strength to failure. Bending moment was not different among treatment groups; however, phytase supplementation had a marginal effect ($p = 0.09$) on bending rigidity. Phytase supplementation increased bending rigidity approximately 10%, which agreed with a previous study (29). This non-significant increase in bending rigidity was not due to animals being larger as evidenced by the growth performance results and suggests that this change in bone function was a treatment effect. Furthermore, the final status of assayed P-response measures was similar between pigs fed BD and BD + phytase. Therefore, any benefit of supplemental phytase was unlikely a response to an increase in P availability, especially in the 0.35% P-adequate diet implemented in this study.

**Table 3.5** Femur geometrical and biomechanical measurements after 5 weeks of the feeding trial[1]

| | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| Phytase, U/kg | 0 | 3500 | 0 | 3500 | | Main effect, p-value | |
| Strontium, mg/kg | 0 | 0 | 50 | 50 | SEM | Phytase | Strontium | Phy x Sr[2] |
| **Geometry** | | | | | | | | |
| Weight, g | 126.3 | 127.7 | 125.1 | 131.3 | 7.9 | 0.4 | 0.8 | 0.6 |
| Length, mm | 125.7 | 126.5 | 125.4 | 126.7 | 2.9 | 0.5 | 1.0 | 0.9 |
| Width (a), mm | 17.3 | 17.1 | 17.2 | 18.1 | 1.3 | 0.6 | 0.5 | 0.4 |
| Height (b), mm | 16.9 | 17.2 | 17.9 | 18.2 | 1.8 | 0.7 | 0.3 | 0.9 |
| Cortical thickness (ct. $th._{ml}$), mm | 2.8 | 2.5 | 2.8 | 3.1 | 0.6 | 1.0 | 0.3 | 0.2 |
| Cortical thickness (ct. $th._{ap}$), mm | 3.5 | 3.6 | 3.6 | 3.7 | 0.6 | 0.9 | 0.7 | 1.0 |
| **Moment of Inertia**, $mm^4$ | | | | | | | | |
| $I_{ap}$ | 3770 | 3710 | 4170 | 4780 | 1180 | 0.7 | 0.3 | 0.6 |
| $I_{ml}$ | 3680 | 3390 | 3580 | 4480 | 1440 | 0.6 | 0.4 | 0.3 |
| Ellipticity index ($I_{ap/}$ $I_{ml}$) | 1.0 | 1.1 | 1.2 | 1.1 | 0.2 | 1.0 | 0.8 | 0.4 |
| **Whole Bone Mechanics** | | | | | | | | |
| Maximum displacement, mm | 5.7 | 5.0 | 6.2 | 5.3 | 0.7 | 0.04 | 0.3 | 0.7 |
| Breaking energy, J | 5.5 | 4.3 | 5.9 | 5.7 | 0.9 | 0.1 | 0.06 | 0.3 |
| Bending moment, N-m | 25.5 | 25.8 | 24.0 | 27.0 | 2.9 | 0.3 | 0.9 | 0.4 |
| Bending rigidity, $N\text{-}m^2$ | 2.2 | 2.4 | 2.1 | 2.4 | 0.3 | 0.09 | 0.9 | 0.7 |

[1] Values are means, n = 5
[2] Phy, phytase; Sr, strontium

The bone cross sectional geometry did not differ with phytase supplementation, and thus did not contribute to the observed increase in bending rigidity. Therefore, changes in material properties, such as femur mineral content, may be responsible for the observed non-significant increase in femur bending rigidity. This hypothesis is supported by DXA analysis, which showed an increase ($p < 0.05$) in BMC with phytase supplementation at week 5.

The basal corn-soybean meal diet used in this study contained approximately 7 mg/kg of strontium as determined by ICP-AES (Inductively Coupled Plasma - Atomic Emission Spectrometer) analysis. Strontium was supplemented at 50 mg/kg (0.005 percent) in the diet, which is approximately 7 times greater than the concentration in a corn-soybean meal diet for pigs. Yet, a 50 mg/kg dose is conservative when compared to the reported effective anti-osteoporotic doses of strontium, such as 2 g/day in humans, 625 mg/kg/day in rats, and 750 mg/day in monkeys (74-76). The low-level of Sr used in this study may account for the responses observed on whole bone strength and geometry. However, a 50 mg/kg dose of strontium was reported to increase ($p < 0.05$) Sr mineral concentration in femur and metatarsal (29). Therefore, this dose seems to be effective in increasing plasma and bone mineral concentration, yet may not lead to significant functional changes in the femur at 5 weeks.

At 10 weeks, strontium supplementation had a main effect ($p < 0.05$) on bone mineral density, however caution should be given in interpreting DXA results in strontium studies. DXA analysis typically overestimates BMC and BMD by

10% in animals treated with strontium (77,78). This is due to the higher atomic number of strontium (Sr, Z = 38) compared to calcium (Ca, Z = 20). Any observed increase in BMC and BMD is primarily a physical effect due to the increased attenuation of X-rays when some of the calcium in bone is replaced by strontium (79). Therefore, DXA analysis may overestimate increases in bone density, because Sr is denser than Ca, and cannot be interpreted as a measure of mineralization in animals supplemented with strontium. At 5 weeks, BMD was not elevated in animals supplemented with strontium, which does not agree with a previous strontium supplementation study (29). Nonetheless, an increase in BMD at 5 or 10 weeks is most likely due to the presence of strontium and does reflect an increase in bone mineralization. Concurrent geometric and biomechanical analysis of femurs at 10 weeks is necessary to better determine the benefits of low-dose strontium supplementation on skeletal tissue in swine.

Overall, phytase supplementation had a marginal effect on bone rigidity. In the previous study (Chapter 2), we observed a more robust effect of supplemental phytase on femur bending rigidity ($p < 0.05$) as well as slight changes in femur geometry. Two differences between this study and the previous study may account for the discrepancy in the observed results. These include the length of the feeding trial and time of mechanical testing. The study in Chapter 2 was a feeding trial for 6-weeks, while this study was conducted for a total of 5-weeks. The additional week of growth, particularly when the swine are at their greatest feed: gain ratio. Furthermore, in the Chapter 2 study three-point bending was performed within a week after bones were collected compared to a year later in this study. It seems likely that

the delay in testing may have compromised the bones and led to differences in observed bone properties between studies.

Strontium supplementation had no significant impact on femur characteristics, which may due to a combination of factors such as the duration of the feeding trial and the low-dose supplemented in the diet. Further examination of femurs from swine at 10 weeks of the feeding trial with three-point bending will demonstrate any impact this supplement has on whole bone strength. In addition, investigating strontium supplementation at a higher dose may better demonstrate the ability of this mineral to impact the skeletal system. In summary, the impact and magnitude of dietary phytase and strontium supplementation on bone is still inconclusive.

# CHAPTER FOUR
# THE IMPACT OF DIETARY PHYTASE SUPPLEMENTATION ON FEMURS OF MICE FED A PHYTATE-FREE NUTRITENT ADEQUATE DIET

## 4.1 Abstract

Phytase, an acid phosphatase, has been shown to improve skeletal properties in swine, poultry, and rats when supplemented in a phosphorus deficient diet. This study was designed to test if dietary microbial phytase improved bone properties when supplemented in a nutrient adequate, phytate-free diet, in wild-type (WT) and superoxide dismutase knockout (SOD1 -/-; Cu, Zn-SOD; EC 1.15.1.1) mice. The SOD1 -/- mice are a mouse model of reduced skeletal integrity, such as maximum breaking moment and bending rigidity. A total of 38 mixed gender WT mice were examined and fed a Torula yeast and sucrose diet, BD, (n = 17, seven females and ten males) or BD + phytase (n = 21, thirteen females and eight males). A total of 17 mice SOD1 -/- were fed BD (n = 7, three females and four males) or BD + phytase (n = 10, five females and five males). For both WT and SOD1 -/- mice, littermates were split between dietary treatments. Phytase supplementation did not alter bone parameters as measured with three-point bending or body weight in the WT or SOD1 -/- mouse. A gender difference was observed in SOD1 -/- mice with females having a greater ($p < 0.05$) bending moment and ($p = 0.09$) maximum bending moment, which was not a consequence of increase cross sectional size. Overall, phytase seemed to require the presence of its intrinsic substrate, phytate, to positively impact skeletal tissue in the growing mice.

## 4.2 Introduction

The two previous swine studies examined the impact of phytase supplementation on whole bone strength early in life to determine if phytase can be used to reduce musculoskeletal disease. This study was conducted to better understand the mechanism of phytase and determine whether phytase, the enzyme, or the products of phytate degradation are responsible for the observed effects on bone biomechanics. We investigated the impact of dietary phytase supplementation on whole bone strength in a phytate-free Torula yeast and sucrose diet in WT and SOD1 -/- mice. Positive results on skeletal parameters would suggest that phytase impacts bone properties via a non-phytate mechanism, and therefore the phosphorus and minerals released from phytate degradation are not responsible for the beneficial effects on skeletal tissue. Furthermore, phytase supplementation in SOD1 -/- mice was examined to demonstrate if phytase can "rescue" the compromised bones of these transgenic mice. SOD1 catalyzes the dismutation of superoxide, a common free radical in the body, to oxygen and hydrogen peroxides, classifying it as a cellular antioxidant (80). Previous studies have demonstrated that SOD1 -/- mice have compromised skeletal tissue, such as a decrease in bending moment and rigidity, compared to WT mice (81). Therefore, positive results would further demonstrate the ability of dietary phytase to improve bone properties without phytate and also restore compromised bone.

## 4.3 Methods

### *4.3.1 Experimental design*

WT and SOD1 −/− (superoxide dismutase; Cu, Zn-SOD; EC 1.15.1.1) mice were derived from 129/SVJ×C57BL/6 lines and provided by Dr Y. S. Ho, Wayne State University (Detroit, MI, U.S.A.) (82). All experiments were approved by the Institutional Animal Care and Use Committee at Cornell University and conducted in accordance with NIH (National Institutes of Health) Guidelines for Animal Care. Mice were housed in shoebox cages in a room at a constant temperature (22 °C) with a 12 h light/dark cycle and were given free access to food and distilled water.

Wild-type mice were weaned at 3 weeks of age and were derived from one of two females (WT) mated to the same male (WT) to reduce genetic variability. At weaning, pups were fed ad libitum a Torula yeast sucrose based diet (BD) or BD supplemented with microbial phytase (BD + phytase, 3500U/kg, OptiPhos, JBS United, Sheridan, IN) for 12 weeks. SOD1 -/- mice were weaned at 3 weeks of age and were derived from one of two females (SOD 1-/+) mated to the same male (SOD1 -/-). Pups were genotyped after weaning and in the interim were fed BD. At 4 weeks of age SOD1 -/- pups were fed ad libitum the same BD or BD + phytase as WT mice and remained on these diets for 12 weeks. All other minerals and nutrients were adequate in the diet. A total of 38 mixed gender WT mice were examined and fed BD (n = 17, 7 females and 10 males) or BD + phytase (n = 21, 13 females and 8 males). Due to the breeding constraints of the transgenic SOD1 mice, a reduced number of

mice were examined. A total of 17 mice SOD1 -/- were fed BD (n = 7, 3 females and 4 males) or BD + phytase (n = 10, 5 females and 5 males). For both WT and SOD1 -/- mice littermates were split between dietary treatments.

Body weights were measured weekly using a digital scale. At the end of 12 weeks, animals were killed by $CO_2$ inhalation as approved by the IACUC at Cornell University and conducted in accordance with NIH Guidelines for Animal Care. The right femur from each animal was collected for mechanical testing.

### *4.3.2 Whole bone strength*

Immediately after sacrifice, femurs were excised and muscle and tissue were removed. Femurs were placed in freezer bags at -20°C until mechanical testing could be performed. Three-point bending was accomplished using a Servohydraulic testing machine (858 MiniBionix, MTS Systems, Eden Prairie, MN). Loading rate was set at 0.1 mm/s and span length was 6mm.

## 4.4 Results

### *4.4.1 Body weight*

Phytase supplementation did not alter the body weights of WT or SOD1 -/- mice during the 12-week feeding trial (Table 4.1 and 4.2).

**Table 4.1** Body weights of WT mice during the 12-week feeding trial[1]

| Gender | Male[2] | | Female[3] | | | Main effect, p-value | | |
|---|---|---|---|---|---|---|---|---|
| Diet | BD[4] | Phytase | BD[4] | Phytase | SEM | Diet | Gender | Diet x Gender |
| Wk 0 | 10.2 | 10.3 | 9.9 | 9.0 | 0.7 | 0.2 | 0.04 | 0.2 |
| Wk 1 | 14.5 | 15.8 | 13.5 | 13.0 | 0.7 | 0.2 | < 0.001 | 0.02 |
| Wk 2 | 19.8 | 20.1 | 16.1 | 15.9 | 1.1 | 1.0 | < 0.001 | 0.6 |
| Wk 3 | 22.3 | 22.5 | 18.3 | 17.5 | 1.1 | 0.6 | < 0.001 | 0.4 |
| Wk 4 | 24.0 | 24.3 | 20.1 | 19.0 | 1.0 | 0.5 | < 0.001 | 0.2 |
| Wk 5 | 25.2 | 25.5 | 21.6 | 20.6 | 1.1 | 0.5 | < 0.001 | 0.3 |
| Wk 6 | 26.5 | 26.8 | 22.2 | 21.7 | 1.3 | 0.9 | < 0.001 | 0.6 |
| Wk 7 | 27.4 | 27.7 | 23.3 | 22.6 | 1.2 | 0.8 | < 0.001 | 0.5 |
| Wk 8 | 28.6 | 28.8 | 24.3 | 23.5 | 1.4 | 0.7 | < 0.001 | 0.5 |
| Wk 9 | 29.5 | 29.9 | 24.1 | 23.7 | 1.6 | 1.0 | < 0.001 | 0.6 |
| Wk 10 | 29.7 | 30.1 | 24.9 | 24.3 | 1.5 | 0.9 | < 0.001 | 0.6 |
| Wk 11 | 30.3 | 30.7 | 25.3 | 24.9 | 1.6 | 1.0 | < 0.001 | 0.6 |
| Wk 12 | 31.0 | 30.9 | 26.0 | 26.1 | 1.9 | 1.0 | < 0.001 | 0.9 |

[1] Values are means
[2] BD, n =10; Phytase, n = 8
[3] BD, n = 7; Phytase, n = 13
[4] BD, basal diet

**Table 4.2** Body weights of SOD1 -/- mice during the 12-week feeding trial[1]

| Gender | Male[2] | | Female[3] | | | Main effect, p-value | | |
|---|---|---|---|---|---|---|---|---|
| Diet | BD[4] | Phytase | BD[4] | Phytase | SEM | Diet | Gender | Diet x Gender |
| Wk 0 | 14.4 | 13.1 | 12.6 | 11.3 | 2.2 | 0.3 | 0.1 | 1.0 |
| Wk 1 | 16.4 | 14.8 | 14.3 | 13.8 | 2.1 | 0.3 | 0.2 | 0.6 |
| Wk 2 | 19.5 | 17.4 | 17.5 | 16.6 | 2.7 | 0.3 | 0.3 | 0.6 |
| Wk 3 | 22.4 | 20.5 | 19.1 | 18.9 | 3.2 | 0.6 | 0.2 | 0.6 |
| Wk 4 | 23.2 | 22.7 | 19.9 | 20.5 | 3.2 | 1.0 | 0.1 | 0.7 |
| Wk 5 | 23.9 | 23.7 | 20.7 | 21.6 | 3.3 | 0.8 | 1.0 | 0.7 |
| Wk 6 | 24.0 | 24.30 | 21.1 | 22.1 | 3.3 | 0.7 | 0.1 | 0.8 |
| Wk 7 | 25.2 | 25.2 | 22.7 | 24.1 | 3.9 | 0.7 | 0.4 | 0.7 |
| Wk 8 | 26.3 | 26.4 | 24.6 | 24.8 | 3.3 | 0.9 | 0.4 | 1.0 |
| Wk 9 | 27.7 | 26.9 | 25.9 | 26.3 | 3.5 | 0.9 | 0.5 | 0.8 |
| Wk 10 | 28.5 | 27.2 | 27.1 | 26.6 | 3.3 | 0.6 | 0.5 | 0.8 |
| Wk 11 | 28.6 | 28.0 | 28.1 | 27.7 | 3.7 | 0.8 | 0.8 | 1.0 |
| Wk 12 | 29.5 | 27.6 | 28.0 | 29.2 | 3.5 | 0.8 | 1.0 | 0.4 |

[1] Values are means
[2] BD, n = 4; Phytase, n = 5
[3] BD, n = 3; Phytase, n = 5
[4] BD, basal diet

### *4.4.2 Whole bone strength*

Femurs were tested with three-point bending and were loaded to failure. Phytase supplementation did not affect maximum bending moment rigidity, bending rigidity, or displacement in the WT or SOD1 -/- mice (Table 4.3 and 4.4). In addition, we did not observe any evidence of decreased skeletal characteristics in the SOD1 -/- mice with the 3-point bending test as previously reported. However, we did observe significant gender differences in SOD1 -/- mice. Bending rigidity significantly increased ($p < 0.05$) and bending moment marginally increased ($p = 0.09$) in female SOD1 -/- mice.

## 4.5 Discussion

Phytase supplementation did not alter body weight or whole bone strength as measured by three-point bending in the WT or SOD -/- mouse. Therefore, it appears that phytase requires phytate to impact skeletal tissue, at least in the mouse. However, we did observe a gender difference in SOD1 -/- mice in bending rigidity and bending moment. This was not due to the female mice having an increased femoral cross sectional size. Typically, male mice, partially due to greater size, exhibit higher skeletal parameters, such as bone mineral mass fraction, bone density, and strength (83,84). Therefore, the loss of superoxide dismutase seems to benefit the skeletal tissue in female mice in this study, but these results may be limited due to the small sample size of female SOD1 -/- mice used in each dietary treatment (BD, n = 4 females and BD + phytase, n = 3 females).

**Table 4.3** Biomechanical results from three-point bending on WT femurs[1]

| Gender | Male[2] | | Female[3] | | | Main effect, p-value | | |
|---|---|---|---|---|---|---|---|---|
| Diet | BD[4] | Phytase | BD[4] | Phytase | SEM | Diet | Gender | Diet x Gender |
| Maximum Bending Moment, N-mm | 43.8 | 45.9 | 43.6 | 44.6 | 3.6 | 0.4 | 0.7 | 0.8 |
| Bending Rigidity, N-mm$^2$ | 1.2 | 1.3 | 1.3 | 1.3 | 0.2 | 0.4 | 0.5 | 0.3 |
| Difference in Displacement, mm | 0.4 | 0.3 | 0.4 | 0.3 | 0.2 | 0.2 | 0.6 | 0.6 |

[1] Values are means
[2] BD, n =10; Phytase, n = 8
[3] BD, n = 7; Phytase, n = 13
[4] BD, basal diet

**Table 4.4** Biomechanical results from three-point bending on SOD1 -/- femurs[1]

| Gender | Male[2] | | Female[3] | | | Main effect, p-value | | |
|---|---|---|---|---|---|---|---|---|
| Diet | BD[4] | Phytase | BD[4] | Phytase | SEM | Diet | Gender | Diet x Gender |
| Maximum Bending Moment, N-mm | 39.3 | 38.5 | 50.5 | 45.2 | 9.9 | 0.6 | 0.09 | 0.7 |
| Bending Rigidity, N-mm$^2$ | 1.2 | 1.0 | 1.7 | 1.4 | 0.4 | 0.2 | 0.02 | 0.9 |
| Difference in Displacement, mm | 0.3 | 0.3 | 0.1 | 0.3 | 0.1 | 0.2 | 0.1 | 0.2 |

[1] Values are means
[2] BD, n = 4; Phytase, n = 5
[3] BD, n = 3; Phytase, n = 5
[4] BD, basal diet

Interestingly, we did not observe any differences in whole bone strength or difference in displacement in the transgenic mice compared to WT mice. Previous studies have demonstrated a 41% decrease in bending moment and a 38% decrease in bending rigidity in SOD -/- mice compared to WT mice (81). To determine the true skeletal integrity of the SOD -/- mice, it is necessary to conduct re-examination of the whole bone strength of these transgenic mice as well as an increase in sample size of each sex to confirm the gender difference observed in this experiment.

# CHAPTER FIVE
# CONCLUSION

## 5.1. Phytase Supplementation in Swine and Mouse

We demonstrated the potential of dietary phytase to improve whole bone strength, specifically bending rigidity, when supplemented in a high phosphorus diet in young pigs. These results agree with previous findings and suggest that skeletal tissue may benefit from the presence of phytase that is not exclusively due to the phosphorus release from the degradation of phytate (29). However, our second swine study (Chapter Three) examined phytase and strontium supplementation over a 10-week feeding period and improvements in skeletal parameters and whole bone strength were not as robust as those observed in the first study (Chapter Two). This discrepancy between the two studies may be due to a few obvious differences between the two studies as discussed in Chapter Three, and include the length of the feeding trial, the season the study was conducted, and the time of mechanical testing. Therefore, the use of supplemental phytase in young animals as a means to prevent musculoskeletal diseases requires further investigation.

Furthermore, the dietary phytase feeding trial in the mouse demonstrated that phytase-mediated bone improvements require the presence of phytate, the intrinsic substrate of phytase, in the diet. We did not observe any improvements in bone parameters in the compromised skeleton of SOD1 -/- mice with phytase supplementation.

### 5.2 Possible Mechanism for Phytase in Improving Whole Bone Strength

The presence of phytase and phytate in the diet increases mineral bioavailability. Minerals liberated from phytate may improve bone health through their interactions with metalloenzymes involved in bone metabolism, such as alkaline phosphatase (AKP) and tartrate-resistant acid phosphatase (TRAP). For example, zinc is an essential trace mineral for animal development, as demonstrated by reduced bone growth in animal fed diets deprived of zinc (85). Microbial phytase-fed piglets increased plasma and bone zinc concentrations. Herein, we observed similar increases ($p < 0.05$) in both of these parameters (86). AKP is found on the surface of osteoblasts and is marker for bone formation. AKP is a Zn-metalloenzyme and contains two Zn active centers necessary for its enzymatic activity, althougth the precise biochemical function of this enzyme is still unclear (87). Experiments investigating levels of dietary Zn on bone health have shown that increases in this element are accompanied by increases in AKP and decreases in bone resorption (88,89). Therefore, dietary Zn appears to adjust the bone remodeling cycle to favor bone mass accumulation. However, there are conflicting reports in regards to the impact of zinc on AKP and bone health, which may be due to differences in the age of animal used in studies and discrepancies between in vitro and in vivo responses (88,90).

TRAP, an iron dependent phosphatase, is another example of a metalloenzyme involved bone metabolism that may be affected by an increase in dietary iron. TRAP is secreted by osteoclasts to resorb the bone matrix and allow for new mineralization by osteoblasts. TRAP contains a diferric center at

its active site that conveys catalytic activity of the enzyme (91,92). In addition, post-transcriptional control of the TRAP gene may also be regulated by iron (93). Therefore, increases in dietary iron may directly impact TRAP and alter bone metabolism in a positive manner. Overall, the participation of metalloenzymes in bone growth and remodeling may respond to changes in their regulatory minerals and influence skeletal tissue dynamics. We hypothesize that dietary phytase impacts skeletal tissue via liberation of bone-affecting metoalloenzyme minerals from phytate, creating a novel mineral environment that may be advantageous during skeletal tissue development.

The release of inositol phosphates from phytate degradation is another possible mechanism for phytase to improve whole bone strength. Inositol phosphates are important cellular signaling molecules and are involved in a numerous pathways. The increase in inositol derivative from phytate degradation may hyperactivate a pathway to improve bone health. For example, the inositol-1-4-5 phosphates pathway is important for intracellular calcium release, a prerequisite for the expression of bone matrix proteins, such as collagen and fibronectin (69,70). The presence of inostiol phosphates may further activate such an inositol pathway, improving the material composition of bone, thus improving whole bone strength.

### 5.3 Future Direction

Additional studies should extend the feeding trial to determine if improvements in bone by phytase supplementation are sustainable as the animals mature. Also, it may be important to examine dietary phytase supplementation in older animals, or an osteoporotic animal model in order to identify the skeletal growth period when supplementation is most effective. Overall, it is critically important that we gain better insight into the nutrition requirements for the optimal growth and maintenance in order to develop simple diet-based strategies that will favor optimal skeletal development and growth, leading to better bone health in the animals and humans.

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