Effects of dietary phosphorus and calcium levels and phytase supplementation on bone metabolism of egg-laying hens

by

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ABSTRACT

The importance of this Ph.D. thesis work is to assess the effects of dietary available phosphorus (avP) and calcium (Ca) levels and phytase supplementation in egg-laying hens and broilers. Two trials were conducted to evaluate the effects of marginal, moderate, and severe reduction in dietary avP and Ca levels with or without phytase supplementation on performance, eggshell quality, and bone metabolism of egg-laying hens and one trial was conducted to study the phytate-degrading efficacy of two phytases at two doses in broilers. In trial 1, 456 laying hens (4 per cage) were fed one of three diets from 30 to 70 weeks of age (woa). The diets were (1) a positive control (PC), based on primary breeder recommendation for nutrient specification, (2) a negative control (NC; the PC with avP and Ca levels marginally reduced by 0.146 and 0.134% of the diet, respectively); and (3) the NC supplemented with 300 FTU/kg phytase (NC+PHY). Egg production, BW, feed intake, FCR, and eggshell quality from 30 to 70 woa, and apparent ileal digestibility of P and Ca, and the majority of bone quality measured were not affected by diet. In trial 2, 100 hens were fed one of five diets from 68 to 78 woa. The diets were (1) a PC with 0.35% avP and 3.5% Ca, (2) a NC1, the PC moderately reduced in avP and Ca by 0.187 and 0.159% of the diet, respectively, and (3) a NC2 which had the PC severely reduced in avP and Ca by 0.231 and 0.275% of the diet, respectively. Other diets for the trial 2 were the NC1 and NC2 supplemented with 600 FTU/kg phytase (4) NC1+PHY and (5) NC2+PHY. Egg production and FCR were maintained by the NC1 but were 11.9% lower and 12.3% higher, respectively, by the NC2 than the PC, which was alleviated by the supplemental phytase. Diet effects on feed intake and eggshell quality traits followed a similar pattern. Body weight was 2.9% lower for NC1, and 6.1% for NC2 than the PC and the phytase use in the two diets alleviated the decreased BW. Cortical bone mineral density was 1.6% higher in PC hens than in NC2 hens, and 3D medullary

bone volume to non-bone and bone mineral density tended to be lower (P<0.068) in the NC2 hens than in hens fed other diets. The marginal reduction in avP and Ca levels in the NC diet in trial 1 maintained performance, eggshell quality, and bone traits, indicating that the mineral levels in the diet were not deficient, and therefore the efficacy of phytase to alleviate the adverse effect of the deficiency could not be evaluated. The moderate reduction of avP and Ca levels in the NC1 of trial 2 maintained performance with decreased BW and bone quality, which were alleviated by phytase addition in the diet to determine efficacy of phytase in mineral-reduced diet on bone metabolism. The severe reduction of avP and Ca levels in the NC2 decreased performance, eggshell quality and bone properties, and phytase supplementation alleviated the adverse effects all parameters with only partial alleviation on the densitometry and micro-architectural bone traits. For the phytate-degradation trial, 1,890 broilers were fed one of seven diets. The diets included a (1) PC; the PC reduced in avP and Ca by 0.146 and 0.134% of the diet respectively (2; NC1) or by 0.174 and 0.159% of the diet, respectively (3; NC2), and (4 and 5) NC1 + PHYA or PHYB at 500 FTU/kg and (6 and 7) NC2 + PHY A or PHYB at 1,000 FTU/kg. Result of the study validated that the efficacy of phytases to dephosphorylate phytate and increase P and Ca availability differ and that the higher dose was more effective than the lower dose. Overall, high and deficient dietary avP and Ca levels lowered quality of bone tissues; however, phytase supplementation in avP- and Ca-deficient diet degrades phytate to increase the minerals availabilities in a dose- and phytase type-dependent manner and subsequently maintain fortified bone in poultry.

PREFACE

This thesis, an original work by Abiodun Bello, was completed with a series of research trials, lab and statistical analysis, and thesis writing. The trials were conducted with approval of the Animal Care and Use Committee: Livestock of the University of Alberta and the animal handling procedure followed were consistent with the Canadian Council on Animal Care guidelines (CCAC, 2009). Three trials were conducted and presented in Chapter 2, 3, 4, and 5 of this thesis. The protocols for the Trials I, II, and III were AUP00000161, AUP00000161, and AUP00001880, respectively. Data from Trial I were presented on Chapter 2 and 4, Trial II presented on Chapter 3 and 4, and Trial III in Chapter 5 The barn work and the lab analysis for each of Trials I, II, and III were conducted between 08/2013 to 09/ 2016. Part of the data generated from this work had been published in the 2014, 2015, 2016, and 2017 proceedings of Poultry Science Association Annual and in the 2016 World Poultry Congress scientific meetings.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

2D - 2-dimensional

3D - 3-dimensional

AA - amino acids

AIA - acid insoluble ash

AIDCa - apparent ileal digestibility of calcium

AIDP - apparent ileal digestibility of phosphorus

Arg - Arginine

ATP - adenosime triphosphapte

avP - available phosphorus

BBS - bone breaking strength

BMD - bone mineral density

BCSA - bone cross-sectional area

BMC - bone mineral content

BPo - bone porosity

BSp - bone separation

BTh - bone thickness

BFD - bone fractial dimention

BMMI - bone maximum moment of inertia

BSP - *Buttiauxella sp.* Phytase

BVol - bone volume

BVTV - bone volume to tissue volume

BW - body weight

Ca - calcium

CAD – Canadian dollar

CBMD - cortical bone mineral density

CBMC - cortical bone mineral content

CTAn - computed tomography analyzer

d - day

doa - day of age

ECP - *Escherichia coli* phytase

FCR - feed conversion ratio

FTU - phytase activity unit

h - hour

Ile - Isoleucine

Lys - Lysine

IP - inositol phosphate

ME - metabolizable energy

Met - Methionine

Met+Cys - Methionine + Cysteine

Micro-CT - micro computed tomography

Na - Sodium

NC - negative control

P - phosphorus

PC - positive control

PHY - phytase

QCT - quantitative computed tomography

ROI - region of interest

Thr - Threonine

Trp - Tryptophan

Val - Valine

VOI - volume of interest

wk - week

woa - week of age

1. LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1. Functions of bone in laying hens

Bone is essential for various functions in laying hens. The skeleton of birds, as in other vertebrates, is composed of bones with different shapes, sizes, and tissue compositions. Despite these diversities, the primary functions of bone are to provide the body with structural support and to aid locomotion (Kerschnitzki et al., 2014). Each bone may also have specific secondary and even tertiary functions, in addition to its primary role. The skull, vertebrae, rib-sternum bones, and pelvic girdle provide the body with protective functions by housing the brain, spinal cord, thoracic and pelvic viscera, respectively. Most of the long bones, including the femur, tibia, tarsometatarsus, and humerus provide a hematopoietic function (Glick, 1986). Also in vertebrates, specific bones, the malleus, incus, and stapes, transmit sound in the middle ear (Turner, 2006). Additionally, bone house non-collagenous proteins including proteoglycans and glycosaminoglycans, and marrow adipose tissue. Proteoglycans and glycosaminoglycans form extracellular matrices and are involved in the healing of bone fractures in mice (Song et al., 2006) and mucosal wounds in humans (Oksala et al., 1995). However, the relationship between the non-collagenous proteins within and outside the bone is not clear. Bone also plays an essential role in the regulation of blood Ca and P levels via the mechanism of bone remodeling (Shahnazari et al., 2006). Bone remodeling is the mechanism of sequential bone formation by the bone-forming cells, osteoblasts, and bone resorption by the bone-resorbing cells, osteoclasts (Johnsson et al., 2015). Bone in laying hens, as in other vertebrates, provide more than structural support or facilitation of locomotion.

Avian bones are essential for flight. Broiler chickens are not capable of flight (Paxton et al., 2013) while laying hens are capable of short distance flight (Nasr et al., 2012). Overall, bones in birds that capable of flight are strong to support their body mass, yet light to aid flight. The avian skeleton is composed of fewer bones and weighs less as a proportion of BW than the mammalian skeleton (Dumont, 2010). Dumont (2010) also reported that bones in birds are light weight but proportionally denser compared to rodents bones. The light weight of the avian skeleton is in part due to pneumatization, the presence of hollow space within specific bones of birds. The skull, clavicle, keel, humerus, lumber and sacral vertebrae, and pelvic girdle in birds are partly air-filled (Jacob, 2015). Heavy meat producing birds such as broilers are largely incapable of flight, in part because of their poorly feathered, heavy body and their adipose tissue-filled bone (Paxton et al., 2013). Overall, because of the high deposition of adipose tissue, the majority of broiler bones are poorly pneumatized and this contributes to the inability to fly. However, laying hens can fly short distances, in part because of their well-feathered, light body and the greater skeletal pneumatization (Nasr et al., 2012).

Medullary bone tissue in egg-laying birds functions as a labile mineral storage in support of eggshell formation (Dacke et al., 1993). The formation of this bone is dependent on a surge in plasma estrogen in preparation for seasonal egg formation in wild birds (Squire et al., 2017) and persistent and long-term egg formation in commercial laying hens (Fleming et al., 1998). The primary component of an eggshell is calcium carbonate (Murakami et al., 2007), and its inflow into the shell gland is necessary for eggshell formation. Hence, the medullary bone tissue provides Ca for the formation of eggshell in the absence of dietary Ca (Dacke et al., 1993). Egg formation takes about 24 to 25 h (Samiullah et al., 2016), and requires a high rate of medullary bone

remodeling within the endosteal cavity to support the eggshell formation (Kerschnitzki et al., 2014).

1.1.2. Current bone-related issues in laying hens

In laying hens, skeletal physiology is uniquely linked to eggshell formation. However, while all bones are predominately composed of cortical and trabecular tissues, bones like femur, tibia, and tarsometatarsus and scapula also contain medullary tissue (Kerschnitzki et al., 2014). The cortical and trabecular bone tissues provide strength, and although medullary bone may also provide resistance to bone fracture, it is primarily a non-structural, labile Ca reserve in support of eggshell formation (Fleming et al., 1998). The metabolism of cortical and trabecular bone tissues is essential to ensure healthy bone, while the metabolism of the medullary bone tissue is vital for persistent and long-term egg production cycle in laying hens. Laying hens are kept from 18 to 70 wk of age (woa) to produce approximately 305 eggs in Canada (Agriculture and Agri-Food Canada, 2017), and beyond 100 woa to produce over 500 eggs over the typically longer production cycles used elsewhere in the world (Pelicia et al., 2009). Although medullary bone is formed and resorbed to support eggshell formation, the structural bone can be resorbed but cannot be formed in active egg-laying hens (Dacke et al., 1993). Over long laying cycles, the gradual resorption of structural bone tissues without the possibility of being replaced can cause osteoporosis in hens. Osteoporosis was observed in caged and aged laying hens managed on long-term Ca- and avP-adequate diets (Wilson and Thorp, 1998). Osteoporosis was also observed in laying hens fed a Ca-deficient diet from 18 to 45 woa (Cransberg et al., 2001). If dietary Ca and P levels are severely deficient, laying hens may be susceptible to bone fracture or caged lager fatigue. Caged layer fatigue in laying hen is a severe loss of bone mass and mineralization (Fasanmi et al., 2014), and is characterized by the inability to walk, brittle bone, paralysis, and death (Freire et al., 2003).

Earlier this century, approximately 30% of commercial laying hens suffered bone fractures or caged layer fatigue over their laying cycles, particularly at the end of the laying cycle (Whitehead and Fleming, 2000). Over the years, primary breeders have genetically selected for hens with high egg-production and resistance to osteoporosis and bone fracture (Bishop et al 2000; Guo et al., 2017). However, the observation of osteoporosis and bone fracture in laying hens (Lay et al., 2011; Fasanmi et al., 2014; Kajlich et al., 2016; Guo et al., 2017) indicated that the conditions were still issue of concern in laying hens in both cage or cage-free systems. The drive to decrease the susceptibility of laying hens to osteoporosis and bone fractures has led to managing hens in a cage-free environment to allow them to exercise. Housing laying hens in facility that allows them to engage in physical activity has been attributed to increase in bone mass (Jahja et al., 2012). Cage-free systems have been adopted in the majority of European countries and gradually in some places in America. Although hens are permitted the freedom to walk around, socialize, and engage in flight for exercise, significant bone-related issues are still very common in hens managed in cage-free systems. The ability to fly also subjects hens to keel deformities and bone breakage by flying into objects within the pen. The keel is the bone primarily affected by deformity or fracture in cage-free hens, and over 40% of laying hens suffer from keel-related issues at one point or the other during the laying cycles (Freire et al., 2003; Kajlich et al., 2016). Hence, regardless of housing system, bone-related issues remain a problem for laying hens.

1.2. BONE FORMATION AND DEVELOPMENT IN LAYING HENS

The formation and development of bone in laying hens are similar to that of other avian species. Mammalian embryos are formed in the maternal host. However, avian embryos develop outside of the hen. Formation and development of the skeletal system in chicken embryos begins during incubation (Hammond et al., 2007) and continues into the pullet phase for laying hens.

Bone formation and development in laying hens involves both membranous and endochondral ossification, each of which requires calcification of soft tissue (Johnsson et al., 2015). Also, healing of bone fractures involves membranous ossification. However, bone formation, development, and healing of bone fracture involve osteoblasts and osteoclasts.

1.2.1. Osteoblasts and osteoclasts

Osteoblasts are mono-nucleated cells that form bone matrix. Osteoblasts differentiate directly from mesenchymal cells for membranous ossification and from chondroblasts, which also differentiate from the mesenchymal cells, for endochondral ossification (Cheng et al., 2016). The bone matrix is primarily made up of collagen and non-collagen proteins, water, and hydroxyapatite, the mineral matrix (Rath et al., 1999). Osteoblasts form the bone matrix in two steps, the formation of osteoid and subsequently, mineralization. The osteoid is a mix of collagen type I, which is the main component of the bone matrix, and osteocalcin and proteoglycan (Jiang et al., 2013). The mineralization step involves the formation of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], over the collagenous bone component to complete formation of the bone matrix (Shahnazari et al., 2006). The activities of osteoblasts to take in Ca and P for mineralization of bone matrix are regulated by the parathyroid hormone and estrogen receptors on the cells (Ohashi et al., 1991). Osteoclasts are multi-nucleated cells that break down the bone matrix to release the organic and inorganic components (Kerschnitzki et al., 2014). The precursors of osteoclasts are mono-nucleated blood cells, monocytes, and macrophages (Dacke et al., 1993). The differentiation of mature osteoclasts from these precursors is regulated by the activities of 1,25-dihydroxycholecalciferol, parathyroid hormone, and estrogen receptors on osteoblasts (Schlesinger et al., 1997). Osteoclasts in the bone of laying hens have estrogen-specific receptors, through which the bone cells are continuously regulated (Pederson et al., 1997). Similarly, the activities of mature osteoclasts in bone resorption are regulated by osteoblasts and 1,25-

dihydroxycholecalciferol, parathyroid hormone, calcitonin, and prostaglandin (Dacke, 1999). While 1,25-dihydroxycholecalciferol and parathyroid hormone stimulate bone resorption for the release of Ca and P into circulation, calcitonin and prostaglandin inhibit osteoclastic activity to stop bone resorption (Sugiyama and Kusuhara, 1996). Structurally, active osteoclasts are characterized by the high number of nuclei, cell spread area, and ruffled border exhibited by the cell (van de Velde et al., 1984). Osteoclasts attach to the bone surface with the ruffled border and initiate resorption through acidification of the bone tissue (Schlesinger et al., 1997). The synchronized activities of osteoclasts and osteoblasts determine the rate of bone remodeling, and over time, the rate of bone turnover.

1.2.2. Membranous ossification

Membranous ossification involves a direct calcification of condensed mesenchymal cells. Formation and development of bones such as the cranium, ribs, and vertebrae are mainly dependent on membranous ossification (Figure. 1.9.1). Formation of membranous bones initiates with the mesodermal stem cell layer giving rise to neural crest-derived mesenchymal cells, which subsequently condense into a localized, cloudy aggregation of cells (Eames et al., 2004) and eventually differentiate into osteoblasts. The differentiated osteoblasts finally start producing osteoid in the shape of the future bone. Mineralization of the osteoid begins in the developing embryo by the 11th d of incubation with the mobilization of Ca from the eggshell through the chorio-allantoic and influx of P from the yolk through the blood vessels (Coleman, 1970). The mobilization of this mineral is dependent on 1,25-dihydroxycholecalciferol (Ono and Tuan, 1991). As with other avian bones, bones formed by membranous ossification also grow in size as bone development continues.

1.2.3. Endochondral ossification

Endochondral ossification involves a stepwise conversion of condensed stem cells into cartilage and eventually to the ossified bone. Ossification of long bones such as the femur, tibia, and tarsometatarsus starts at the mid-diaphysis and is primarily dependent on endochondral ossification (Figure. 1.9.1). Endochondral ossification begins with the aggregation of the mesenchymal cells of the lateral mesodermal plate or the sclerotome of the paraxial mesoderm-derived somites into a pack of cells, which condense and eventually differentiate into cartilage-forming cells, chondroblasts. The chondroblasts undergo division and hypertrophy to form cartilage (Roach, 1997). The cartilage formed in a typical long bone has three distinct longitudinal layers, each of which differs from the other based on cell density (Thomazy and Davies, 1999). As development continues, the three longitudinal layers of cartilage become more distinct from each other, giving rise to two outer cylindrical bone models and an inner rod-shaped model (cross sections illustrated in Figure 1.9.2). The majority of the cartilage cells in the two outer cylindrical bone models differentiate into osteoblasts, with the outer one developing into the periosteum and the inner one developing into the endosteum of the growing long bone (Cheng et al., 2016). The osteoblasts of the developing periosteum and endosteum of the long bones then begin formation of osteoid, the collagen type I bone matrix. In a non-organized pattern, some of the cartilage cells within the two outer cylindrical bone models disintegrate to create blood vessels and lacuna space within the future bone (Roach, 1997).

The periosteum and endosteum are lined with longitudinal and perpendicular blood vessels allowing for a constant exchange of minerals and hormones between bone and the systemic circulation (Seymour et al., 2012). The endosteum develops into cortical bone tissue in the embryo, which continues into the pullet phase. Similarly, the cartilage on the proximal and distal ends of long bones develops into trabecular bone in the embryo, which also continues into the

pullet phase. The bone osteoid is gradually mineralized, beginning at the mid-diaphysis in long bones from approximately 10 d of incubation (Romanoff, 1960). The process of Ca influx into the forming bone is the same for both endochondral and membranous ossification, and while some P is supplied from the yolk, phosphate released as part of the by-product of cartilage disintegration is also used in the ossification process (Roach, 1997). During ossification of the endosteum, some osteoblasts are trapped within bone tissues, are mineralized within the bone matrix, and lose their bone-forming function. These cells are referred to as osteocytes and serve as communication channels between two or more osteoblasts through extra-cellular receptors (Franz-Odendaal et al., 2006). Contrarily, the inner rod-shaped cartilage disintegrates gradually starting around the mid-diaphysis and continues to other part of the bone's future shape randomly to eventually create the endosteal space within each bone (Roach, 1997; Thomazy and Davies, 1999). Cartilage disintegration occurs through apoptosis, the programmed death of the cartilage cells and gradual disappearance of the residual cartilage, chondromucoid, water, and other substances within the space (Roach, 1997). The hollow space created is later partially filled by non-collagenous protein including proteoglycans and glycosaminoglycans and bone marrow during the pullet phase, and by medullary bone tissue during the egg-laying period. An overview of the sequence endochondral bone formation and development is illustrated in Figure 1.9.3.

1.3. BONE METABOLISM IN LAYING HENS

Overall, skeletal tissue metabolism in laying hens is tightly regulated by blood Ca and P concentrations and estrogen, calcitonin, parathyroid hormone, prostaglandin, and 1,25-dihydroxycholecalciferol (Dacke et al., 1993). While all of the bones in egg-laying birds are comprised of cortical and trabecular tissues, long bones such as the femur, tibia, and tarsometatarsus, and flat bones such as the scapula also contain medullary bone tissue. In the femur of laying hens, cortical and medullary bone tissues are primarily concentrated in the mid-

diaphysis (Dacke et al., 1993; Kerschnitzki et al., 2014) while the trabecular bone tissue is mainly located towards the proximal and distal ends (Barak et al., 2010). However, the distribution and volume of the three bone tissues vary in each bone. It is important to elaborate on the activities of blood Ca and P concentrations and the hormones, and the activities of osteoblasts and osteoclasts in the different bone tissues in the skeleton of active laying hens to understand bone metabolism in laying hens.

1.3.1. Blood concentration of Ca, P and regulatory hormones

Calcium is required for the formation of bone and eggshell, as well as the formation and contraction of muscle fiber, and is an essential factor in intrinsic and extrinsic blood coagulation mechanisms, among other functions (Dacke et al, 1993; Dacke, 1999). In laying hens, Ca is concentrated in bone and is mobilized into the shell gland for eggshell formation. Shahnazari et al. (2006) reported that total Ca levels were 5.72 mM in plasma, 424 mg/g of bone ash, and 728 mg/g of eggshell ash in laying hens fed Ca-adequate diets. Likewise, P is also required for the formation of bone, serves as an essential component of ATP, but is minimally involved in eggshell formation (Kebreab et al., 2009). Approximately 80% of body P is in bone (Taylor, 1960). Maintenance of adequate blood levels of Ca and P is essential to help ensure blood Ca-P balance. The levels of Ca and P in blood increase through dietary intake of the minerals and bone resorption. On the other hand, blood Ca and P levels decrease through the mineral deposition into medullary bone, the use of Ca in eggshell formation, and urinary excretion of excess P (Pelicia et al., 2009). While excess Ca may also be excreted through urine in non-laying hens, the high need of Ca for formation of eggshell, bone, and other functions influences the pattern and decreases the amount of urinary Ca excretion in active laying hens (Buss et al., 1980). The absorption of Ca and P into systemic circulation, the blood mineral levels balance, and their deposition rate into bone are regulated by the synchronized activities of 1,25-dihydroxycholecalciferol and parathyroid

hormone (Dacke, 1999). Similarly, the influx of Ca into the shell gland for eggshell formation and the urinary excretion of excess circulating P are also tightly controlled by these hormones.

Fundamentally, Ca is deposited in bone as calcium phosphate (Kerschnitzki et al., 2014), and in the eggshell as calcium carbonate (Murakami et al., 2007). Eggshell formation takes 18 to 20 h out of the approximately 25 h required for egg formation (Samiullah et al., 2016). Approximately 125 mg per h of total Ca is mobilized from the diet or bone resorption into the blood to meet the demand for about 2.5 g of Ca required to form an eggshell (Taylor, 1970). Decreased serum Ca increases the secretion of parathyroid hormone into the blood, while increased blood Ca decreases the secretion of parathyroid hormone (Dacke, 1999). Elevated serum parathyroid hormone increases renal and intestinal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol (Pelicia et al., 2009). The 1,25-dihydroxycholecalciferol aids the intestinal absorption and systemic mobilization of Ca and P. Relative to laying hens fed a Ca-adequate diet, those fed Ca-deficient diets had higher blood parathyroid hormone levels (Singh et al., 1986) and a higher rate of renal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol (Elaroussi et al., 1994). However, an increase in blood parathyroid hormone level increases the urinary excretion of P. Elaroussi et al. (1994) reported over 70% higher renal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol in hens at 22 woa relative to 120 woa, indicating that efficiency of hens to metabolize Ca and P decreases with age.

1.3.2. Cortical, trabecular, and medullary bone tissues

Cortical bone is the compactly arranged bone tissue that primarily provides strength to the skeleton of laying hens. Cortical bone is composed of osteons aggregated together to form longitudinal rod-like structures, the lamellae, which are held together to form the concentric lamella (Bala et al., 2015), and is found in all vertebrate bones (Reich and Gefen, 2006).

However, the shape of cortical tissue varies from bone to bone. While the bone tissue exhibits a cylindrical shape in the femur and tibia, the shape is flat in the scapula and pelvic bone. Following membranous or endochondral formation of cortical bone, it undergoes continuous resorption and formation to allow for growth of the bone during the pullet phase (Whitehead, 2004). Cortical bone is remodeled throughout the lifetime of birds, except in active laying birds. In active laying hens, while the cortical bone tissue can be resorbed, it cannot be re-formed. The attainment of sexual maturity is triggered by a surge in estrogen release, which halts cortical bone formation and induces the osteoblasts to form medullary bone instead (Dacke et al., 1993). Hence, the formation of the cortical bone tissue stops until estrogen level drops during a molt, and the hens go out of lay (Whitehead, 2004). However, because osteoclast activity in the endosteum continues at random locations, the cortical bone is still susceptible to resorption. Hence, any factor that causes the resorption of cortical bone tissue in active laying hens can pre-dispose the birds to osteoporosis and bone fracture.

Trabecular bone is a cancellous structure that also provides strength and it is found in virtually all bones in the laying hen (Reich and Gefen, 2006). However, although trabecular bone is located in the endosteal space, the location and shape differ from bone to bone. Trabecular bone is primarily concentrated towards the proximal and distal ends of long bones and within the short, irregular, and flat bone types (Barak et al., 2010). The activities of osteoblasts and osteoclasts in trabecular bone tissue are similar to those in cortical bone from the onset of sexual maturity and the surge in estrogen level. Structurally, the high concentration of trabecular bone tissue at the proximal and distal ends of long bones helps to rigidify the bone structure (Passi and Gefen, 2005). Hence, while the cortical bone tissue is the primary strength-providing and load-bearing bone tissue, trabecular bone also provides internal fortification and strength. The trabecular bone

tissue is located in close proximity of the medullary bone tissue, the labile mineral reserve for eggshell formation (Whitehead and Fleming, 2000). Hence, the high concentration of osteoclasts in the medullary bone may also resorb trabecular bone, indicating a high possibility of gradual loss of structural bone tissue over time. Because trabecular bone is not formed in active laying hens (Fleming et al., 1998), the gradual loss of the bone volume over laying cycle of hens is typical. Thoracic vertebra trabecular bone volume decreased by 25% from 25 to 60 woa in both caged and cage-free laying hens fed nutrient-adequate diets (Wilson et al., 1992). There is no indication that medullary bone is formed in the vertebral column. Hence, the trabecular bone is also gradually lost in bones that do not contain medullary bone.

Medullary bone occurs as spicules formed within the endosteal cavity of bones in some oviparous species. The medullary bone is mostly deposited in long bones such as the tibia, femur, and ulna, but is also found in the scapula, ribs, pelvic bones, metatarsus, and tarsus (Jacob, 2015). Formation of medullary bone in hens is induced by the sexual maturation- and light stimulation-dependent increase in circulating estrogen level (Dacke, 1993). In fact, male birds injected with estrogen developed medullary bone tissue (Miller and Bowman, 1981). Due to a surge in estrogen, osteoblasts switch from formation of cortical and trabecular bone to formation of medullary bone within the endosteum. Medullary bone is primarily concentrated at the mid-diaphysis, but it is also associated with trabecular bone at the distal and proximal epiphysis of long bones because its formation is not specific to a particular site (Dacke et al., 1993). Injection of estrogen into egg-laying Japanese quail increased the number of osteoblasts and decreased the number of osteoclasts within the medullary bone in the femur (Ohashi et al. 1987), indicating higher formation and lesser resorption of bone. Because of the physiological need for the medullary bone to support eggshell formation, it undergoes rapid remodeling, showing a high rate of bone metabolism.

Through constant remodeling and high Ca mobilization of the medullary bone, laying hens are among the most efficient in metabolizing calcium among vertebrates (Bar, 2009). Laying hens are fed high levels of dietary Ca, which is transported directly to the shell gland or stored as medullary bone, depending on whether the eggshell formation process is active or not (Dacke et al., 1993). Laying hens are exposed to 15 to 16 h of light per day, during which they have unrestricted access to feed, and 8 to 9 h of darkness while they only have limited access to feed (Lohmann Tierzucht, 2011). Formation of the complete egg takes about 25 h; however eggshell formation in the shell gland takes about 20 h (Murakami et al., 2007). Limited intake of feed during the 8 to 9 h of darkness results in a shortage of dietary Ca supply relative to requirements. Out of the 20 h of eggshell formation, Ca is provided from the dietary intake for 12 to 16 h of the light period and the bone for 4 to 8 h of darkness (Kerschnitzki et al., 2014). Hence, maintenance of the medullary bone tissue in laying hens with adequate daily intake of Ca and avP is essential to maintain long-term egg production.

1.4. ASSESSMENT OF BONE QUALITY IN LAYING HENS

Approximately 40% of the Ca required to make an eggshell comes from the bone (Kerschnitzki et al., 2014). Because bone Ca released for eggshell formation is partly from cortical and trabecular bone tissues, which are not formed in active laying hens, evaluation of the structural bone quality is essential to monitor welfare of the birds. Modern egg-laying hens produce egg approximately every 25 hours and lay about 320 eggs in a 52 week laying cycle (Lohmann Tierzucht, 2011). Hens fed Ca- and P-reduced diets maintained long-term egg production and eggshell quality at the expense bone quality (Hughes et al., 2009; Cufadar et al., 2011). Hence, while changes in egg production and eggshell quality are not sufficient to assess the effects of marginal reductions in dietary Ca and avP, assessment of bone quality parameters in laying hens is vital to understand diet effects on bone metabolism.

1.4.1. Bone ash, Ca, and P content and breaking strength

Whole femur ash, Ca and P contents have been commonly used to assess bone quality in laying hens (Cransberg et al., 2001; Hughes et al., 2009; Cufadar et al., 2011; Regmi et al., 2015). The femur is comprised of cortical, trabecular, and medullary bone tissues, and age-related increases of medullary bone may mask treatment-related changes in the other bone tissues (Korver et al., 2004). Because measurements of femur ash, Ca and P contents provide little information on changes in the structural integrity of the bone in laying hens, they are not sufficient to evaluate bone strength, resistance to osteoporosis, and overall bone health. Bone breaking strength is a mechanical assessment of the force required to fracture a long bone, and has been commonly used to evaluate bone quality in laying hens (de Lima et al., 2010; Cufadar et al., 2011). Bone breaking strength can be assessed using either three or four breaking points. Three-point bone breaking strength is assessed with an application of a single-point perpendicular force on the mid-diaphysis of a bone rested on two supports on a strength testing apparatus. The three-point breaking strength specifically tests fracture resistance of cortical and medullary bone tissues, which are concentrated at the mid-diaphysis (de Lima et al., 2010; Cufadar et al., 2011). Four-point bone breaking strength is assessed with application of perpendicular force at two points along the length of a bone rested on two supports on a strength testing apparatus. The three-point bone breaking strength is the easier and the commonly used bone fracture resistance assessment. Overall, bone breaking strength does not provide information on the structural changes of the strength-providing trabecular bone tissues at the proximal and distal metaphyses. The bone at the proximal and distal ends of long bones are less compact but spongier with the relatively higher concentration of the trabecular bone tissue at the site (Figure 1.9.4.). Hence, the bone of distal and proximal epiphysis are more susceptible to fracture than the bone of the mid-diaphysis (Reich and Gefen, 2006). While four-point breaking strength quantifies qualities of the bone as a whole, the assessment

does not provide information on specific changes on each of cortical, trabecular, and medullary bone tissues. Therefore, the relevance of bone breaking strength generally to the risk of osteoporosis is limited. Overall, while bone ash, P, Ca and breaking strength are easy and fast to assess, it is crucial to interpret effects of treatments on these bone parameters in laying hens in light of their respective limitations.

1.4.2. Bone densitometry

Bone densitometry is the use of imaging technology to measure bone mineral density and cross-sectional area of bone (Korver et al., 2004). By multiplying bone mineral density and bone cross-sectional area, bone mineral content can also be calculated (Saunders-Blades et al., 2009). Bone mineral content measures the bone contained within a given volume of bone tissue (Saunders-Blades et al., 2009). Bone densitometry has been measured using different imaging technologies to assess bone quality in laying hens. Bone densitometry measurement by quantitative computed tomography (**QCT**) provides data on bone cross-sectional areas, and mineral densities and content of total, cortical, and trabecular space bone tissues (Korver et al., 2004). Bone densitometry in laying hens has also been assessed with digitized fluoroscopy and ultrasound (Fleming et al., 2004), dual-energy X-ray absorptiometry (Hester et al., 2004), and QCT, (Korver et al., 2004; Kim et al., 2007; Saunders-Blades et al., 2009). However, of these, only QCT can be used to assess total, cortical, and trabecular space bone separately. The trabecular space bone is comprised of both trabecular and medullary bone tissues (Saunders-Blades et al., 2009), although Kim et al. (2007) reported QCT-measured trabecular and medullary bone tissues separately. Kim et al. (2007) assessed the distal and proximal metaphyses for trabecular bone tissue, but did not account for how the medullary bone fraction is closely housed and intertwined with the trabecular bone at the assessed sites was delineated from the structural bone. Because the trabecular and medullary bone tissues are intertwined within the endosteal

space (Whitehead and Fleming, 2000), it is appropriate to refer to the QCT-measured tissue within the endosteum as bone within the trabecular space. Trabecular bone volume was measured within the mid-diaphysis of laying hens femurs using micro-computed tomography (**micro-CT**; Shahnazari et al., 2006), indicating that although medullary bone is mainly concentrated in the mid-diaphysis and trabecular bone towards the metaphyses ends, the two bone tissues are intertwined within the endosteal cavity. Molting via a 9-d feed withdrawal from 86 woa hens completely stopped egg production and decreased the mineral density of both medullary and trabecular bone tissues (Kim et al., 2007). Molting of laying hens decreases blood estrogen causing a complete caseation in egg production and medullary bone formation, but the formation of structural bone is resumed (Dacke et al., 1993). Hence, the parameter reported by Kim et al. (2007) as the trabecular bone tissue likely also contained a considerable fraction of medullary bone. The decrease in what was reported as trabecular bone mineral density, along with medullary bone mineral density following 9 d of feed withdrawal shown by Kim et al. (2007) evidenced that the reported trabecular bone in the study was masked by medullary bone. Overall, QCT is useful to measure total and each of cortical, and the trabecular space bone tissues in the bone laying hens.

1.4.3. Bone micro-architecture

Bone micro-architecture details the volume, thickness, porosity, numbers, arrangements, separation spaces, size, and distribution of structures within each of cortical, trabecular, and medullary bone tissues (Shahnazari et al., 2006; Kerschnitzki et al., 2014). Bone micro-architecture is measured using micro-CT, an imaging technology that also provides densitometry information on the bone tissue (Bouxsein et al., 2010). Micro-CT uses the attenuation of X-ray energy through the bone to cast 2-dimensional images, which are reconstructed into 3-dimensional images (Bouxsein et al., 2010; Wu et al., 2015). Micro-CT can be used to separate structural from

non-structural bone fractions, which makes the technology suitable for evaluating bone quality in egg-laying hens. The use of micro-CT in bone quality assessment involves the segmentation of a region of interest, delineation of the volume of interest, and greyscale thresholding with associated software to aid the separate analyses of cortical, trabecular, and medullary bone tissues. Micro-CT software allows delineation of a region of interest from 3-dimensional bone scans and a volume of interest within a bone region of interest (Bouxsein et al., 2010). Also, this software is also enabled with threshold tool with which segmentation of bone from non-bone objects or two intertwined bone tissues from each other is possible. In a greyscale of 0 ~ 255, the threshold of 70 ~ 255 was used to segment bone from non-bone objects within a region of interest in lumbar vertebrae of mice (Wu et al., 2015). Micro-CT was used to assess the volume, thickness, arrangements, separation spaces, size, and connectivity density of tissues within each of the cortical and medullary bones in the mid-diaphysis of the tibia in laying hens (Shahnazari et al., 2006). Specifically, changes in medullary bone tissue were assessed in laying hens with micro-CT (Kerschnitzki et al., 2014). Also, micro-CT was used to analyze trabecular bone in femur, tibia, and humerus of laying hens (Martinez-Cummer et al., 2006; Vaughan et al., 2016; Regmi et al., 2017). However, the 0 ~ 255 greyscale thresholding used for the separation of each bone tissue in these previous studies were not provided.

1.4.4. Serum biochemical bone markers

The blood concentrations of some bone formation or resorption by-products are used as biomarkers to quantify the levels of bone turnover. Osteocalcin is a non-collagenous protein produced by osteoblasts and released into the blood as a by-product of bone formation. Serum osteocalcin has been used to quantify osteoblastic activity in poultry (Jiang et al., 2013 and Regmi et al., 2015). Osteocalcin plays a role in bone mineralization by aiding the interaction between osteoblasts and osteoclasts in mice, however, the exact functions of osteocalcin in these processes

have yet to be validated (Wei and Karsenty, 2015). Blood osteocalcin has been positively correlated to bone mineral density and osteoblast numbers in humans (Bharadwaj et al., 2009). Pyridinoline is a collagen type I crosslink compound that is broken down by osteoclasts and released into systemic circulation. Serum pyridinoline level has been assayed to assess osteoclastic activity in laying hens (Regmi et al., 2015). Serum pyridinoline level was positively correlated to resorption of type 1 collagen in humans (Urena et al., 1995). Overall, serum osteocalcin and pyridinoline enable estimation of bone formation and resorption levels, respectively, making them important markers of bone metabolism in poultry.

1.5.1. Dietary levels and digestibility of Ca and P

Calcium and P are added to poultry diets at macro levels. Dietary Ca and P are the two most abundant minerals in the body of laying hens (Pelicia et al., 2009). Calcium and P are often discussed together because of the inter-dependency of the dietary, absorption, and excretion levels and the post-absorption physiological usage of the two minerals (Kebreab et al., 2009). Commercially, dietary Ca and avP levels are formulated based on the recommendation of primary breeders (e.g., Lohmann Tierzucht, 2011; H&N International, 2016). High dietary Ca levels are fed to laying hens to support the eggshell formation and medullary bone remodeling. Recommended dietary Ca for laying hens ranges from 3.50 to 4.20 g/day (Lohmann Tierzucht, 2011). Deficient dietary Ca and avP are detrimental to performance and eggshell quality of laying hens (Cransberg et al., 2001) and excess dietary Ca level also adversely affects Ca and P digestibility (Pelicia et al., 2009). The high dietary Ca level in laying hen diets results in the formation of Ca-phytate complexes in the gastrointestinal tract (**GIT**) of birds, which decreases dietary Ca and P availability and utilization (Pelicia et al., 2009; Beutler, 2009). Structures of phytate and Ca-phytate complex are presented in Figure 1.9.5. The higher the level

of dietary Ca, the greater the formation of the Ca-phytate complexes and the associated adverse effects in the GIT of birds. Increasing dietary Ca from 30 to 40 g/kg (Van der Klis et al., 1997) or from 25 to 55 g/kg (Beutler, 2009) linearly decreased P and Ca digestibility in laying hens by increasing the formation of Ca-phytate complexes. Also, the Ca-phytate complexes and increased pH in the GIT of birds caused by high Ca level decrease the availability of amino acids and energy (Beutler, 2009).

1.5.2. Absorption of Ca and P in the gastrointestinal tract

Dietary Ca and P share similar absorption routes and depend on 1,25-dihydroxycholecalciferol for absorption and utilization (Blahos et al., 1987). Calcium and P are absorbed from the GIT of birds through both transcellular and paracellular mechanisms. The majority of transcellular Ca and P absorption in chickens occurs in the duodenum and upper jejunum, while paracellular absorption takes place throughout the small intestine (Elaroussi et al., 1994). Transcellular absorption is an active process that depends on intestinal calbidins (proteins with 4 active Ca-binding domains) and an ATP-powered Ca pump (Bronner et al., 1986). The paracellular mechanism on the other hand, involves passive absorption. However, both mechanisms are dependent on 1,25-dihydroxycholecalciferol (Blahos et al., 1987). The relative amounts of Ca and P absorbed by either the transcellular or paracellular mechanisms are dependent on the amount of Ca and P available for absorption. With adequate availability of dietary Ca and P, most absorption is via the transcellular route (Blahos et al., 1987). However, with reduced availability of these minerals, the amount of the transcellular absorption is decreased. Laying hens fed a Ca-deficient diet were able to induce a compensatory increase in absorption of Ca and P (Elaroussi et al., 1994) through the paracellular route, particularly in the distal jejunum and ileum. Elaroussi et al. (1994) reported that hens fed a Ca-deficient diet had greater renal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol to

increase Ca absorption than hens fed Ca-adequate diets. Overall, dietary Ca and P are directly linked to the blood levels of the minerals and the circulating 1,25-dihydroxycholecalciferol are essential for the absorption and metabolism of the minerals.

1.5.3. Effects of dietary Ca and available P levels on performance, eggshell quality, and bone properties

The effects of dietary Ca and avP levels on egg performance, eggshell quality, and bone properties of laying hens have been well studied. Overall, the levels of avP and Ca typically used in commercial diets are higher than the minerals requirement in egg-laying hens. Recommendations for dietary avP and Ca levels for laying hen are 0.38 to 0.42 g/day and 4.1 to 4.5 g/day, respectively (Lohmann Tierzucht, 2011). In comparison to the primary breeder-recommended levels, marginal to moderate reductions in dietary avP and Ca did not affect laying hen performance, eggshell quality, nor bone properties. Layer diets with 0.15 or 0.20% avP and 3.0 or 3.8% Ca maintained egg production, feed conversion ratio, and eggshell breaking strength and thickness (Pelicia et al., 2009; Boling et al. 2000). However, Boling et al. (2000) reported that the 0.15% avP diet decreased BW from 41 to 50 woa and bone ash at 70 woa, while the 0.2% avP diet did not. Also, diets severely deficient in avP or Ca decreased volumes and mineral densities of trabecular bone and increased medullary bone and incidence of osteoporosis (Knott et al., 1995; Fleming et al., 1998; Cransberg et al., 2001). Also, a Ca-deficient diet increased blood pyridinoline (Knott et al., 1995). Therefore, laying hens fed diets marginally to moderately deficient in avP and Ca can maintain performance and eggshell quality, but with reduced BW and bone properties. A decrease in egg production and eggshell quality, along with BW and bone quality in laying hens indicate severe dietary avP or Ca deficiencies. Compared to a Ca-adequate diet, a diet with 3.25% Ca decreased bone mass and mineralization and BW of laying hens (Cransberg et al., 2001). Performance parameters such as egg production, egg mass, and feed

conversion ratio and eggshell quality such as shell breaking strength, thickness, and specific gravity are of direct economic importance to the egg industry. BW and bone qualities such as bone mineral density, porosity, and thickness are also of economic importance to the industry. However, they are also of welfare importance to the industry as decreases in BW and bone quality may indicate reduction in adipose tissue and structural bone essential for body reserve (Cransberg et al., 2001). Overall, laying hens possess a high efficiency to physiologically adapt to nutrient deficiency by making use of their soft tissue reserve to support internal egg content formation and use of structural bone to support eggshell formation (Boling et al., 2000). Hence, BW, bone densitometry and micro-architecture, and serum biochemical bone markers are essential for a clearer interpretation of the effects of dietary Ca and avP on laying hens.

1.6. PHYTATE AND PHYTASE IN LAYING HEN DIETS

1.6.1. Phytate in plant-based feed ingredients and diets

Approximately 80 to 90% of laying hen diets are comprised of plant-based feed ingredients, such as cereals and oilseed meals. The majority of phosphorus in these plant-based fed ingredients are bound to phytate (Tahir et al., 2012). Phytate binds P to render it unavailable for absorption. Structurally, phytate, also known as inositol hexaphosphate (IP_6), is characterized by a myo-inositol ring and six phosphates, the metabolites of which are IP_5, IP_4, IP_3, IP_2, IP_1 (Fig. 1.4; Goa et al., 2013; Li et al. 2016). The structure of phytate in different plant-based feed ingredients is similar. However, phytate levels in different plant-based feed ingredients vary depending on cultivation type, season of the year, use and type of fertilizer, soil water holding capacity, plant watering, and period of harvest (Reedy et al 1982; Tahir et al., 2012). While phytate levels in plant-based feed ingredient range from 1 to 10%, phytate levels tend to be higher in cereals than in oilseeds, and in highly fibrous than in less fibrous feed ingredients, and in biofuel by-products such as dried distiller grains than in feed ingredients such as corn (Schlemmer

et al., 2009). Hence, the level of phytate in diets fed to laying hens is dependent on the level of cereals and oil-seed ingredients and the type and levels of ingredients.

Phosphorus is abundant in plant-based feed ingredients, but about 55 to 80% is bound to phytate and is unavailable for absorption by monogastric animals (Tahir et al., 2012). Hence, depending on diet composition, approximately 70% of P in a typical diet is unavailable for absorption by birds (Tahir et al., 2012). In light of this, inorganic P sources such as monocalcium phosphate and dicalcium phosphate are used to meet the dietary avP requirement (Hughes et al., 2009; Pelicia et al., 2009; Saunders-Blades et al., 2009). However, because these inorganic sources of P are getting scarce and are expensive, the dietary use of monocalcium phosphate and dicalcium phosphate substantially increase feed cost (Ponnuvel et al., 2013; Wealleans et al., 2016). Also, because each of the six phytate-bound phosphates is negatively charged, phytate also complexes with Ca as well as most other cations, starch, and amino acids (Amerah et al., 2014; Sommerfeld et al., 2018). Calcium is low in most plant-based feed ingredients, and a considerable fraction of the dietary Ca is also complexed with phytate, hence rendered unavailable for absorption by monogastrics. Ingredients such as calcium carbonate, oyster shell, and bone meal are added in laying hens diet to meet the high dietary requirement for Ca (Hughes et al., 2009; Pelicia et al., 2009; Saunders-Blades et al., 2009). Overall, phytate directly binds P and complexes Ca to decrease the availability of both minerals for absorption and increases excretion into the environment.

1.6.2. Phytase supplementation in laying hen diets

Phytase is an enzyme supplemented in diets to release the phytate-bound P. The ability of phytase to increase utilization of P, Ca, other minerals, and nutrients such as amino acids, lipids, and starch in laying hens diets is well established (Beulter, 2009; Englmaierova et al., 2015). A limited amount of phytase is secreted in the brush border of the GIT of birds (Maenz and Classen,

1998). Since its first commercial use in animal nutrition in 1991 (Lei et al., 2013), phytase production has attracted a significant number of commercial manufacturers. Phytase is produced from various microbial sources, mostly bacteria or fungi. The bacterial sources of phytase include *Escherichia coli*, *Buttiauxella* species, and *Citrobacter braakii*, while the fungal sources include *Aspergillus niger* and *Peniophora lycii,* among others. Phytases also vary in biochemical and biophysical properties (Greiner and Konietzny, 2011). An *in vitro* assessment of seven bacterial or fungal phytases showed variation in pH range of activity and phytate-degrading potentials (Menezes-Blackburn et al., 2015). Menezes-Blackburn et al. (2015) also reported that phytate-degrading activity of the 7 phytases averaged 105.6% at pH 3 and 2.9 at pH 7, indicating that phytase activity significantly decreases with increased GIT pH.

Phytase degradation of phytate begins in the crop and continues through the proventiculus, gizzard, duodenum, jejunum, and ileum of poultry (Beeson et al., 2017, Sommerfeld et al., 2018). The dietary phytase degrades phytate to liberate phosphate and decrease the Ca-P complex formation, thereby increasing Ca and P availability. Overall, optimal pH for maximum activity for the majority of commercial phytases ranges between 3 and 5 (Brejnholt et al., 2011). In laying hens, pH was 4.67 in the crop, 4.45 in the proventriculus and gizzard, and 5.74 in the small intestine (Englmaierova et al., 2014), while Beutler (2009) reported values of 6.07 in the duodenum, 6.15 in the jejunum, and 8.13 in the ileum. In broilers, pH is 5.3 in crop, 1.9 in the proventriculus, 2.5 in the gizzard, 6.1 in the duodenum, 6.5 in the jejunum, and 6.8 in the ileum (Angel et al., 2010). This indicates that the sites of optimal phytase activity in laying hens and broilers are the crop, proventriculus, and gizzard. Overall, phytate degradation by phytase differs in each GIT segment of broilers (Beeson et al., 2017, Sommerfeld et al., 2018), and laying hens (Gao et al., 2013), as the enzyme activity is directly related to the pH. In laying hens, over 53% of

dietary phytate degradation occurred in the crop, proventriculus, and gizzard, while 41% of dietary phytate was degraded in the duodenum, jejunum, and ileum with 500 FTU/kg phytase supplementation (Gao et al., 2013). Hence, high phytate-degrading efficiency in the low pH environment of the upper GIT in laying hens is essential for the overall efficacy of a commercial phytase.

Commercially, phytase is typically added to laying hen diets at 300 or 600 phytase unit/kg (FTU/kg) of feed, with the higher dose allowing for greater reduction in dietary avP and Ca levels relative to the lower dose. Overall, the use of phytase in laying hen diets allows for the use of high-phytate and low-cost feed ingredients for partial substitution of relatively low-phytate and high-cost feed ingredients. Also, the drive to decrease dietary supplementation of expensive inorganic P has in part led to the use of dietary phytase. Hence, phytase supplementation in diets with reduced inorganic P supplementation, along with the use of cheaper ingredients reduces overall diet cost (Ponnuvel et al., 2013) and excretion of Ca and P to increase nutrient efficiency of the birds and sustainability of the environment (Lim et al., 2003). The cost of phytase supplementation is approximately $ 0.5 to 1.6 USD/ton of feed (Wealleans et al., 2016). The supplementation of phytase in avP-reduced diets saves approximately $ 3 to 5 USD/ton of feed (Wealleans et al., 2016). In addition to increased P availability, supplementation of phytase in poultry diets also increases Ca utilization (Beutler, 2009).

Phytase supplementation in avP- and Ca-reduced diets liberates the phytate-bound P to decrease the formation of Ca-phytate complexes, hence increase the availability of the minerals for absorption. The phytase supplementation in avP- and Ca-reduced diets increases apparent digestibility of the minerals (Van der Klis et al., 1997; Sommerfeld et al., 2018), and performance of laying hens (Boling et al., 2000). Similar effects of phytase on bone ash (Hughes et al., 2009)

and breaking strength (de Lima et al., 2010) have been reported in laying hens. Although the ability of phytase to alleviate the adverse effects of reductions in dietary avP and Ca of laying hens is well established, only limited information is available on the effects on bone quality. Knowledge of how phytase supplementation in avP- and Ca-deficient diet alleviates the mineral deficiency in bone quality will be vital to understanding the process by which the enzyme influences overall performance in laying hens.

The mechanism by which phytase influences Ca and P bioavailability, and subsequently affects bone quality traits in laying hens fed avP- and Ca-deficient diets is not well understood. Overall, information on the influence of phytase supplementation in a Ca- and avP-reduced diets on cortical, trabecular, and medullary bone densitometry and micro-architectural traits, as well as serum bone biomarkers in laying hens, is limited.

1.7. RESEARCH APPLICATION

The effects of dietary Ca and avP levels on bone metabolism of laying hens have been well studied, and the mechanisms involved in the impact of dietary minerals on bone are quite clear. Phytase supplementation in Ca- and avP-reduced diets increased bone ash (Hughes et al., 2009) and breaking strength (de Lima et al., 2010). Understanding the mechanisms of how phytase supplementation in Ca- and avP-deficient diets alleviate the adverse effects on bone quality of laying hens requires details beyond bone ash and breaking strength. Assessment of densitometry and micro-architectural traits in cortical, trabecular, and medullary bone tissues, as well as serum bone biomarkers, are essential to determine how supplemental phytase alleviates adverse effects of avP- and Ca-deficiencies. The densitometry and micro-architecture of each bone tissue were measured using QCT and micro-CT imaging technology, respectively. The serum levels of osteocalcin and pyridinoline released into the blood as markers of bone formation or resorption, respectively, were measured. Overall, this Ph.D. thesis was focused on the effect phytase

supplementation in Ca- and avP-reduced diet on bone quality in laying hens. Also, the efficacy of two phytases at two doses to degrade phytate in the crop, proventriculus + gizzard, and distal ileum of broilers was investigated.

1.7.1 Thesis objectives

1) The primary objective of this Ph.D. thesis was to evaluate the effects of phytase supplementation in Ca- and avP- reduced diets on bone biology, laying performance and eggshell quality of laying hens.

2) To validate the relationship between phytate degradation by phytase and digestibility of Ca and P, and bone quality in broilers.

1.7.2 Thesis hypotheses

Four hypotheses were tested in this Ph.D. thesis:

1) The inclusion of *Buttiauxella sp* phytase laying hen diets from 30 to 70 wk of age would alleviate the adverse effects of reduced dietary Ca and avP.

2) Phytase supplementation in Ca- and avP-deficient diets would increase availability of P and Ca to maintain the micro-architecture of femur cortical, trabecular, and medullary bone tissues in laying hens fed Ca- and avP-reduced diets at similar levels as hens fed Ca- and avP-adequate diets.

3) Long-term phytase supplementation in Ca- and avP-deficient diets would increase availability of P and Ca maintain osteoblast and osteoclast activities, as indicated by serum levels of osteocalcin, pyridinoline, PTH, Ca, and P, at similar levels as a Ca- and avP-adequate diet in 78-wk-old egg-laying hens.

4) Degradation of phytate in each of the crop, proventriculus + gizzard, and distal ileum would be dependent on phytase source and dose, and would be directly linked to P and Ca digestibility and bone quality in broilers.

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1.9 FIGURES

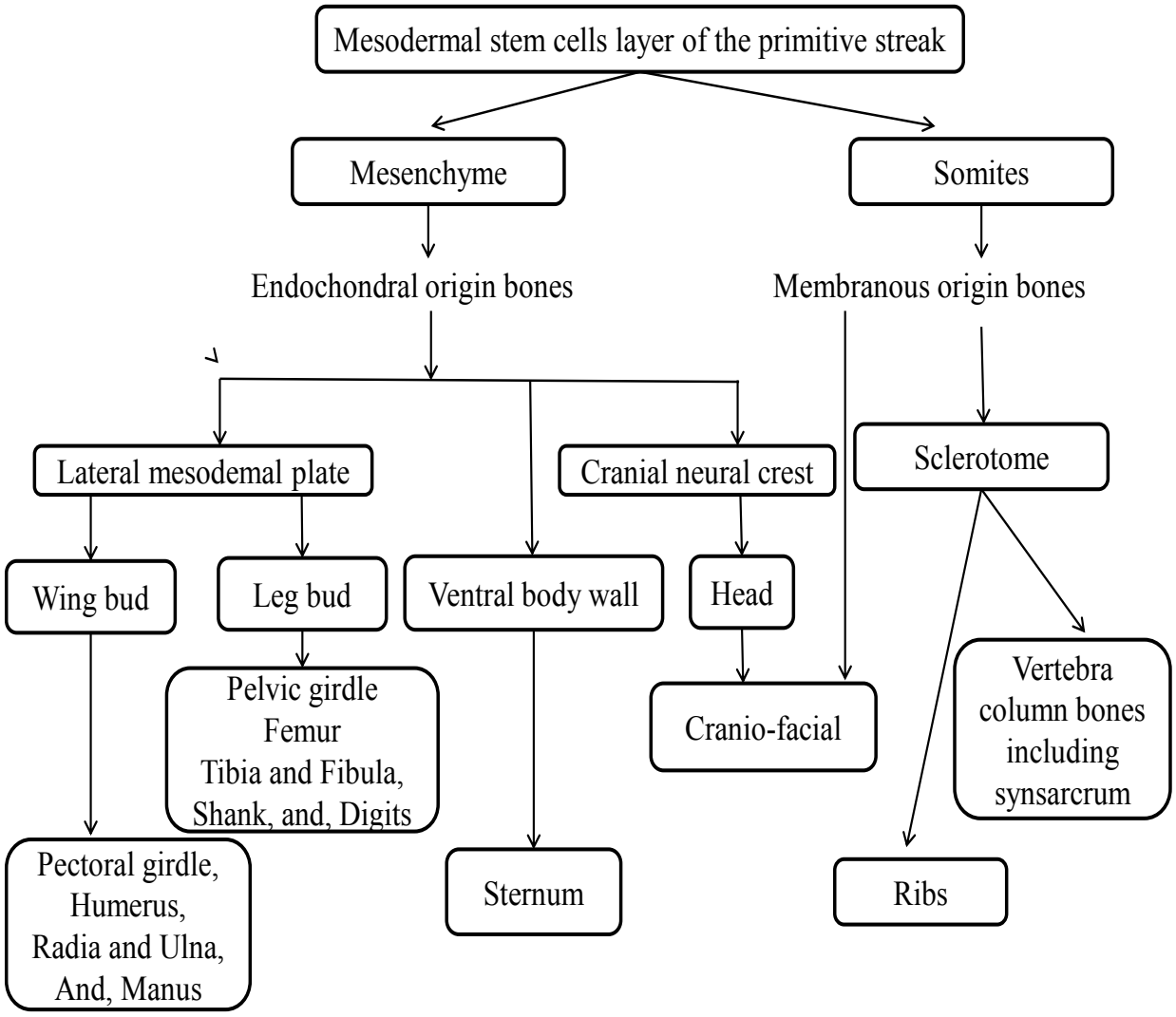
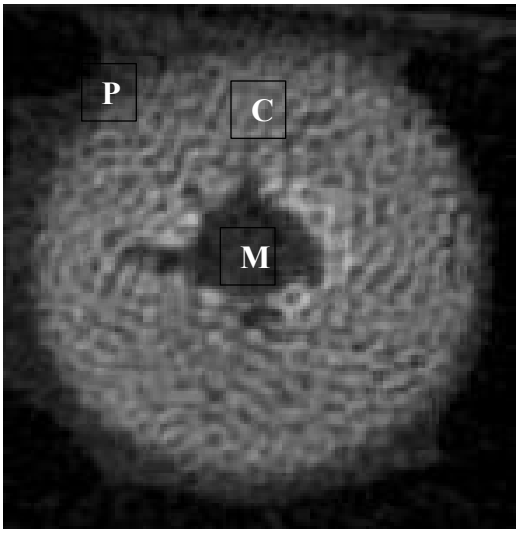
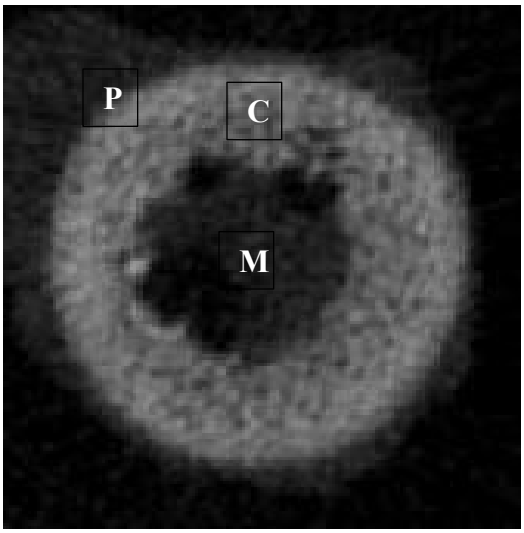


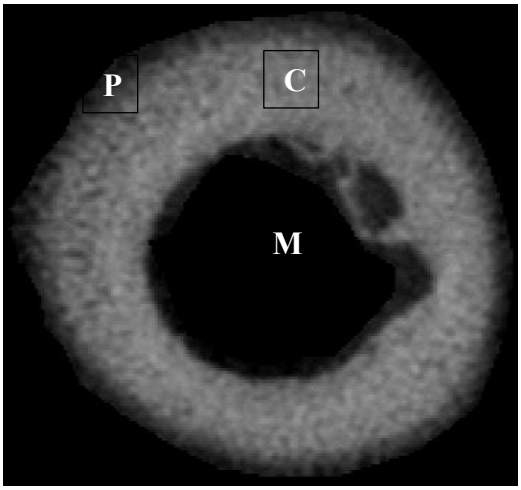
Figure 1.9.1: Skeletal origin, formation, and development of an embryonic chick (Modified from Romanoff, 1960).



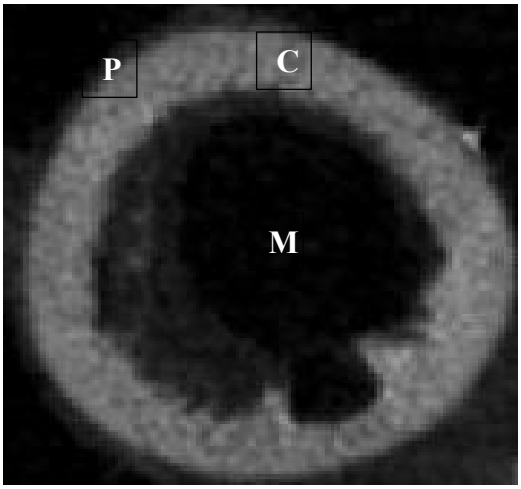
A. Day 15 of incubation



B. Day 20 of incubation



C. Day of hatch



D. Day 7 of post-hatch

Figure 1.9.2: Micro-computed tomography images of mid-diaphysis cross-sections of cartilage differentiation into a thin outmost layer, P (periosteum), and the thick ring-shape, C (cortical bone tissue) and apoptosis of cartilage cells to form M (medullary space) in femur of broiler embryo (in A and B) and chick (in C and D; modified from Torres, 2013).

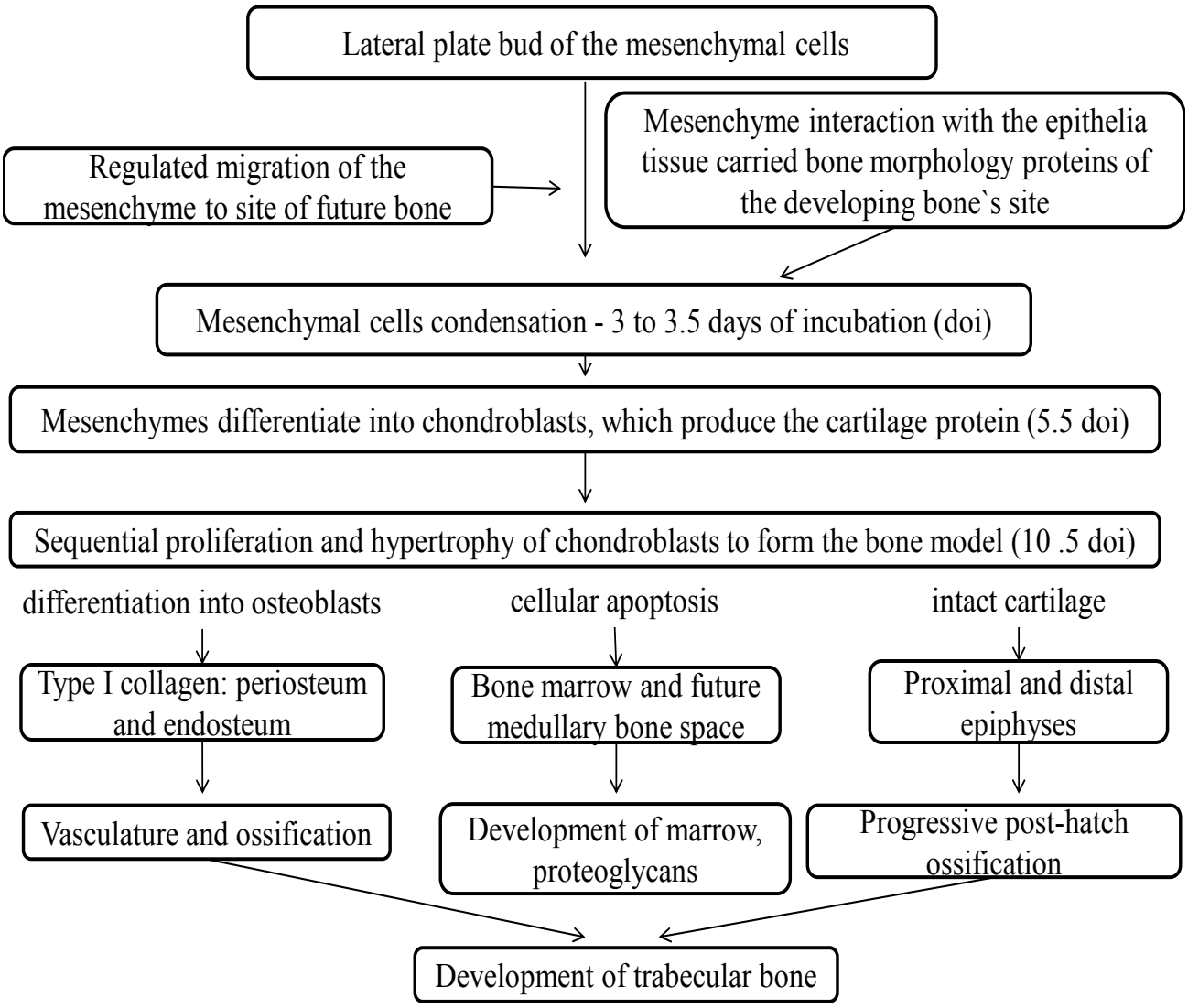


Figure 1.9.3: Schematic overview of embryonic chick`s femur initiation, formation, and development (Modified from Romanoff, 1960).

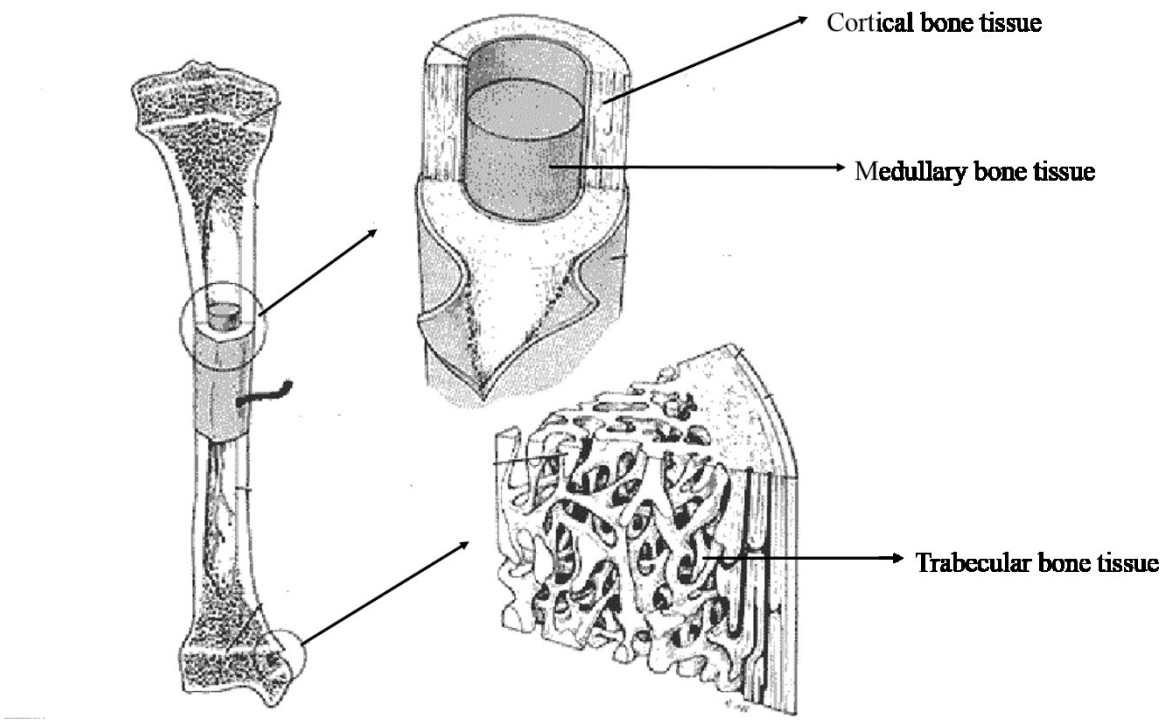


Figure 1.9.4: Illustration of cortical, trabecular, and medullary bone tissues within a long bone of egg-laying hen (Modified from Verma, 2007; https://en.engormix.com/mycotoxins/articles/effects-of-mycotoxins-in-poultry-t33686.htm).

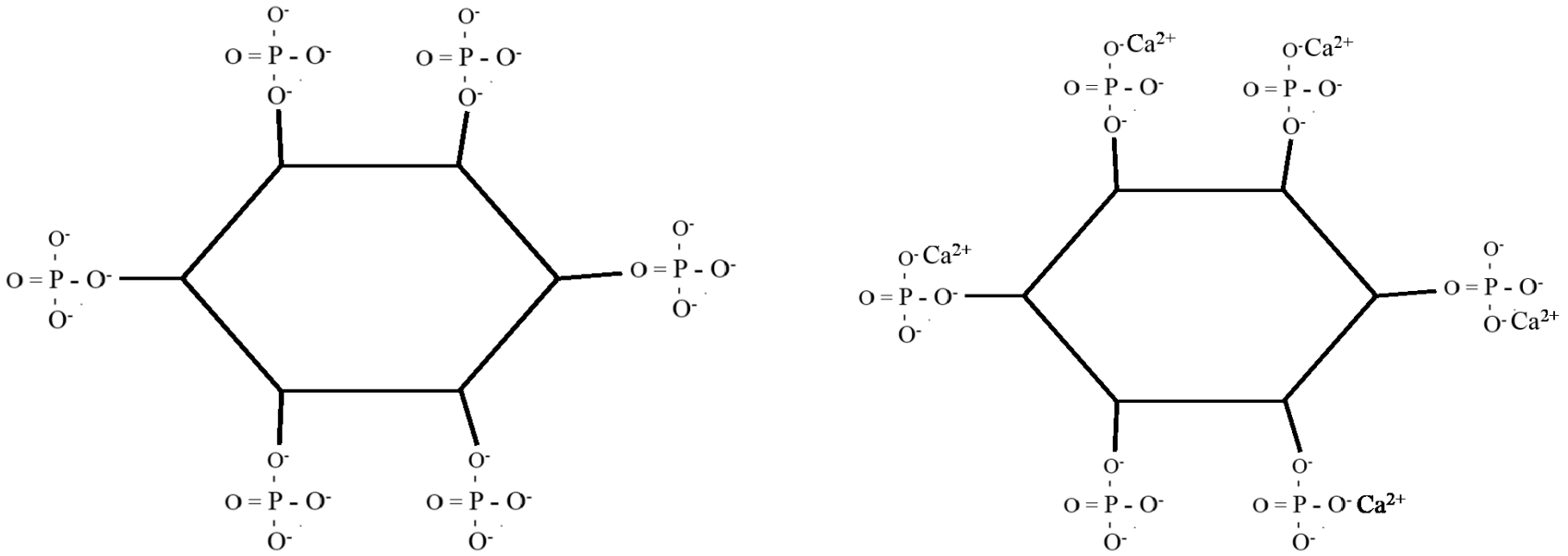


Figure 1.9.5: Structure of phytate with six phosphate molecules chelated to the myo-inositol ring (A) and positively charged Ca ions complexed with phytate by binding to the negatively charged phosphates.

2. LONG-TERM EFFECTS OF *BUTTIAUXELLA* SP. PHYTASE ON PERFORMANCE, EGGSHELL QUALITY, APPARENT ILEAL CA AND P DIGESTIBILITY, AND BONE PROPERTIES OF WHITE EGG LAYERS

ABSTRACT

Adequate dietary Ca and available phosphorus (avP) levels are essential to long-term egg production and bone health in laying hens. The effects of dietary Ca and avP levels and a *Buttiauxella sp*. phytase (BSP) were studied in Lohmann LSL Lite hens from 30 to 70 wk of age (woa). Hens (n=456; 4 per cage) were fed either a primary breeder recomendation based diet (positive control; PC); the PC with avP and Ca levels marginally reduced by 0.146 and 0.134% of the diet, respectively without (NC) or with 300 FTU/kg BSP (NC+BSP). Egg production, BW, feed intake, FCR, and eggshell quality from 30 to 70 woa, and apparent ileal digestibility of P (AIDP) and Ca (AIDCa), and bone quality at 32, 48, and 70 woa were measured. Most parameters were not affected by diet, indicating that the avP and Ca levels in the NC diet were not clinically deficient. Hen BW from 34 to 70 woa tended to be 2.9% greater (P=0.076) for PC and NC+BSP compared to NC. Cortical bone mineral content (CBMC) at the mid-diaphysis tended to be 10% and 9% higher (P=0.065) in the NC+BSP hens than in NC hens at 48 and 70 woa, respectively. AIDP of NC+BSP was 24% greater (P=0.034) than of NC at 32 woa and tended to be 18% greater (P=0.082) than AIDP of PC at 48 woa, and 25% lower than of NC and PC at 70 woa (P=0.028). AIDCa was 25% lower for NC+BSP than PC at 48 woa only (P=0.037). The sufficiency of avP and Ca in the NC diet limited the opportunity to determine a phytase effect. Although the supplemental BSP tended to increase BW and 48 and 70 woa CBMC and increased 32 woa AIDP, the efficacy of BSP could not be determined due to the lack of an NC effect on performance, shell quality, and the majority of bone traits parameters.

Commercial laying hens can maintain health and productivity at lower than recommended levels of dietary Ca and avP; phytase supplementation may allow even further reductions.

Keywords: Available phosphorus, bone, calcium, laying hen performance, phytase

2.1 INTRODUCTION

Adequate dietary Ca and available phosphorus (avP) levels are essential to maximize long-term egg production and bone health in laying hens. Dietary Ca and avP at deficient or excess levels decrease hen performance and compromise the welfare of the bird (Pelicia et al., 2009). Also, the relationship between dietary Ca and avP is complicated because the absorption rate and metabolic regulation of these minerals in hens are inter-related (Bougouin et al., 2014). Interestingly, recommendations for laying hen diets increase in Ca and decrease in avP with increasing hen age. Increasing dietary Ca from 2.5 to 5.5% linearly decreased digestibility of P, Ca, energy, and protein, due to an increase in Ca-phytate complex formation in the gastrointestinal tract of laying hens (Beutler, 2009). Hence, while dietary Ca deficiency decreases performance, eggshell and bone quality in laying hens, excess dietary Ca also decrease availability of P and Ca for absorption.

Approximately 60% of P in plant-based feed ingredients is bound to myo-inositol as myo-inositol phosphates (phytate) and is unavailable for absorption in the gastrointestinal tract of birds (Tahir et al., 2012). The low availability of P in plant-based feed ingredients increases the need for dietary inorganic P supplementation. However, increased dietary supplementation of inorganic P also increases P excretion (Lim et al., 2003) and feed cost (Ponnuvel et al., 2013), which drives the use of phytase in poultry diets. Commercially-available phytases are sourced from various bacteria or fungi, and different commercial phytases have different catalytic and biochemical properties (Menezes-Blackburn et al., 2015). Phytase supplementation

in Ca- and P-deficient diets increased mineral availability, egg production and shell quality in laying hens (Lim et al., 2003). *Buttiauxella sp.* phytase (BSP) possesses a high phytate-degrading efficacy, rapidly decreasing dietary phytate concentration in the gastrointestinal tract of broilers by 60 to 90% (Chapter 5; Li et al., 2016). Also, dietary BSP increased the availability of Ca, P, and amino acids in pigs (Velayudhan et al., 2015; Adedokun et al., 2015). Similarly, dietary BSP also increased ileal digestibility of P in laying hens between 23 and 26 weeks of age (woa; Barnard et al., 2014). However, only limited information is available on the long-term effects of dietary BSP in laying hens.

In addition to the positive effects on P and Ca digestibility, performance and shell quality, dietary phytase supplementation increased bone Ca and P concentrations, breaking strength, and ash content in laying hens fed Ca- and P-deficient diets (Hughes et al., 2009; de Lima et al., 2010). Long bones such as the femur and tarsometatarsus in laying hens are composed of cortical, trabecular, and medullary bone tissues. The cortical and trabecular bone fractions are strength-providing structural tissues, whereas the medullary bone fraction is primarily a labile store of Ca in support of eggshell formation (Fleming et al., 1998a). Hence, understanding the effect of phytase on bone quality in laying hens requires a separate evaluation of each bone tissue. Quantitative computed tomography (QCT) has been used to evaluate bone mineralization of the various bone fractions in laying hens (Saunders-Blades et al., 2009). The objectives of the current study were to assess the long-term effects of dietary BSP on egg production, BW, feed intake, FCR, shell quality, bone density and apparent ileal digestibility of P (AIDP) and Ca (AIDCa) in Lohmann LSL Lite layers from 30 to 70 woa. The hypothesis was that the long-term supplementation of BPS would prevent adverse effects of dietary Ca and avP reductions in laying hens.

2.2 MATERIALS AND METHODS

This study was approved by the Animal Care and Use Committee: Livestock of the University of Alberta and was consistent with the Canadian Council on Animal Care guidelines (CCAC, 2009). Lohmann LSL Lite white laying hens (n=456) were selected from the University of Alberta flock at 27 woa and were randomly housed with four hens in each of 114 cages (48 × 43 × 41 cm for width, depth, and height, respectively) in a two-tier battery cage system located in an environmentally-controlled facility. All the hens were fed a nutrient-adequate diet from 27 to 30 woa for adaptation to the cages. The average BW and egg production of the hens at 30 woa was 1.62 kg and 97.6%, respectively. Standard management recommended by the primary breeder company (Lohmann Tierzucht, 2011) was maintained throughout the study.

2.2.1 Experimental treatments

At 30 woa, three experimental diets were each assigned to 38 cages and fed in two dietary phases: phase I from 30 to 48 woa and phase II from 49 to 70 woa. The diets were a positive control (PC), formulated based on the primary breeder recommendations; the PC diet with avP and Ca marginally reduced by 0.146 and 0.134% of the diet, respectively, (negative control; NC); and the NC diet supplemented with BSP at 300 FTU/kg (NC+BSP). One phytase unit (FTU) is the activity of phytase needed to release one μmol of inorganic P per minute from sodium phytate at pH 5.5 and 37°C (Li et al., 2016). The BSP used was Axtra®PHY (Danisco Animal Nutrition, DuPont Industrial Biosciences, Marlborough, UK), sourced from *Buttiauxella sp.* and expressed in *Trichoderma reesei*. The reduction in avP by 0.146% and Ca by 0.134% of the diet in the NC diet compared to the PC was based on the expected levels of phosphoric and extra-phosphoric contributions of 300 FTU/kg BSP. Phytase activity in each experimental diet was analyzed using Method 30024 of ISO (2009). Dietary ingredient composition, calculated

nutrient composition, and analyzed Ca and total P levels, and phytase activities are presented in Table 2.6.1.

2.2.2 Performance data

Hen-day egg production data were collected from each of the cages and mortality-corrected hen-day egg production calculated on a four-wk basis. Also on a four-wk basis, individual hen BW and average daily feed intake were determined, and mortality-corrected feed conversion ratio (FCR; kg feed/dozen eggs produced per cage) was calculated.

At 30, 38, 46, 54, 62, and 70 woa, eggs produced within 24 h were collected from eight randomly pre-specified cages per treatment for determination of fresh specific gravity (Holder and Bradford, 1979) and eggshell breaking strength (kgF), thickness (mm), and relative shell weight (%). Eggshell breaking strength was measured with an Instron universal testing instrument (Table Model 4411, Instron Corp., Canton, MA) loaded with a 200 N weight. Eggshell thickness was measured with a micrometer screw gauge (Model SR44, Mitutoyo, Miyazaki, Japan). Relative shell weight was calculated as eggshell weight as a percentage of fresh egg weight.

2.2.3 Bone quality analysis

At the start of the experiment, hens in two pre-identified cages per treatment (4 hens × 2 cages × 3 diets) were selected at random. At 30, 38, 46, 54, 62, and 70 woa, each of the hens was anesthetized and analyzed *in vivo* for tarsometatarsus bone densitometry analysis. Each hen was anesthetized using 2 mg/kg BW of a 20 mg/mL of Rompun and 20 mg/kg of BW of 100 mg/mL of Ketamine (Korver et al., 2004). Also at 32, 48, and 70 woa, hens in two pre-identified cages per treatment (2 hens × 8 cages × 3 diets) were euthanized using cervical dislocation, for the collection of the right femurs. The femurs were analyzed *ex vivo* for bone

densitometry, breaking strength (BBS) and ash content. Bone densitometry was measured using QCT (Stratec XCT scanner; Korver et al., 2004). The tarsometatarsus was scanned *in vivo* at the mid-diaphysis (50% of the bone length), and the femur was scanned *ex vivo* at both the mid-diaphysis and proximal metaphysis (30% from the proximal end) for assessment of bone densitometry in each of total, cortical, and trabecular space bone tissue. Because the trabecular and medullary bone tissues are both found within the marrow space, the two bone tissues are inseparable with QCT (Korver et al., 2004). Hence, in this paper, trabecular space bone measurements represent both trabecular and medullary bone tissues. Tarsometatarsus and femur bone mineral density (BMD; mg/cm^3) and bone cross-sectional area (BCSA; mm^2) were measured by QCT. Bone mineral content (BMC; mg/mm), was determined by multiplying BCSA by BMD to indicate the amount of mineral (mg) contained within a one mm-thick x-ray longitudinal scan of bone tissue (Saunders-Blades et al., 2009). Femur BBS was analyzed using the same Instron instrument as was used in the eggshell breaking strength assessment, except with a 200 N load cell (Riczu et al., 2004). The compression force required to break each bone was recorded as BBS (kgF). Subsequently, the broken femur sample was oven-dried, weighed, and ashed at 500°C for 48 h in a muffle furnace to determine percentage bone ash content (Bello et al., 2014).

2.2.4 Digestibility assays

The diets fed to hens in eight pre-identified cages per treatment from 30 to 32, 46 to 48, and 68 to 70 woa were supplemented with 2% Celite® as an indigestible marker. At 32, 48, and 70 woa, all the hens fed the Celite-supplemented diets were euthanized for digesta collection by gentle squeezing of the distal ileum. Digesta samples were pooled within cage and freeze-dried. The Celite-supplemented diets (n=9: 3 diets×3 periods) and the digesta samples (n=72: 3

diets×8 cages×3 periods) were analyzed for acid insoluble ash (AIA) and P and Ca concentrations for determination of AIDP and AIDCa, respectively. For the AIA, Ca, and P analyses, approximately 2 g of diet sample was ashed at 500°C for 48 h, and approximately 1 g of digesta sample was ashed at 500°C for 24 h in a muffle furnace (Table-model 30400; Barnstead Thermolyne Corp., Dubuque, IA). For AIA analysis, ash samples were digested using 5 ml of 4 N HCl, and the supernatants were removed by vacuum aspiration. The residues were washed with 5 ml of deionized distilled water (ddH_2O), vortexed, centrifuged at 3,000 rpm for 8 minutes, and the supernatants were removed by vacuum aspiration thrice. The residues were subsequently oven-dried for 4 h, placed in a muffle furnace at 500°C for 12 h, and weighed to determine the amount of AIA (Scott and Boldaji, 1997). For Ca and P analyses, the diet, and digesta ash samples were digested using 10 ml of 3 N HCl and 3 to 4 drops of HNO_3, and the supernatants separated using filtration and diluted with ddH_2O to 50 ml volume. The supernatants were analyzed for P and Ca concentrations using Methods 935.13 and 964.06, respectively (AOAC, 1990). For the P analysis, 5 ml of 20 g/L of ammonium molybdate tetrahydrate and 1 g/L of ammonium metavanadate solution were added to 2 ml of the supernatant and diluted with ddH_2O to 25 ml. The P concentration was measured using a spectrophotometer (SOFTmax Pro3.1.1, Molecular Devices Corporation, Sunnyvale, CA) at 400 nm. The P levels were calculated using a linear standard curve generated with 0.0002, 0.0004, 0.0008, 0.0016, 0.0032, 0.0064, and 0.0128 mg P/ml. For the Ca analysis, 2 ml of 95 g/L of anhydrous KCl solution was added to 50 µL of the supernatant and diluted with ddH_2O to 25 ml. The solution was measured for Ca concentration using a nitrous oxide-acetylene fueled flame atomic absorption spectrometer (Varian; Model 240FS AA; Agilent Technologies, CA, US) at 422.7 nm wavelength. The Ca levels were calculated using a standard linear curve

generated with 0.002, 0.004, 0.008, 0.016, 0.032, 0.064, and 0.128 µg of Ca/ml. For determination of AIDP and AIDCa, the formula used was:

Digestibility = 100 - [100 × (% AIA in diet /% AIA in digesta) × (% mineral in digesta /% mineral in diet)].

2.2.5 Statistical analysis

The cage of four hens was the experimental unit in a completely randomized design for all parameters except BW and bone quality properties, for which the individual hen was the experimental unit. The AIDP, AIDCa, hen-day egg production, BW, feed intake, FCR, and eggshell and bone quality parameters were each analyzed for diet × age interaction and diet and age main effects. Body weight was included as a covariate when significant, for the analyses of BBS, bone ash content and BCSA, BMD, and BMC of the total, cortical, and trabecular space bone tissues in the tarsometatarsus mid-diaphysis and the femur mid-diaphysis and proximal metaphysis. The MIXED procedure of SAS 9.3 (2013) was used in the analysis of each parameter. Effects were considered significant when $P \leq 0.05$ and means were subsequently separated using LSMEANS.

2.3 RESULTS

2.3.1 Performance and eggshell quality parameters

There was no diet × age interaction for BW (Table 2.6.2). However, BW tended to be higher (P=0.076) in the PC and NC+BSP hens than in NC hens from 34 to 70 woa. Across diets, BW was higher at 42 and 58 woa than at 30, 34, 46, and 62 woa and higher at 38, 50, 54, and 66 woa than at 30 and 34 woa. Body weight was also higher at 46 and 62 woa than at 30 woa; and higher at 34 and 70 woa than at 30 woa. Egg production, feed intake, FCR, egg specific gravity, and eggshell breaking strength, thickness, and relative weight were not affected by diet × age

interaction or diet main effect. However, egg production was higher from 30 to 42 woa than at the other ages; generally, egg production declined with hen age. Daily feed intake was higher at 42 and 54 woa than at other ages, and higher at 34 and 70 woa than at 46, 50, and 62 woa. Daily feed intake was also higher at 38 and 66 woa than 46 and 50 woa, and higher at 46 woa than at 50 woa. Feed conversion ratio was lower at 46, 50, and 66 woa than any other ages. Feed conversion ratio was also lower at 34, 38, and 62 woa than at 42, 54, 58, and 70 woa and lower at 42 and 54 woa than at 58 and 70 woa. Eggshell breaking strength was higher at 30 and 38 woa than at 46 and 62 woa and was intermediate at 54 and 70 woa. Egg specific gravity, shell thickness, and relative shell weight decreased with increasing hen age.

2.3.2 Bone densitometry, breaking strength, and ash

In vivo tarsometatarsus densitometry was not affected by the diet × age interaction (Table 2.6.3). Tarsometatarsus total BMD was lower in both PC and NC+BSP hens than in the NC hens, with no diet effect on any of the other bone densitometry parameters. The tarsometatarsus trabecular space BMD was higher from 46 to 70 woa than at 38 woa and was intermediate at 30 woa. Also, tarsometatarsus trabecular space BMC was higher at 62 and 70 woa than at 30 and 38 woa, and intermediate at 46 and 54 woa.

Ex vivo total BMD in the femur proximal metaphysis was higher in the NC hens than in both the NC+BSP and PC hens at 48 woa, with no difference between diets at either of the other ages (Table 2.6.4). Total BMD in the femur mid-diaphysis was higher in the NC hens than in the NC+BSP and PC hens at 48 woa, but higher in NC+BSP hens than in the NC hens at 70 woa, with no difference due to diet at 32 woa. While trabecular space BCSA in the femur proximal metaphysis was not affected by diet × age interaction effect, trabecular space BCSA in the femur mid-diaphysis was higher in NC+BSP and PC hens than in the NC hens at 48 woa, and

was higher in NC hens than in the NC+BSP hens at 70 woa, with no diet effect at 32 woa. The diet × age interactions on cortical BMC in the femur proximal metaphysis (P=0.078) and mid-diaphysis (P=0.065) were nearly significant, and each followed the similar pattern as for the mid-diaphysis total BMD. There were no diet × age interactions none diet main effects on any of the other bone densitometry parameters measured (Table 2.6.5). The trabecular space BMD and total and trabecular space BMC in the femur proximal metaphysis and mid-diaphysis increased with age. Total BCSA at proximal metaphysis and mid-diaphysis were each higher (P=0.020) or tended to be higher (P=0.061), respectively, at 32 woa than at other ages. None of the other *ex vivo* bone densitometry parameters was affected by age main effect.

There were no diet × age interactions or diet main effects on femur ash content (Table 2.6.6). Femur breaking strength tended to be higher in the NC+BSP hens compared to the NC hens at 32 woa but was not affected by diet at other ages (P=0.073).

2.3.3 Apparent ileal digestibility of P and Ca

The AIDP was higher in the NC+BSP hens than in NC hens and intermediate in the PC hens at 32 woa (Table 2.6.7). The AIDP tended to be higher (P=0.082) in NC+BSP hens than in PC hens at 48 woa. However, the AIDP was higher in each of PC and NC hens than in NC+BSP hens at 70 woa. The AIDCa was higher in the PC hens than in NC+BSP hens at 48 woa and intermediate in NC hens, but was not affected by diet at other ages.

2.4 DISCUSSION

2.4.1 Performance and eggshell quality parameters

There was no diet effect on BW at 70 woa, and the BW of the NC hens (1,685 g) at 70 woa was within the 1,611 to 1,746 g range provided for Lohmann LSL Lite hens at that age (Lohmann Tierzucht, 2011), implying that the NC hens were not adversely affected by the Ca

and avP reduction. Reducing dietary avP from 2.5 g/kg to 2.0 g/kg from 20 to 70 woa maintained hen BW, whereas 1.5 g/kg consistently decreased hen BW after 41 woa (Boling et al., 2000). This indicates that 2.0 g/kg of avP was not deficient over a 50-wk laying cycle. However, a nearly significant decrease in BW of the NC hens was observed in the current study relative to the PC, which may have indicated a response to the reduction of avP and Ca and P in the NC relative to the PC. Also, the lack of diet effects on egg production, feed intake, FCR, and eggshell quality similarly indicated that the avP and Ca levels in the NC diets were not deficient in the NC hens. Likewise, the average 30 to 70 woa egg production of the NC hens (93.2%) was higher than the management guide objective for the laying hens (88.5%; Lohmann Tierzucht, 2011), which also supported the sufficiency of the NC diet avP and Ca levels to maintain laying rate. Dietary avP at 1.5 to 2.5 g/kg (Boling et al., 2000; Pelicia et al., 2009) and Ca at 30 to 35 g/kg (Cufadar et al., 2011; An et al., 2016) did not decrease performance and eggshell quality of laying hens. Evidently, the dietary avP and Ca levels of 2.57 and 39.6 g/kg respectively, in phase I and 2.58 and 40.3 g/kg respectively, in phase II of the NC treatment (Table 2.6.1) were sufficient to maintain laying hen performance and eggshell quality from 30 to 70 woa. The effects of diet on performance and eggshell quality were also consistent with the general lack of adverse effects on bone quality (Tables 2.6.3 and 2.6.6) and AIDP and AIDCa by the NC diet relative to the PC diet. Similarly, the overall effect of age on BW, performance, and eggshell quality of hens (Table 2.6.2) indicate that the parameters were reasonably consistent with results of previous studies (Hurnik et al., 1977; Swiatkiewicz et al., 2015). The consistent effects of age on the parameters and the lack of diet by age interactions on laying hen performance and eggshell quality also indicate that the reductions in avP and Ca levels in the NC diet did not clinically affect the hens. The lack of effects of the NC diet also indicates that the primary

breeder recommendations include considerable safety margins for avP and Ca. Overall, the sufficiency of avP and Ca levels in the NC diet limited the opportunity to observe the effects of BSP. In contrast, supplemental BSP alleviated the decrease in egg production, BW, and eggshell quality of laying hens fed a severe avP- and Ca-deficient diet (Chapter 3). Hence, negative control diets in such studies must be deficient to be able to evaluate phytase efficacy in laying hens. It is possible to provide commercial laying hens with lower safety margins for avP and Ca without compromising long-term performance and eggshell quality, particularly when phytase is used.

2.4.2 Bone quality traits

Bone mineral density (mg/mm) and BCSA (mm^2) reported for each of total, cortical, and trabecular space bone in this study were measured by QCT, while BMC (mg/mm) was determined by multiplying BMD by BCSA (Saunders-Blades et al., 2009). The analysis of each bone densitometry trait including BMC as well as BBS and bone ash was conducted with hen BW as a covariate when significant, and therefore reported BMC means reported may not reflect the product of the table values for BMD and BCSA. The lack of interaction or diet main effects on the majority of the bone quality parameters assessed (Tables 2.6.3, 2.6.4, 2.6.5, and 2.6.6) demonstrated that the avP and Ca levels in the NC diet were not deficient. Relative to a diet with 3.5 g/kg non-phytate P, lowering dietary non-phytate P to 2.5 g/kg had no effect on bone ash, but a further reduction to 1.5 g/kg decreased bone ash in a 40 wk laying hen study (Hughes et al., 2009), indicating that deficient dietary levels of avP decrease bone quality. Osteoclast activity in structural bones of actively laying hens fed avP- and Ca-deficient diets is increased relative to those fed adequate diets (Cransberg et al., 2001), resulting in the loss of bone tissue. Hence, the lack of a decrease in cortical bone of the NC hens relative to the PC

hens also supported that NC diet was not deficient in avP and Ca. The 2.7% increase in total tarsometatarsus BMD (Table 2.6.3), the increased or nearly significant increase in ($P \leq 0.080$) femur densitometry parameters at 48 woa (Table 2.6.4) by the NC relative to other diets indicated an increase in bone mass and mineralization by the avP and Ca reduction. Similarly, Hughes et al. (2009) reported that a diet with 38 g/kg Ca and 2.5 g/kg NPP increased bone ash relative to the same diet with 3.5 g/kg NPP at 42 woa, which indicated a compensatory increase in medullary bone formation. A possibility may be that the increase in total bone mineralization in the NC hens was related to the decline observed in feed intake from 42 to 50 woa. Severely deficient Ca intake lowered blood Ca levels to increase blood parathyroid hormone level and renal 1,25-dihydroxycholecalciferol metabolism, which eventually resulted in increased circulating P and Ca levels (Elaroussi et al., 1994) to support eggshell and medullary bone mineralization through increased P and Ca absorption. Cortical bone is resorbed but not formed in actively laying hens (Fleming et al., 1998a), so the increased total BMD and nearly significantly increased cortical BMC in the femur proximal metaphysis and mid-diaphysis of the NC hens at 48 woa (Table 2.6.4) relative to the other treatments were not expected. Hence, the reason for increased bone mass and mineralization in hens fed the NC diet is unknown. The overall lack of adverse effects of the NC diet relative to the PC diet on bone densitometry traits (Tables 2.6.3, 2.6.4, and 2.6.5) and breaking strength and ash content (Table 2.6.6) at each age also suggest that the NC diets were not deficient. However, the increase or nearly significant increase ($P \leq 0.078$) in femur densitometry measures at 70 woa and BBS at 32 woa by the NC+BSP relative to NC indicated the potential of phytase supplementation to increase bone mass and mineralization. Phytase increases availability and bone utilization of P and Ca, and hen productivity, however, the effects of phytase on laying hen bone quality is observable only

when deficiencies in avP or Ca are present, and sufficient phytase and substrate are present to provide sufficient available P (Hughes et al., 2009; Dersjant-Li et al., 2018). In Ca- and avP-sufficient diets, the hen will not benefit from the additional bioavailable Ca and aP, and so liberation of these minerals by phytase should have no effect on bird performance. With moderate deficiencies, hens may increase the efficiency of absorption by upregulating transport from the gut and decreasing renal excretion to maintain an adequate systemic supply of Ca and P (Dacke et al., 1993). However, with severe deficiencies, the increase in Ca and P retention efficiency may not be sufficient to overcome the inadequate supply from the diet. In this case, and assuming sufficient amounts of avP and Ca can be released from phytate contained in the feed, phytase can be used to increase the amount of metabolically available P and Ca to the bird. Experimentally, for a phytase effect to be observed, an NC diet must therefore provide less Ca or avP than the hen needs, and to observe a phytase response, there must be phytate bound avP and Ca that can be released to the bird (Boling et al., 2000; Dersjant-Li et al., 2018). In practice, the degree of dietary Ca and aP reduction that can be achieved to reduce diet cost will depend on the ability of the phytase to release sufficient quantities of mineral to replace that removed to reduce diet costs. Feeding a severely avP- and Ca-deficient diet decreased total and cortical BMD and BMC relative to an avP- and Ca-adequate diet, and BSP alleviated the decrease in bone densitometry (Chapter 3). Hence, with the sufficiency of avP and Ca in the NC diet of the current study, the increase or nearly significant increase in bone quality in the NC+BSP hens relative to NC hens are not sufficient to validate the efficacy of this phytase on bone quality of laying hens. Overall, these findings show that the dietary avP and Ca recommendations for commercial layer diets are substantially over-estimated relative to requirements, especially when supplemented with exogenous phytase.

The total bone parameters assessed with QCT include cortical, trabecular, and the medullary bone tissues; the bone contained within the trabecular space includes both trabecular and medullary bone tissues (Korver et al., 2004). The overall increase in total and trabecular space BMD and BMC with age, and the lack of similar effects on overall cortical densitometry (Tables 2.6.3 and 2.6.5) indicate an age-related increase in the deposition of medullary bone, the only bone tissue that is formed in actively laying hens (Fleming et al., 1998a). Hens fed adequate avP and Ca had increased medullary bone volume but decreased trabecular bone volume in the proximal tarsometatarsus from 15 to 70 woa (Fleming et al., 1998b). However, diets severely deficient in Ca (<3%) decreased tibia BBS within 14 d in 22 wk-old hens (Elaroussi et al., 1994), and decreased cortical bone ash with an increase in medullary bone volume in the femur within 10 d from the start of lay (Taylor and Moore, 1954). While the cortical bone tissue was maintained from 30 to 70 woa, the age-related increase in medullary bone tissue likely masked any effects of age on trabecular bone. Therefore, the age-related decrease in trabecular bone tissue observed by Fleming et al. (1998b) cannot be affirmed in the current study. The continued deposition of medullary bone as hens age could mask treatment-related changes in the amount of strength-providing structural bone. Bone ash measures the total amount of mineral contained in the bone, but does not provide any specific information on the quantity of the strength-providing cortical and trabecular bone tissues. Hence, while evaluation of bone ash may provide information on the effect of experimental treatments on total bone mineralization, it would not be sufficient to predict changes in bone strength of laying hens. Also, the overall greater diet and age effects observed in the femur can be related to the proportionally larger volume of each of cortical, trabecular, and medullary bone tissues in the femur relative to the tarsometatarsus in laying hens (Taylor and Moore, 1954). Medullary bone

ash, Ca and P concentrations were lower in the tarsometatarsus than in the femur of laying hens, indicating proportionally higher bone remodeling activity (Taylor and Moore, 1954). Hence, with the proportionately higher volumes of cortical, trabecular and medullary tissues, the femur may provide a greater opportunity to observe treatment effects on skeletal quality relative to the tarsometatarsus.

2.4.3 Apparent ileal digestibility of P and Ca

The lack of adverse effects of the NC diet on productivity performance and the majority of bone quality traits assessed relative to the PC and the NC+BSP at each age indicated that avP and Ca in the NC diets were not deficient. AIDP in the NC diet increased at 32 woa, and tended to be increased at 48 woa with phytase supplementation. However, the phytase supplementation decreased at 70 woa compared to both control diets (Table 2.6.7). Also, AIDCa in the NC diet decreased with phytase usage relative to the PC diet at 48 woa and was similar on AIDCa relative to the NC and PC diets at other ages. Dietary phytase increased availability of P but not of Ca when used in a diet with 2.5 g/kg avP and 40 g/kg Ca (Lim et al., 2003). However, phytase increased both AIDP and AIDCa when used in a diet with 1.5 g/kg avP and 31.8 g/kg Ca (Lui et al., 2007). While the effect of phytase on mineral digestibility was influenced by age, the mechanism for the different effects of diet at each age in the current study are not clear. A meta-analysis of 25 studies showed that increasing dietary Ca level, and increasing hen age are important variables that decrease phytase effects on P retention in laying hens (Bougouin et al., 2014). Although a high dietary Ca level is required to support eggshell formation and bone remodeling, it also decreases P and Ca digestibility. High dietary Ca results in the formation of a high-pH, insoluble Ca-phytate complex in the gastrointestinal tract of birds (Hurwitz and Bar, 1971; Nelson and Kirby, 1987). Increasing dietary Ca from 30 to 40 g/kg (Van der Klis et

al., 1997) and from 25 to 55 g/kg (Beutler, 2009) increased Ca-phytate complex formation, which increased gastrointestinal tract pH to decrease phytase activity, phytate hydrolysis, and P and Ca digestibility in laying hens. Therefore, dietary Ca in the NC and PC diets may have resulted in formation of high levels of Ca-phytate complex that subsequently reduced mineral absorption relative to the phytase-supplemented diet. Phytase supplementation partly decreases Ca-phytate complex formation by dephosphorylating phytate and decreasing the affinity for Ca and phytate to complex. The high Ca levels in the non-phytase supplemented PC and NC diets may have resulted in Ca-phytate complex formation resulting in the associated adverse effect on the hens. In commercial layer diets, Ca is increased while avP is decreased as hens age because of age-related physiological changes in laying hens (Pelicia et al., 2009). Feeding a Ca-deficient diet to laying hens lowered blood Ca and increased renal metabolism of 1,25-dihydroxycholecalciferol, which was decreased by over 70% from 22 to 120 woa (Elaroussi et al., 1994). In addition to the sufficiency of avP and Ca in the NC diet limiting the opportunity to observe phytase effects, the high Ca level in the PC and NC diets relative to the actual requirements (as opposed to the management guide recommendations) may also have contributed to the overall diet effects on digestibility of the minerals at each age.

Clearly, the effect of diet on AIDP and AIDCa at each age differ. The increase in AIDP at 32 woa and the nearly significant increase in AIDP at 48 woa showed that BSP usage in the NC diet increased P digestibility, indicating phytate degradation. Conversely, AIDCa at 48 woa was higher in the PC hens and AIDP at 70 woa was higher in the PC and NC hens relative to the phytase-supplemented hens. Supplementation of a 600 FTU/kg phytase in a corn-soybean meal laying hen diet maintained 61 woa digestibility of P at 21% and Ca at 40% (Beutler, 2009). The result of the current study showed that phytase supplementation in the NC diet maintained AIDP

at 53, 61, and 39% and AIDCa at 48, 54, and 51%, at 32, 48, and 70 woa hens, respectively. Relative to the results of Beulter, (2009), the 300 FTU/kg BSP did not decrease AIDCa and AIDP at 48 and 70 woa, respectively. Hence, the high Ca levels and the absence of supplemental phytase to at the least reduce the formation and adverse effects of Ca-phytate complex in the NC and PC diets may have prompted an age-specific increase in P and Ca absorption mechanism. Laying hens are able to physiologically increase absorption of P and Ca by increasing metabolism of renal and intestinal 1, 25-hydrooxycholecalciferol as a response to decline in circulating levels of these minerals (Elaroussi et al., 1994), which high Ca-phytate complex formation could cause. In the current study, levels of avP in the PC and NC diets were 4.18 and 2.57 g/kg, respectively during phase I (32 and 48 woa) and 4.26 and 2.58 in g/kg during phase II (70 woa), while Ca levels in the diets were 41.0 and 39.6 g/kg respectively during phase I and 41.9 and 40.3 g/kg during phase II (Table 2.6.1). The high dietary Ca, particularly in the phase II diets, may have promoted an increase in Ca-phytate formation, its potential to decrease P availability, and the need to increase the absorptive mechanisms in the highly prolific hens. The increased AIDCa by the PC diet at 48 woa and the increased AIDP by PC and NC diets 70 woa compared to the phytase-supplemented diet may indicate the ability of hens to physiologically adapt to decreases in P and Ca availability by increasing mineral absorption mechanisms. Hen P and Ca metabolism decreases with age (Elaroussi et al., 1994), but the impact of age-related changes in laying hen GIT physiology on the effects of diet on AIDP and AIDCa at each age are not known. Phytase supplementation in the NC diet increased AIDP at 32 woa and tended to increase the parameter at 48 woa. However, because the avP and Ca in the NC diet did not decrease overall performance of the hens relative to the PC, effects of supplemental BSP on P digestibility at the younger age were not sufficient to observe the effect

of the enzyme on P digestibility. Overall, these findings imply that the high Ca levels used in commercial layer diets relative to the actual requirements of the bird may inhibit phytase activity and decrease P and Ca digestibility.

Overall, the lack of diet effects on performance, eggshell quality, and the majority of the assessed bone trait parameters and the lack of differences between PC and NC diets on AIDP and AIDCa strongly suggest that the avP and Ca in the NC diets were not deficient. The sufficiency of the NC diet demonstrate that the primary breeder recommendations include considerable safety margins, and limited the opportunity for supplemental BSP to alleviate adverse effects of P and Ca reductions. Also, the sensitivity and the additional information provided by bone densitometry relative to bone ash and breaking strength in evaluation of effects of dietary avP and Ca levels and supplemental phytase on bone quality of laying hens was established. Because avP and Ca levels in the NC diet were not deficient, the efficacy of the supplemental phytase in laying hens could not be evaluated in the current study. The overall findings of this study emphasize that dietary Ca and avP levels in commercial layers should be re-evaluated, especially when supplemented with exogenous phytase.

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2.6 TABLES

Table 2.6.1: Ingredients, calculated and analyzed nutrients, and phytase activities levels of experimental diets[1]

	Phase I (30 to 48 wk of age)			Phase II (48 to 70 wk of age)		
Diets[2]	PC	NC	NC+BSP	PC	NC	NC+BSP
Ingredients (%, unless stated)						
Yellow corn	59.6	61.0	61.0	66.3	64.3	64.3
Soybean meal	**16.5**	16.3	16.3	11.0	13.8	13.8
Canola meal	12.0	12.0	12.0	12.0	12.0	12.0
Canola oil	0.96	0.52	0.52	-	-	-
Calcium carbonate	8.67	8.74	8.74	8.48	8.54	8.54
Dicalcium phosphate	1.37	0.57	0.57	1.35	0.53	0.53
L-Lysine	-	-	-	-	-	-
DL-methionine	0.10	0.10	0.10	0.07	0.04	0.04
Vitamin-mineral premix[3]	0.50	0.50	0.50	0.50	0.50	0.50
Phytase (g/kg)[4]	-	-	0.02	-	-	0.02
Salt (NaCl)	0.29	0.29	0.29	0.32	0.31	0.31
Celite[5]						
Calculated nutrients						
M.E. poultry (MJ/kg)	11.6	11.6	11.6	11.6	11.6	11.62
Crude protein (%)	17.6	17.6	17.6	16.6	16.6	16.61
Calcium (%)	3.730	3.596	3.596	3.640	3.506	3.506
Total phosphorus (%)	0.643	0.498	0.498	0.620	0.483	0.483
Available phosphorus (%)	0.380	0.234	0.234	0.370	0.224	0.224
Phytate phosphorus (%)	0.281	0.283	0.283	0.271	0.279	0.279
Analyzed nutrients (as fed)						
Calcium (%)	3.643	3.552	3.349	3.831	3.491	3.426
Total phosphorus (%)	0.587	0.453	0.477	0.576	0.432	0.418
Phytase activity (FTU/kg)	67	52	205	65	56	263

[1]Phase I diets were formulated based on an assumed intake of 110 g/hen per day, and phase II diets were formulated based on 115 g/hen per day.
[2]PC = positive control (nutritionally adequate diets based on the primary breeder nutrient recommendations); NC = negative control (the PC diets with Ca and avP reduced by 0.134 and 0.146% of the diet, respectively); and NC+BSP = the NC diets supplemented with *Buttiauxella sp*. phytase at 300 FTU/kg.
[3]Vitamin-mineral premix provided (per kg of complete diet): 88 mg Mn; 100 mg Zn; 80 mg Fe; 15 mg Cu; 1.7 mg I; 12,500 IU vitamin A; 3,125 IU vitamin D; 40 IU vitamin E; 2.5 mg vitamin K; 2.6 mg thiamin; 7.5 mg riboflavin; 37.5 mg niacin; 5 mg pyridoxine; 12.5 mg d-pantothenic acid; 18.8 µg vitamin B_{12}; 0.63 mg folic acid; and 150 µg biotin.
[4]Axtra® PHY, Danisco Animal Nutrition, DuPont Industrial Bioscience, Marlborough, UK.
[5]Celite (Celite Corp., Lompoc, CA) only supplemented in diets fed to hens in eight pre-identified cages per treatment from 30 to 32, 46 to 48, and 68 to 70 wk of age for digesta collection for digestibility assay.

Table 2.6.2: Effect of dietary Ca, available P and phytase on performance, feed efficiency, and eggshell quality of laying hens from 30 to 70 wk of age[1,2]

	Body weight (kg)[3]	Hen-day egg production (%)[3]	Feed intake (g/hen/d)[3]	FCR[3,4]	Shell breaking strength (kgF)[3]	Specific Gravity[3]	Shell thickness (mm)[3]	Relative Shell weight (%)[3,5]
Diets								
PC[6]	1.74	94.5	110	1.38	4.08	1.086	0.34	9.71
NC[6]	1.69	94.1	109	1.39	4.25	1.087	0.35	9.85
NC+BSP[6]	1.74	94.4	109	1.39	4.09	1.087	0.34	9.77
Pooled SEM	0.02	0.24	0.42	0.01	0.13	0.01	0.01	0.11
Weeks of age								
30	1.62^{d}	97.3±0.72^{a}	-	-	4.43±0.21^{a}	1.092^{a}	0.37^{a}	10.41^{a}
34	1.70^{c}	97.0±0.78^{a}	110±0.64^{b}	1.36^{c}	-	-	-	-
38	1.73^{ab}	97.0±0.78^{a}	109±0.71^{bc}	1.35^{c}	4.36±0.15^{a}	1.090^{b}	0.36^{b}	9.90^{b}
42	1.74^{a}	97.2±0.78^{a}	114±0.71^{a}	1.42^{b}	-	-	-	-
46	1.71^{bc}	95.7±0.82^{b}	105±0.71^{d}	1.31^{d}	3.94±0.15^{b}	1.088^{c}	0.34^{c}	9.75^{b}
50	1.73^{ab}	93.4±0.92^{c}	102±0.71^{e}	1.28^{d}	-	-	-	-
54	1.73^{ab}	95.2±0.92^{b}	115±0.83^{a}	1.46^{b}	4.06±0.15^{ab}	1.085^{d}	0.34^{c}	9.66^{cd}
58	1.75^{a}	93.2±0.92^{c}	110±0.83^{bc}	1.51^{a}	-	-	-	-
62	1.71^{bc}	92.2±0.92^{cd}	108±0.83^{c}	1.37^{c}	3.83±0.15^{b}	1.084^{e}	0.33^{d}	9.45^{de}
66	1.73^{ab}	92.5±0.92^{c}	110±0.83^{bc}	1.28^{d}	-	-	-	-
70	1.72^{abc}	91.1±0.92^{d}	111±0.83^{b}	1.52^{a}	4.21±0.16^{ab}	1.080^{f}	0.32^{d}	9.48^{e}
Pooled SEM	0.01	-	-	0.01	-	<0.01	<0.01	0.09
Source of variation	----------*P*-value----------							
Diet	0.076	0.651	0.331	0.847	0.624	0.575	0.586	0.649
Age	<0.001	<0.001	<0.001	<0.001	0.050	<0.001	<0.001	<0.001
Diet×Age	0.991	0.907	0.905	0.529	0.910	0.608	0.816	0.440

[a-f]Treatment means with no common superscript within a column differ significantly ($P \leq 0.05$).

[1]Body weight, egg production, feed intake, and FCR means were calculated from 38, 30, and 22 cages from 30 to 32, 32 to 48, and 48 to 70 wk, respectively, and the eggshell quality parameter means were calculated from 8 cages at eight wk intervals.

[2]Pooled SEM used when variance were equal between treatment while individual SEM were used in the event of unequal variance.

[3]Dietary treatment means for body weight averaged over 34 to 70 wk and each of other parameters averaged over 30 to 70 w.

[4]Feed conversion ratio: feed intake (kg) divided by dozens of eggs produced within four wk intervals.
[5]Egg shell weight as a percentage of egg weight.
[6]PC = positive control (nutritionally adequate diets based on the primary breeder nutrient recommendations), NC = negative control (the PC diets with Ca and avP reduced by 0.134 and 0.146% of the diet, respectively); and NC+BSP = the NC diets supplemented with *Buttiauxella sp*. phytase at 300 FTU/kg

Table 2.6.3: Effect of dietary Ca, available P, phytase, and age on in vivo bone densitometry of total, cortical, and trabecular space bone tissues in tarsometatarsus of white egg laying hens from 30 to 70 weeks of age[1,2]

	Bone mineral density (mg/cm^3)			Bone cross-sectional area (mm^2)			Bone mineral content (mg/mm)^3		
Bone tissues	Total	Cortical	Trabecular space[4]	Total	Cortical	Trabecular space[4]	Total	Cortical	Trabecular space[4]
Diet									
PC[5]	488±4.94[b]	995±3.76	64.1±5.57	28.0	12.3	14.6	13.6	12.3	0.94
NC[5]	502±4.79[a]	1,000±3.61	62.1±5.55	27.4	12.4	14.0	13.8	12.4	0.88
NC+BSP[5]	490±4.97[b]	993±3.73	60.8±5.58	28.0	12.3	14.3	13.6	12.2	0.87
Pooled SEM	-	-	-	0.65	0.34	0.27	0.36	0.36	0.08
Weeks of age									
30	502±6.80	1,001±5.14	60.2±3.98[ab]	27.4	12.4	14.0	13.7	12.4	0.84[b]
38	488±6.72	994±5.07	55.6±3.95[b]	27.7	12.3	14.3	13.5	12.3	0.79[b]
46	493±6.88	1,000±5.19	60.9±3.99[a]	27.4	12.2	14.2	13.5	12.2	0.86[ab]
54	488±6.88	995±5.20	63.0±3.99[a]	27.8	12.2	14.4	13.6	12.2	0.91[ab]
62	491±7.22	986±5.45	67.5±4.08[a]	28.2	12.5	14.43	13.8	12.3	0.98[a]
70	494±7.02	998±5.31	66.7±4.03[a]	28.2	12.5	14.47	14.0	12.5	0.97[a]
Pooled SEM	-	-	-	0.52	0.24	0.33	0.26	0.24	0.06
Source of variation	----------*P*-value----------								
Age	0.777	0.463	0.016	0.530	0.468	0.890	0.280	0.575	0.015
Diet	0.046	0.458	0.919	0.837	0.954	0.402	0.962	0.904	0.760
Diet×Age	1.000	0.999	0.982	0.999	0.999	0.999	1.000	1.000	0.978
Covariate (BW)	0.002	<0.001	0.482	0.145	<0.001	0.519	<0.001	<0.001	0.440

[a-b]Treatment means with no common superscript within a column differ significantly($P \leq 0.05$).

[1]Each dietary treatment mean was calculated from 2 cages (of 4 hens each) at each age.

[2]Individual SEM were used in the event of unequal variance and pooled SEM used when variance were equal between treatments.

[3]Calculated by multiplying the respective bone cross-sectional area by bone mineral density to yield the amount of mineral (mg) contained in one mm-thick x-ray scan.

[4]Trabecular space bone tissue represented trabecular and medullary bone tissues.[5]PC = positive control (nutritionally adequate diets based on the primary breeder nutrient recommendations), NC = negative control (the PC diets with Ca and avP reduced by 0.134 and 0.146% of the diet, respectively); and NC+BSP = the NC diets supplemented with *Buttiauxella sp.* phytase at 300 FTU/kg.

Table 2.6.4: Effect of dietary Ca, available P, phytase, and age on *ex vivo* bone densitometry of total, trabecular space, and cortical bone tissues in femur proximal metaphysis and mid-diaphysis of white egg laying hens from 30 to 70 weeks of age[1]

	Total bone mineral density (mg/cm^3)			Trabecular space bone cross sectional area (mm^2)			Cortical bone mineral content $(mg/mm)^2$		
Weeks of age	32	48	70	32	48	70	32	48	70
Proximal metaphysis[4]									
PC[5]	469	511[b]	553	31.8	29.6	26.7	16.8	16.7	17.8
NC[5]	473	555[a]	539	30.9	24.6	27.1	16.7	18.2	16.8
NC+BSP[5]	486	508[b]	572	30.4	29.7	24.7	16.8	16.6	18.5
Pooled SEM		14.32			1.55			0.62	
Source of variation	----------				*P*-value			----------	
Diet×Age		0.050			0.160			0.087	
Covariate (BW)		0.011			0.002			<0.001	
Mid-diaphysis[4]									
PC[5]	522	574[b]	624[ab]	24.8	22.5[a]	20.7[ab]	17.3	17.1	18.1
NC[5]	526	614[a]	593[b]	23.6	18.5[b]	21.3[a]	17.2	18.7	17.2
NC+BSP[5]	539	571[b]	650[a]	23.7	23.1[a]	17.0[b]	17.3	17.2	18.9
Pooled SEM		15.88			1.35			0.57	
Source of variation	----------				*P*-value			----------	
Diet×Age		0.032			0.026			0.060	
Covariate (BW)		0.002			0.126			<0.001	

[a-b]Treatment means with no common superscript for each parameter differ significantly ($P \leq 0.05$).
[1]Each dietary treatment mean was calculated from 16 hens at each age.
[2]Calculated by multiplying the respective bone cross-sectional area by bone mineral density to yield the amount of mineral (mg) contained in a one mm-thick x-ray scan.
[3]Trabecular space bone cross-sectional area represented trabecular and medullary bone tissues.
[4]Femur proximal metaphysis measured at 30% of the bone length from the proximal end and mid-diaphysis measured at the mid-point of the bone.

[5]PC = positive control (nutritionally adequate diets based on the primary breeder nutrient recommendations), NC = negative control (the PC diets with Ca and avP reduced by 0.134 and 0.146% of the diet, respectively); and NC+BSP = the NC diets supplemented with *Buttiauxella sp.* phytase at 300 FTU/kg.

Table 2.6.5: Effect of dietary Ca, available P, phytase, and age on *ex vivo* bone densitometry of total, trabecular space, and cortical bone tissues in femur proximal metaphysis and mid-diaphysis of white egg laying hens from 30 to 70 weeks of age[1,2]

	Bone mineral density (mg/cm^3)		Bone cross-sectional area (mm^2)		Bone mineral content $(mg/mm)^2$	
	Cortical	Trabecular space[3]	Total	Cortical	Total	Trabecular space[3]
Proximal metaphysis[4]						
Diet						
PC[5]	1,076±7.12	219± 6.10	48.0	16.0	24.4	6.39
NC[5]	1,067±6.93	222±6.10	47.6	16.2	24.8	6.08
NC+BSP[5]	1,077±6.93	225±6.25	47.6	16.1	24.7	6.25
Pooled SEM	-	-	0.52	0.46	0.41	0.24
Weeks of age						
32	1,070±6.93	183±6.10[c]	49.0[a]	15.7	23.3[c]	5.61[b]
48	1,076±7.04	227±6.19[b]	46.9[b]	16.0	24.5[b]	6.39[a]
70	1,074±7.01	256±6.16[a]	47.3[b]	16.7	26.2[a]	6.73[a]
Pooled SEM	-	-	0.52	0.47	0.42	0.25
Source of variation	----------*P*-value----------					
Age	0.811	<0.001	0.020	0.297	<0.001	0.006
Diet	0.522	0.781	0.864	0.922	0.810	0.665
Diet×Age	0.675	0.623	0.346	0.207	0.205	0.596
Covariate (BW)	0.632	0.441	<0.001	<0.001	<0.001	0.757
Mid-diaphysis[4]						
Diet						
PC[5]	1,150±8.05	200±6.67	39.7	15.3	22.7	4.51
NC[5]	1,135±7.93	196±6.57	39.8	15.7	23.0	4.09
NC+BSP[5]	1,143±7.93	201±6.57	39.4	15.7	23.0	4.17
Pooled SEM	-	-	0.45	0.4	0.37	0.21
Weeks of age						
32	1,142±7.93	151±6.57[c]	40.5	15.2	21.4[c]	3.61[b]
48	1,142±7.97	204±6.59[b]	39.1	15.6	22.79[b]	4.37[a]
70	1,144±8.02	241±6.64[a]	39.3	16.0	24.38[a]	4.79[a]
Pooled SEM	-	-	0.46	0.41	0.37	0.21
Source of variation	----------*P*-value----------					
Age	0.973	<0.001	0.061	0.403	<0.001	0.001
Diet	0.418	0.818	0.782	0.719	0.801	0.337
Diet×Age	0.734	0.999	0.650	0.104	0.196	0.177
Covariate (BW)	0.291	0.262	0.001	<0.001	<0.001	0.630

[a-c]Treatment means with no common superscript within a column differ significantly ($P \leq 0.05$).
[1]Each diet mean was calculated from 16 hens at each age.
[2] Calculated by multiplying the respective bone cross-sectional area by bone mineral density to yield the amount of mineral (mg) contained in one mm-thick x-ray scan.
[3]Trabecular space bone tissue represented trabecular and medullary bone tissues.

[4]Femur proximal metaphysis measured at 30% of the bone length from the proximal end and mid-diaphysis measured at the mid-point of the bone.
[5]PC = positive control (nutritionally adequate diets based on the primary breeder nutrient recommendations), NC = negative control (the PC diets with Ca and avP reduced by 0.134 and 0.146% of the diet, respectively); and NC+BSP = the NC diets supplemented with *Buttiauxella sp.* phytase at 300 FTU/kg.

Table 2.6.6: Effect of dietary Ca, available P and phytase on bone breaking strength and ash content in femur of laying hens from 30 to 70 wk of age[1]

	Bone ash (%)			Bone breaking strength (kgF)		
Diet	32	48	70	32	48	70
PC[3]	45.5	50.9	52.3	19.1	16.4	16.8
NC[3]	45.4	52.0	50.3	17.8	18.0	15.1
NC+BSP[3]	46.8	48.7	54.0	20.3	16.1	16.8
Pooled SEM	1.28	1.32	1.29	0.84	0.86	0.84
Source of variation	----------*P*-value----------			----------*P*-value----------		
Diet×Age		0.758			0.073	
Covariate (BW)		<0.001			<0.001	

[1]Each dietary treatment mean was calculated from 16 hens at each age.
[2]PC = positive control (nutritionally adequate diets based on the primary breeder nutrient recommendations), NC = negative control (the PC diets with Ca and avP reduced by 0.134 and 0.146% of the diet, respectively); and NC+BSP = the NC diets supplemented with *Buttiauxella sp.* phytase at 300 FTU/kg.

Table 2.6.7: Effect of dietary Ca, available P and phytase on apparent ileal digestibility of P (AIDP) and Ca (AIDCa) in laying hens from 30 to 70 wk of age[1]

	AIDP (%)			AIDCa (%)		
Weeks of age	32	48	70	32	48	70
PC[2]	43.4[ab]	49.6	50.8[a]	48.9	71.7[a]	44.1
NC[2]	39.9[b]	53.0	54.2[a]	53.1	60.2[ab]	47.8
NC+BSP[2]	52.7[a]	60.7	39.2[b]	47.7	53.7[b]	50.6
Pooled SEM	3.45	3.45	3.53	5.20	5.22	4.62
Source of variation	----------*P*-value----------					
Diet: *P*-value	0.034	0.082	0.028	0.592	0.037	0.814

[a-b]Dietary treatment means with no common superscript within a column differ significantly ($P \leq 0.05$).

[1]Each dietary treatment mean was calculated from 8 cages (of 4 hens each) at each age.

[2]PC = positive control (nutritionally adequate diets based on the primary breeder nutrient recommendations), NC = negative control (the PC diets with Ca and avP reduced by 0.134 and 0.146% of the diet, respectively); and NC+BSP = the NC diets supplemented with *Buttiauxella sp*. phytase at 300 FTU/kg.

3. EFFECTS OF DIETARY Ca, AVAILABLE P AND PHYTASE SUPPLEMENTATION ON PERFORMANCE AND BONE QUALITY IN AGED WHITE EGG-LAYING HENS

ABSTRACT

Dietary phytase supplementation increases P and Ca availability to subsequently alleviate the adverse effects of the mineral reductions in bone quality of laying hens. Effects of a *Buttiauxella sp.* phytase (BSP) supplemented in diets reduced in available P (avP) and Ca on performance, BW, eggshell quality, serum biochemical bone markers, bone densitometry, and economic analysis were evaluated in egg-laying hens from 68 to 78 weeks of age (woa). Twenty hens were fed one of five diets (n=100), including a positive control (PC) with 0.35% avP and 3.5% Ca and the PC with avP and Ca levels moderately reduced by 0.187 and 0.159% of the diet, respectively (NC1, 53 and 4.5% less than the PC, respectively) or severely reduced by 0.231 and 0.275% of the diet, respectively (NC2, 66 and 7.9% less than the PC, respectively). Other diets were the NC1 or NC2 diets supplemented with BSP at 600 FTU/kg (NC1+BSP or NC2+BSP, respectively). Egg production and feed conversion ratio were maintained by NC1 but were 11.9% lower and 12.3% higher, respectively, with the NC2 than the PC, which was alleviated by the supplemental BSP. Diet effects on FI and eggshell quality traits followed a similar pattern. Body weight was 2.9% lower for NC1, and 6.1% for NC2 than the PC and BSP alleviated the decreased BW. Serum pyridinoline (bone resorption marker) was 20 to 27% higher in NC2 hens than in the other groups, with no effects on other bone markers. Total and trabecular space bone mineral density in the proximal metaphysis were 8.4 and 15.2% lower for NC1, respectively, and 12.1 and 26.7% lower for NC2, respectively than PC. Supplemental BSP completely alleviated the decreased bone densitometry measures in NC1, but only partially in NC2. Phytase supplementation to NC1 and NC2 decreased feed cost/dozen eggs produced to increase profit.

The NC1 hens maintained performance with decreased BW and bone quality, and phytase addition maintained productivity, BW and bone quality of the hens. The Ca and avP deficiencies in the NC2 hens relative to other groups were partially alleviated by the 600 FTU/kg BSP.

Keywords: Available phosphorus, bone, calcium, digestibility, *Buttiauxella* sp. phytase, layers

3.1 INTRODUCTION

Phytase is commonly supplemented laying hens diets to increase the bioavailability of P and Ca. Dietary phytase supplementation increases Ca, and P digestibilities (Beutler, 2009), and egg production, BW, eggshell quality, and bone quality in egg-laying hens (Pelicia et al., 2009; Hughes et al., 2009), and increases blood myo-inositol concentration in broilers (Sommerfeld et al., 2018). The effects of phytase in diets reduced in available P (**avP**) and Ca on laying hen bone quality are mainly reported in terms of bone breaking strength and ash content (Boling et al., 2000; de Lima et al., 2010). Bones, particularly long bones, in egg-laying hens are comprised of cortical, trabecular, and medullary bone tissues. Cortical and trabecular tissues are the strength-providing structural bone (Reich and Gefen, 2006) while medullary bone is a non-structural tissue which stores mineral to support egg-shell formation (Fleming et al., 1998). In actively laying hens, the cortical and trabecular bone tissues are not formed but may be resorbed, while the medullary bone tissue can be formed and resorbed to support eggshell formation (Dacke et al., 1993). Hence, changes in bone ash as a result of experimental treatment may have no relationship to the bone strength of egg-laying hens, depending on the specific changes to structural and non-structure bone tissues. Bone breaking strength (**BBS**) is assessed with the application of a perpendicular force on the mid-diaphysis of a long bone, a site primarily concentrated with cortical and medullary bone tissues (de Lima et al., 2010). However, the proximal and distal ends of long bones have less of the compact cortical bone but more of the

spongy trabecular bone (Reich and Gefen, 2006). The bone metaphysis is more susceptible to fracture than the mid-diaphysis, which is usually the location of bone breaking strength testing (Reich and Gefen, 2006). While BBS provides information on fracture resistance specific to the mid-diaphysis, (de Lima et al., 2010; Regmi et al., 2015), the assessment does not provide information changes on trabecular bone, which is the strength-providing bone tissue most susceptible to fracture (Reich and Gefen, 2006). Overall, BBS and bone ash, P, and Ca contents are commonly used to measure bone quality as they are easy, fast, and inexpensive to measure relative to bone imaging technology such as quantitative computed tomography (**QCT)**. Therefore, while bone ash and BBS are important parameters that provide information on bone mineralization and resistance to fracture, respectively, it is vital to interpret the results in light of their limitations.

Densitometry of each bone tissue and assessment of the circulating concentrations of bone biochemical markers can help to understand the effects of dietary phytase on bone biology. Quantitative computed tomography has been used to evaluate bone mass and mineralization in different bone tissues in egg-laying hens (Saunders-Blades et al., 2009). Serum osteocalcin (bone formation marker) and pyridinoline (bone resorption marker) and factors related to bone metabolism including serum parathyroid hormone (**PTH**), Ca, and P have been used to assess bone formation and resorption (Jiang et al., 2013 and Regmi et al., 2015). However, little information is available on the effects of dietary Ca and avP levels and phytase supplementation on bone metabolism in laying hens.

Phytase is commonly used in commercial laying hen diets at 300 or 600 FTU/kg of feed (Abudabos, 2012), with the level of phytase activity being related to the degree to which avP and Ca can be reduced. The use of dietary phytase allows for a decrease in dietary supplementation

of expensive and limiting inorganic P sources (Kim et al., 2017). Also, the use of phytase in egg-laying hen diets allows for the use of high-phytate and low-cost feed ingredients for partial substitution of other relatively low-phytate and high-cost feed ingredients (Ponnuvel et al., 2013). The increase in the level of high-phytate ingredients in phytase-supplemented diets provides more substrate for the enzyme to degrade. Dietary phytase supplementation, reduced use of inorganic P and use of cheaper ingredients reduces overall diet cost (Ponnuvel et al., 2013). However, there is limited information available on the economic importance of phytase in laying hen diets with moderate to severe reductions of avP and Ca. Hence, it was hypothesized that phytase supplementation in diets moderately or severely reduced in avP and Ca would alleviate the adverse effects of the deficiencies on performance, bone densitometry, serum osteocalcin and pyridinoline concentrations, and production economics in laying hens. The objective of this study was to evaluate the effects of moderate and severe reductions of dietary Ca and avP and phytase supplementation on bone densitometry; serum osteocalcin, pyridinoline, PTH, Ca, and P concentrations; and production economics.

3.2 MATERIALS AND METHODS

The Animal Care and Use Committee: Livestock of the University of Alberta approved the protocol of the current study and the animal handling procedure followed were consistent with the Canadian Council on Animal Care guidelines (CCAC, 2009). One hundred H&N Nick Chick egg-laying hens were obtained at 68 weeks of age (**woa**) from a flock of healthy laying hens in the Poultry Research Centre of the University of Alberta flock. The hens were housed individually (48 × 43 × 41 cm for width, depth, and height, respectively; Specht Canada Inc., Stony Plain, AB, Canada) in a double-tier cage system located in an environmentally-controlled facility. The hens were monitored from 66 to 68 woa to ensure all hens were actively laying. The

hens were managed as recommended by the primary breeder company (H&N International, 2016).

3.2.1 Treatments

Each of five dietary treatments was fed to 20 hens from 68 to 78 woa. The diets included a positive control (**PC**) with 0.35% avP and 3.5% Ca, and the PC with avP and Ca levels moderately reduced by 0.187 and 0.159% of the diet, respectively (**NC1**; 53 and 4.5% less than the PC, respectively) or severely reduced by 0.231 and 0.275% of the diet, respectively (**NC2**; 66 and 7.9% less than the PC, respectively). Other diets were the NC1 or NC2 diets supplemented with BSP at 600 FTU/kg (**NC1+BSP** or **NC2+BSP**, respectively). The reduction in avP and Ca levels in the NC1 was based on the expected P- and Ca-releasing activity of the supplemented BSP at 600 FTU/kg. The avP and Ca reductions used in the NC2 diet were greater than the levels expected to be released by 600 FTU/kg BSP; the rationale was to assess the efficacy of BSP at 600 FTU/kg of diet with a severe reduction of dietary avP and Ca. The formulated ingredients, calculated nutrient composition, and analyzed Ca and total P levels, and phytase activity in diets are shown in Table 3.6.1. The phytase used in the study was Axtra®PHY, (Danisco Animal Nutrition, DuPont Industrial Biosciences, Marlborough, UK), which was sourced from *Buttiauxella sp.* and expressed in *Trichoderma reesei*. The phytase activity in each of the five diets was analyzed using Method 30024 of ISO (2009).

3.2.2 Performance and eggshell quality

Egg production was assessed daily and calculated on a weekly basis. On a two-wk basis, individual hen BW and average daily feed intake were determined, and feed conversion ratio (**FCR**) was calculated for each hen based on kg of feed consumed divided per dozen eggs produced. Also at each of 68, 70, 72, 74, 76, and 78 woa, eggshell breaking strength and specific gravity of all eggs produced on those days were determined as described in Chapter 2.

3.2.3 Serum collection and bone biochemical analyses

At 78 woa, 12 hens per treatment (n=60) were bled between 7:00 AM and 11:30 AM. Serum samples were separated from the blood (Jiang et al., 2013) and stored at -80°C until the time of analyses. The serum samples were subsequently thawed and analyzed for osteocalcin and pyridinoline using commercial ELISA kits (Quidel Corporation, San Diego CA, USA), with procedures described by the manufacturer. The commercial ELISA kits used for the osteocalcin and pyridinoline have been used in assessement of bone formation and resorption indicators, respectively (Regmi et al., 2015). For PTH, a chicken-specific ELISA kit (Cusabio, Baltimore, MD USA), was used following the manufacturer's recommended procedures. Also, serum Ca and P levels were analyzed as described in Chapter 2.

3.2.4 Bone quality sample collection and analyses

At 78 woa, all hens were euthanized by cervical dislocation and the left femurs excised for subsequent bone densitometry assessment using QCT. The femurs (n=100) were analyzed at the mid-diaphysis (at 50% of bone length) and proximal metaphysis (30% from the proximal end) for total, cortical, and trabecular space bone mineral densities (**BMD**; mg/cm^3) and cross-sectional areas (**BCSA**; mm^2). Bone mineral content (**BMC**; mg/mm) was calculated by multiplying BMD by BCSA to determine the amount of mineral (mg) contained within a one mm-thick x-ray scan perpendicular to the bone length (Saunders-Blades et al., 2009; Chapter 2).

3.2.5 Economic analysis

An economic analysis was conducted based on the quantity and cost of feeds and egg production. Total quantity of feed intake per hen throughout the ten-wk study was determined and multiplied by the cost of feed consumed for overall feed cost. Based on the current price of $1.77 CAD per dozen eggs at the time of the experiment (Statistics Canada, 2017), profit was calculated by subtracting overall feed cost from total egg income over the 10 wk of study. One

Canadian dollar was estimated as price of BSP used per ton of diet was considered in the feed cost for the BSP-supplemented diets (Wealleans et al., 2016). Price differences for each of NC1, NC2, NC1+BSP, and NC2+BSP relative to the PC were calculated to estimate the effects of the moderate and severe avP and Ca reduction and the phytase usage on economic of egg production.

3.2.6 Experimental design and statistical analysis

The individually-housed hen was the experimental unit in a completely randomized experimental design. The egg production data was not normally distributed, and was log transformed prior to analysis. However, treatment means for the egg production analysis was presented as the original values. The MIXED procedure of SAS 9.3 (SAS, 2013) was used for analysis of the log-transformed egg production, feed intake, FCR, BW, and eggshell breaking strength and specific gravity in repeated measure analysis for the diet×age interaction and diet and age main effects. In the repeated measure analysis for each of the parameters, the fixed variables (diet×age interaction and diet and age main effects) were analyzed with age as the repeated subject and cage as the random subject. Also, the MIXED procedure of SAS 9.3 (2013) was used for analysis of diet main effect for each of the biochemical bone markers, the bone densitometry traits, and the economic parameters. When significant, BW was included in the analyses of the bone densitometry parameters as covariate. Effects were significant when $P \leq 0.05$. LSMEANS was used for means comparison.

3.3 RESULTS

3.3.1 Performance and eggshell quality parameters

From 68 to 71 woa, egg production was not affected by dietary treatment (Table 3.6.2). Egg production was not different between PC, NC1, and NC1+BSP hens except at 77 woa, when egg production with either the NC1 or NC2 diets was increased by BSP supplementation.

However, the NC2 diet reduced egg production relative to all other treatments from 72 to 78 woa (P<0.001). Feed intake from 68 to 69 woa was lower in NC1 and NC2 than PC, and from 70 to 71 woa was lower in NC1 than in PC and was intermediate in the NC2 and respective BSP-supplemented diets (P=0.002; Table 3.6.3). Feed intake from 72 to 73 and from 74 to 75 was maintained by NC1 but decreased by NC2 relative to PC and NC2+BSP, but no differences between diets were observed from 76 to 78 woa. FCR was maintained by NC1 relative to PC and NC1+BSP throughout the study and was maintained from 68 to 69 and 70 to 71 woa but decreased from 72 to 73, 74 to 75, and 76 to 78 woa by the NC2 relative to the PC and NC2+BSP (P<0.001). Eggshell breaking strength was maintained by NC1 and NC2 relative to PC and the respective BSP-supplemented diets at all ages except at 70, 76, and 78 woa, however, was higher in NC2+BSP than NC2 at 68 woa (P=0.001; Table 3.6.4). At each of 70, 76, and 78 woa, the eggshell breaking strength were decreased by the NC2 relative to the PC and NC2+BSP. Egg specific gravity was not different between diets at 68 and 74 woa, but was lower for NC2 than in PC and NC2+BSP at 70, 76, and 78 woa (P=0.009). At 72 woa, NC2 had lower specific gravity than PC, but was intermediate in NC2+BSP. Hen BW was not affected by diet at each of 68 and 70 woa (P=0.001; Table 3.6.5). BW was decreased by NC1 relative to PC at each of 76 and 78 woa and was intermediate in the NC1+BSP at both ages. BW of NC2 hens was lower than the PC hens at each of 72, 74, 76, and 78 woa and was intermediate in NC2+BSP hens at 72 woa, while phytase addition to the NC2 diet completely alleviated the adverse effects of reduced dietary avP and Ca.

3.3.2 Serum biochemical bone markers and bone densitometry

Serum pyridinoline concentration was maintained by NC1 relative to PC and NC1+BSP and was increased by NC2 relative to the other diets (P=0.002; Table 3.6.6). There was no diet

effect on any other bone biochemical marker measured. Proximal metaphysis and mid-diaphysis total BMD, and mid-diaphysis trabecular space BMD were each lower ($P \leq 0.002$, 0.004, and 0.001, respectively) for NC1 and NC2 than PC, NC1+BSP, and NC2+BSP (Table 3.6.7). Proximal metaphysis trabecular space BMD was lowered by NC1 and NC2 relative to PC, but supplemental BSP completely alleviated the adverse effect of mineral reductions in the NC1 diet only (P=0.001). The trabecular space BCSA tended to be higher (P=0.083) for NC1 and NC2 relative to PC, NC1+BSP, and NC2+BSP. The effects of diet for proximal metaphysis and mid-diaphysis total BMC, and mid-diaphysis trabecular space BMC were each similar (P=0.004, 0.008, and 0.002, respectively) to the effect pattern for trabecular space BMD in the proximal metaphysis. Also, the proximal metaphysis trabecular space BMC was maintained by NC1 and was decreased by NC2 and NC2+BSP relative to the PC (P=0.004).

3.3.3 Economic analysis

The NC1 diet maintained economic parameters relative to the PC and NC1+BSP diets ($P \leq 0.006$; Table 3.6.8). However, the NC2 decreased feed intake and decreased total return on eggs with reduced egg production to subsequently decrease profit on total egg produced over the 10 wk of the study. The use of phytase in the NC2 diet completely alleviated the adverse effects of the avP and Ca severe deficiency on all economic parameters assessed from 68 to 78 woa.

3.4 DISCUSSION

3.4.1 Effects of moderate and severe deficiencies of dietary available P and Ca

The avP and Ca levels in the NC1 diet maintained performance throughout the ten wk study at the expense of BW and bone mass and mineralization. The NC2 diet decreased productivity after 71 woa, but the NC1 diet maintained productivity relative to PC at all ages. Apparently, BW and bone reserves in the NC2 hens were depleted beyond the level required to maintain their production performance, while BW and bone reserves of the NC1 hens was

adequate to support egg production throughout the study. Diets with 2.0 g/kg avP maintained productivity and BW from 20 to 70 woa, while a diet with 1.5 g/kg avP maintained productivity but decreased BW from 41 woa, and a 1.0 g/kg avP diet decreased productivity from 28 woa and BW from 31 woa, (Boling et al., 2000). The mechanism by which NC1 maintained, and NC2 decreased performance can be explained by the adverse effects of avP and Ca reductions in each of the diets on BW and bone quality of the hens. The moderate reductions in avP and Ca in the NC1 diet were not sufficient to reduce egg production and eggshell quality throughout the study. In actively laying hens, cortical and trabecular bone tissues are not formed but may be resorbed, while the medullary bone tissue can be formed and resorbed to support eggshell formation (Dacke et al., 1993). Hence, structural bones in the NC1 hens would not have been newly formed but may have been resorbed with the medullary bone for eggshell formation. However, the NC1 diet also maintained cortical bone densitometry but decreased BW from 76 woa and femur trabecular space BMD and BMC at the end of the study. Trabecular micro-architecture of the NC1 hens was maintained, and the medullary BMD and volume, and thickness were increased or tended to increase relative to the PC (Chapter 4). The decreased trabecular space BMD and BMC by the NC1 indicated decreased medullary bone mass and mineralization in the NC1 hens (Chapter 4). A diet with 32.6 g/kg Ca fed from 19 to 45 woa maintained egg production and decreased BW and increased osteoporosis incidence (Cransberg et al., 2001), indicating that the NC1 hens maintained egg production by increasing resorption of structural bone after 26 wks. Genetic selection over the years has increased the resistance of modern egg-laying hens to structural bone loss (Fleming et al., 2006). Hence, the NC1 diet may need to be fed for more than ten wk to observe adverse effects on structural bone tissues. The decreased BW from 76 woa,

and the nearly significant decrease in medullary bone at 78 woa (Chapter 4) imply an initial stage of the effect of the moderate reduction of avP and Ca in the NC1 on the performance of the hens.

The decreased egg production and eggshell quality and the maintained structural bone tissues by the NC2 diet indicated that the severe avP and Ca deficiencies forced some of the hens to molt to restore structural bones in the hens. At least 4 out of the 20 hens were molting and stopped laying in the last 3 to 5 wk of the study. Molting in egg-laying hens decreases circulating estrogen level to stop egg production and medullary bone formation, but restore structural bone (Dacke et al., 1993). However, Kim et al. (2007) reported that QCT-measured trabecular bone decreased after nine days of molting. The femur and tibia metaphyses measured as "trabecular bone" in that study would have also contained medullary bone, which was not accounted for by Kim et al. (2007). Although medullary bone is mainly concentrated in the mid-diaphysis, and trabecular bone towards the metaphyses, the two bone tissues are intermingled within the endosteal cavity (Shahnazari et al., 2006; Kerschnitzki et al., 2014; Chapter 4). Since it is illogical for the trabecular bone to decrease and for medullary bone to increase during a molt, the decreased "trabecular" BMD with a 9-d molting from 86 woa hens reported by Kim et al., (2007) indicated that the deposition of structural bone was likely masked by the loss of medullary bone in the distal and proximal metaphyses of long bones. The NC2 hens that began to molt in the current study would have had decreased medullary bone and have begun to replenish structural bone (Fleming et al., 1998). Furthermore, NC2 increased serum pyridinoline concentration, which indicates higher osteoclastic activity with no indication of which of the three bone tissues was resorbed (Regmi et al., 2015). Knott et al. (1995) also affirmed that a Ca-reduced diet increased serum pyridinoline level. Overall, the serum analyzed for the bone biomarker was collected between 7:00 AM to 11:30 AM, around when most hens lay (Kerschnitzki et al., 2014),

and this may have influenced the lack of diet effect on serum osteocalcin concentration, the bone formation marker. Palpation of the eggshell gland of the hens prior to bleeding confirmed that the majority of hens were forming eggshell during the 4.5 h blood collection period. Hens that were forming eggshells at this time should have Ca supplied to the eggshell largely from dietary intake and not bone, and hens that were not forming eggshells at this time should have been forming medullary bone. The increase in serum pyridinoline concentration indicated that the severe reduction in Ca and avP caused the NC2 hens to mobilize bone Ca to support eggshell formation. Also, the lack of diet effects on serum PTH, P, and Ca may be because of the very tight regulation of their respective blood levels (Dacke et al., 1993). Hence, the decreased BW, which started from 73 woa, the medullary bone loss at 78 woa (Chapter 4), and the maintained structural bone implies an advanced, severe deficiency of at least one of avP and Ca in the NC2 hens.

3.4.2 Effects of phytase supplementation in diets moderately and severely reduced in available P and Ca

The NC1 diet maintained egg production, feed intake, FCR, and eggshell quality, and therefore limited the opportunity to observe effects of BSP on performance and eggshell quality. However, phytase supplementation in the NC1 diet alleviated the adverse effect of the moderate avP and Ca reductions on the decreased BW and total and trabecular space BMD and BMC of the hens. The inclusion of phytase in avP- and Ca-reduced diets increases the availability of the minerals in the gastrointestinal tract of laying hens (Van der Klis et al., 1997; Liu et al., 2007; Beutler, 2009), resulting in maintained egg production, BW, and bone mineralization (Boling et al., 2000). These findings imply that supplemental BSP increased P and Ca utilization to maintain medullary bone mass and mineralization and support healthy remodeling of the non-structural bone for eggshell formation. On the other hand, phytase inclusion in the NC2 diet

alleviated the decreased performance, eggshell quality, BW, and bone quality caused by the severe avP and Ca reductions in laying hens. Supplemental BSP also decreased overall bone resorption as indicated by the decrease in serum pyridinoline concentration. However, supplemental phytase did not completely prevent the decreased total and trabecular BMD and BMC relative to the PC. Evidently, the reduction of avP and Ca in the NC2 relative to the PC were beyond the phosphoric and extra-phosphoric potential of 600 FTU/kg of BSP. This finding is consistent with that of Chapter 4, which also showed that the use of the BSP in the NC2 diet did not completely alleviate the detrimental effects on micro-architectural traits of the femur medullary bone relative to the PC. Although 600 FTU/kg BSP prevented the adverse effects of severe avP and Ca reductions on the performance of laying hens, this level of supplementation was insufficient to maintain medullary bone metabolism. This finding implies the need to maintain a balance between the degree of avP and Ca reduction and dietary phytase activity to maintain hen performance and prevent bone loss.

3.4.3 Economic analysis

Inorganic sources of P such as monocalcium phosphate and dicalcium phosphate are expensive and limiting (Neset and Cordell, 2012; Ahmadi and Rodehutscord, 2012). Therefore, dietary phytase supplementation allows for a substantial reduction in the use of these ingredients (Kim et al., 2017), which would be expected to decrease feed cost. Similarly, dietary phytase allows for the inclusion of high-phytate and low-cost feed ingredients as a partial substitution for other relatively low-phytate and high-cost feed ingredients, to also decrease feed cost (Ponnuvel et al., 2013). The lack of a significant decrease in feed cost per dozen egg produced or increase in the estimated profit relative to the NC1+BSP relative to the NC1 diet was because the NC1 maintained egg production throughout the 10 wk study. Although BSP supplementation in NC1

allowed a saving of $5 CAD/ton relative to PC, the lack of difference in egg production of the NC1 hens relative to PC and NC1+BSP hens limited the opportunity for the enzyme to increase profitability. The lack of difference in profit/hen between the NC2+BSP diet and the PC diet was because of the ability of BSP to statistically alleviate the suppressed feed intake and decreased egg production by the severe reductions in the NC2 diet to prevent a loss in profitability. The avP and Ca reductions in the NC2 diet decreased total egg income and profit across the 10 wk by $1.59 CAD, and the overall profit on all eggs produced by $1.45 CAD per hen relative to the PC. Supplementation of BSP in the NC2 diet alleviated the decreased total egg income and profit by $1.37 CAD and $1.23 CAD, respectively. Significantly, inclusion of BSP in the NC2 diet restored the decreased total egg income and profit to similar levels as the PC diet. The increase in profitability with the use of BSP in the NC2 diet was because of the release of phytate-bound P and Ca to alleviate the adverse effects of the severe deficiencies on the productivity of the hens. Therefore, BSP supplementation in the NC2 diet increased productivity to increase profitability relative to the NC2 diet.

Egg-laying hens are able to maintain productivity on nutrient-reduced diets, depending on the degree of nutrient deficiency. (Boling et al., 2000; Silversides et al., 2006; Geraldo et al., 2014). Egg-laying hens were able to physiologically adapt to the nutrient reduction and maintain performance at the expense of BW and bone mineralization, until their BW and bone reserve could no longer support performance and eggshell formation. The NC1 hens fed the moderately avP- and Ca- reduced diet maintained performance and eggshell quality throughout the study, with no evidence of structural bone loss. While the severe reduction of avP and Ca in the NC2 diet decreased hen performance, it did not result in structural bone loss. However, the NC2 hens that went out of lay may have partially replaced lost structural bone as the molt proceeded. On

the other hand, the NC2 hens that were actively laying when the trial was stopped would likely have still been undergoing structural bone loss. Hence, phytase supplementation in diets reduced in avP and Ca supported medullary bone remodeling in actively laying hens. Phytase supplementation allows for a substantial reduction in dietary avP and Ca levels with no adverse effect on bone metabolism of laying hens when the enzyme is adequate to release sufficient mineral to counteract the dietary reductions. Medullary bone is essential in the laying hen as a labile mineral reserve for eggshell formation (Dacke et al., 1993), but provides minimal resistance to bone fracture (Fleming et al., 1998). Phytase in the diet of laying hens may allow for further reductions in avP and Ca levels to decrease feed costs relative to the current practice, with no adverse effects on performance and bone health of the birds.

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3.6 TABLES

Table 3.6.1: Ingredients, calculated nutrients, and analyzed Ca and P levels and phytase activity[1]

	PC[2]	NC1[2]	NC2[2]	NC1+ BSP[2]	NC2+ BSP[2]
Ingredients, %					
Corn	62.8	62.7	62.7	62.7	62.7
Soybean meal	12.3	12.3	12.2	12.3	12.2
Canola meal	12.0	12.0	12.0	12.0	12.0
Oat hulls	2.97	3.99	4.46	3.99	4.46
Calcium carbonate	7.78	7.89	7.71	7.89	7.71
Dicalcium phosphate	1.25	0.24	-	0.24	-
L - Lysine	-	-	-	-	-
DL - Methionine	0.09	0.09	0.09	0.09	0.09
Layer vitamin-mineral premix[3]	0.50	0.50	0.50	0.50	0.50
Salt	0.32	0.32	0.32	0.32	0.32
Buttiauxella sp. phytase[4]	-	-	-	0.04	0.04
Calculated nutrient, % unless stated					
Metabolizable energy, MJ/kg	11.3	11.3	11.3	11.3	11.3
Crude protein	15.9	15.9	15.9	15.9	15.9
Dig. Lys	0.67	0.66	0.66	0.66	0.66
Dig. Met	0.35	0.35	0.35	0.35	0.35
Dig.TSAA	0.54	0.54	0.54	0.54	0.54
Linoleic acid	1.49	1.49	1.49	1.49	1.49
Calcium (%)	3.500	3.341	3.225	3.341	3.225
Total phosphorus	0.593	0.406	0.362	0.406	0.362
Available phosphorus	0.350	0.163	0.119	0.163	0.119
Phytate phosphorus	0.241	0.241	0.241	0.241	0.241
Analyzed nutrients, DM basis					
Calcium	3.785	3.689	3.605	3.256	3.224
Total phosphorus (%)	0.594	0.487	0.480	0.440	0.445
Analyzed phytase activity	<100	<100	421	< 100	537

[1]Diets were formulated based on an assumed intake of 110 g/hen per day.

[2] PC = a diet with 0.35% avP and 3.5% Ca; NC1 = the PC diet reduced in avP and Ca by 0.187 and 0.159% of the diet, respectively: NC2 = the PC diet reduced in avP and Ca by 0.231 and 0.275% of the diet, respectively; and supplementation of 600 FTU/kg BSP in NC1 (NC1+BSP) or NC2 (NC2+BSP).

[3]Vitamin-mineral premix provided (per kg of complete diet): 88 mg Mn; 100 mg Zn; 80 mg Fe; 15 mg Cu; 1.7 mg I; 12,500 IU vitamin A; 3,125 IU vitamin D_3; 40 IU vitamin E; 2.5 mg vitamin K; 2.6 mg thiamin; 7.5 mg riboflavin; 37.5 mg niacin; 5 mg pyridoxine; 12.5 mg d-pantothenic acid; 18.8 µg vitamin B_{12}; 0.63 mg folic acid; and 150 µg biotin.

[4]Axtra® PHY (Danisco Animal Nutrition, DuPont Industrial Bioscience, Marlborough, UK) supplemented in the NC1+BSP and NC2+BSP diets to provide 600 FTU/kg feed.

Table 3.6.2: Effect of dietary Ca, available P levels and phytase on egg production of laying hens from 68 to 78 wk of age[1]

Weeks of age	68	69	70	71	72	73	74	75	76	77	78
	----------%----------										
PC[2]	91.4	86.4	87.1	89.3	92.9[a]	87.9[a]	93.6[a]	89.3[a]	92.1[a]	90.0[ab]	91.0[a]
NC1[2]	92.9	93.6	87.1	91.4	87.9[a]	87.1[a]	86.4[a]	89.5[a]	91.4[a]	84.3[b]	85.0[a]
NC2[2]	96.4	90.7	82.1	85.7	76.4[b]	72.1[b]	71.4[b]	72.2[b]	69.3[b]	69.3[c]	73.3[b]
NC1+BSP[2]	96.4	93.6	92.1	90.7	91.4[a]	90.0[a]	92.5[a]	88.7[a]	85.7[a]	95.7[a]	89.0[a]
NC2+BSP[2]	92.9	92.1	93.6	87.9	87.9[a]	89.3[a]	85.7[a]	87.9[a]	90.0[a]	82.1[b]	86.0[a]
Pooled SEM						4.00					
Source of variation	----------*P*-value----------										
Diet×Age						<0.001					

[a-c]Treatment means with no common superscript within each column differ significantly ($P \leq 0.05$).

[1]Treatment means at each week of age were calculated with 20 replicates per treatment.

[2]PC = positive control (nutrient-adequate diet with 3.5% Ca and 0.35% avP), NC1 = negative control 1 (the PC reduced in Ca and avP by 0.187 and 0.159% of the diet, respectively); NC2 = negative control 2 (the PC reduced in Ca and avP by 0.231 and 0.275% of the diet, respectively); and NC1+BSP and NC2+BSP = the NC diets supplemented with *Buttiauxella sp*. phytase at 600 FTU/kg.

Table 3.6.3: Effect of dietary Ca, available P levels and phytase on feed intake and feed conversion ratio of egg-laying hens from 68 to 78 wk of age[1]

Weeks of age	68-69	70-71	72-73	74-75	76-78
Feed intake, kg/d/hen					
PC[2]	0.113[a]	0.106[a]	0.104[a]	0.110[ab]	0.104
NC1[2]	0.105[b]	0.099[b]	0.102[ab]	0.105[bc]	0.105
NC2[2]	0.103[b]	0.101[ab]	0.097[b]	0.103[c]	0.105
NC1+BSP[2]	0.108[ab]	0.103[ab]	0.105[a]	0.108[abc]	0.107
NC2+BSP[2]	0.108[ab]	0.103[ab]	0.106[a]	0.111[a]	0.108
Pooled SEM			0.002		
Source of variation	----------*P*-value----------				
Diet×Age			0.011		
FCR, kg feed/dozen eggs					
PC[2]	1.500	1.447	1.360[b]	1.419[b]	1.350[b]
NC1[2]	1.354	1.347	1.417[b]	1.444[b]	1.467[b]
NC2[2]	1.282	1.488	1.703[a]	1.865[a]	1.736[a]
NC1+BSP[2]	1.346	1.309	1.362[b]	1.439[b]	1.396[b]
NC2+BSP[2]	1.369	1.342	1.462[b]	1.611[b]	1.506[b]
Pooled SEM			0.083		
Source of variation	----------*P*-value----------				
Diet×Age			0.001		

[a-c]Treatment means with no common superscript within each column for feed intake and feed conversion ratio differ significantly ($P \leq 0.05$).

[1]Treatment means for feed intake and feed conversion ratio at each age range were calculated with 20 replicates.

[2]PC = positive control (nutrient-adequate diet with 3.5% Ca and 0.35% avP), NC1 = negative control 1 (the PC reduced in Ca and avP by 0.187 and 0.159% of the diet, respectively); NC2 = negative control 2 (the PC reduced in Ca and avP by 0.231 and 0.275% of the diet, respectively); and NC1+BSP and NC2+BSP = the NC diets supplemented with *Buttiauxella sp*. phytase at 600 FTU/kg.

Table 3.6.4: Effect of dietary Ca and available P levels and phytase on eggshell quality traits of laying hens from 68 to 78 wk of age[1]

Weeks of age	68	70	72	74	76	78
Egg-shell breaking strength, kgF						
PC[2]	3.41[ab]	3.77[a]	3.68	3.40[ab]	3.37[a]	3.58[a]
NC1[2]	3.41[ab]	3.68[a]	3.55	3.10[b]	3.20[ab]	3.50[ab]
NC2[2]	3.21[b]	2.76[b]	3.44	3.09[b]	2.83[b]	3.02[b]
NC1+BSP[2]	3.73[a]	3.86[a]	3.91	3.46[ab]	3.68[a]	3.70[a]
NC2+BSP[2]	3.87[a]	3.50[a]	3.80	3.67[a]	3.40[a]	3.77[a]
Pooled SEM				0.18		
Source of variation	----------*P*-value----------					
Diet×Age				0.001		
Specific gravity						
PC	1.082	1.084[a]	1.082[a]	1.082	1.082[a]	1.080[a]
NC1	1.081	1.081[ab]	1.079[ab]	1.081	1.082[a]	1.081[a]
NC2	1.080	1.078[b]	1.078[b]	1.080	1.077[b]	1.076[b]
NC1+BSP	1.082	1.083[a]	1.081[ab]	1.081	1.082[a]	1.080[a]
NC2+BSP	1.081	1.084[a]	1.081[ab]	1.083	1.082[a]	1.080[a]
Pooled SEM				0.006		
Source of variation	----------*P*-value----------					
Diet×Age				0.009		

[a-b]Treatment means with no common superscript within each column for egg-shell breaking strength and specific gravity differ significantly ($P \leq 0.05$).

[1]Treatment means for eggshell breaking strength and specific gravity at each week of age were calculated with 20 replicates.

[2]PC = positive control (nutrient-adequate diet with 3.5% Ca and 0.35% avP), NC1 = negative control 1 (the PC reduced in Ca and avP by 0.187 and 0.159% of the diet, respectively); NC2 = negative control 2 (the PC reduced in Ca and avP by 0.231 and 0.275% of the diet, respectively); and NC1+BSP and NC2+BSP = the NC diets supplemented with *Buttiauxella sp*. phytase at 600 FTU/kg.

Table 3.6.5: Effect of dietary Ca and available P levels and phytase on body weight of egg-laying hens from 68 to 78 wk of age[1]

	68	70	72	74	76	78
	----------%----------					
PC[2]	1.659	1.673	1.641[a]	1.639[a]	1.641[a]	1.622[a]
NC1[2]	1.669	1.654	1.600[ab]	1.587[a]	1.557[bc]	1.524[bc]
NC2[2]	1.657	1.632	1.553[b]	1.506[b]	1.482[c]	1.441[c]
NC1+BSP[2]	1.641	1.641	1.606[ab]	1.590[a]	1.584[ab]	1.575[ab]
NC2+BSP[2]	1.662	1.679	1.619[ab]	1.631[a]	1.626[ab]	1.603[a]
Pooled SEM				0.03		
Source of variation	----------*P*-value----------					
Diet×Age				0.001		

[a-c]Treatment means with no common superscript within each column differ significantly ($P \leq 0.05$).

[1]Treatment means at each week of age were calculated with 20 replicates.

[2]PC = positive control (nutrient-adequate diet with 3.5% Ca and 0.35% avP), NC1 = negative control 1 (the PC reduced in Ca and avP by 0.187 and 0.159% of the diet, respectively); NC2 = negative control 2 (the PC reduced in Ca and avP by 0.231 and 0.275% of the diet, respectively); and NC1+BSP and NC2+BSP = the NC diets supplemented with *Buttiauxella sp*. phytase at 600 FTU/kg.

Table 3.6.6: Effect of dietary Ca, available P levels and phytase on serum biochemical bone markers in egg-laying hens at 78 wk of age[1]

	Osteocalcin[2] (ng/ml)	Pyridinoline[3] (nmol/L)	Parathyroid hormone[4] (pg/dl)	Ca (mg/dl)	P (mg/dl)
PC[5]	8.78	4.65[b]	35.5	12.9	2.79
NC1[5]	9.90	5.07[b]	34.0	12.9	2.43
NC2[5]	6.76	6.41[a]	38.6	13.4	1.95
NC1+BSP[5]	7.62	4.90[b]	37.6	13.5	2.32
NC2+BSP[5]	8.58	4.85[b]	36.9	12.9	2.26
Pooled SEM	0.88	0.31	4.36	0.77	0.43
Source of variation	----------*P*-value----------				
Diet	NS	0.002	NS	NS	NS

[a-b]Treatment means with no common superscript for serum pyridinoline differ significantly ($P \leq 0.05$).

[1]Treatment means for each parameter were calculated with 12 replicates.

[2]Indicates bone formation across the cortical, trabecular, and medullary bones.

[3]Indicates bone resorption across the cortical, trabecular, and medullary bones.

[4]Involves in the regulation of bone formation and resorption (remodeling).

[5]PC = positive control (nutrient-adequate diet with 3.5% Ca and 0.35% avP), NC1 = negative control 1 (the PC reduced in Ca and avP by 0.187 and 0.159% of the diet, respectively); NC2 = negative control 2 (the PC reduced in Ca and avP by 0.231 and 0.275% of the diet, respectively); and NC1+BSP and NC2+BSP = the NC diets.

Table 3.6.7: Effect of dietary Ca, available P levels and phytase on bone densitometry of total, trabecular space, and cortical bone tissues in femur proximal metaphysis and mid-diaphysis of white egg-laying hens at 78 weeks of age[1]

	Bone mineral density (mg/cm^3)			Bone cross-sectional area (mm^3)			Bone mineral content (mg/cm)^2		
	Total	Cortical	Trabecular space[3]	Total	Cortical	Trabecular space[3]	Total	Cortical	Trabecular space[3]
Proximal metaphysis[4]									
PC[5]	519^{a}	1,075	259^{a}	47.2	14.8	30.1	24.5^{a}	15.8	7.74^{a}
NC1[5]	475^{bc}	1,077	220^{c}	47.8	14.1	32.9	22.7^{c}	15.2	7.20^{ab}
NC2[5]	456^{c}	1,086	190^{d}	47.2	14.1	32.4	21.5^{c}	15.2	6.06^{c}
NC1+BSP[5]	519^{a}	1,087	253^{ab}	46.7	14.7	30.4	24.2^{ab}	16.0	7.62^{ab}
NC2+BSP[5]	498^{ab}	1,087	230^{bc}	46.1	14.3	30.2	23.0^{bc}	15.5	6.89^{bc}
Pooled SEM	11.74	6.66	10.24	0.53	0.34	0.856	0.55	0.34	0.31
Source of variation	----------*P*-value----------								
Diet	0.002	0.543	0.002	0.234	0.389	0.083	0.002	0.391	0.003
Co-variate (BW)	0.017	0.190	0.112	0.002	0.002	0.910	<0.001	<0.001	0.135
Mid-diaphysis[4]									
PC[5]	572^{a}	1,162	254^{a}	39.9	13.7	24.3	22.8^{a}	15.9	6.11^{ab}
NC1[5]	533^{bc}	1,169	211^{bc}	39.9	13.4	26.1	21.2^{b}	15.6	5.46^{b}
NC2[5]	516^{c}	1,162	181^{c}	39.9	13.7	25.7	20.6^{b}	15.9	4.58^{c}
NC1+BSP[5]	584^{a}	1,164	263^{a}	39.6	14.1	24.3	23.1^{a}	16.4	6.32^{a}
NC2+BSP[5]	560^{ab}	1,162	235^{ab}	38.8	13.6	23.7	21.7^{ab}	15.8	5.39^{bc}
Pooled SEM	12.71	8.36	11.09	0.53	0.37	0.94	0.51	0.36	0.30
Source of variation	----------*P*-value----------								
Diet	0.004	0.966	<0.001	0.540	0.715	0.385	0.008	0.653	0.002
Co-variate (BW)	0.008	0.346	0.209	0.008	<0.001	0.459	<0.001	<0.001	0.485

[a-c]Treatment means with no common superscript within each column at the proximal metaphysis and mid-diaphysis differ significantly ($P \leq 0.05$).

[1]Each dietary treatment mean was calculated from 20 hens at each age.

[2]Calculated by multiplying the respective bone cross-sectional area by bone mineral density to yield the amount of mineral (mg) contained in one mm-thick x-ray scan.

[3]Represents both trabecular and medullary bone tissues.

[4]Femur proximal metaphysis measurement at 30% of the bone length from the proximal end, and mid-diaphysis measurement at the longitudinal center of the bone.
[5]PC = positive control (nutrient-adequate diet with 3.5% Ca and 0.35% avP), NC1 = negative control 1 (the PC reduced in Ca and avP by 0.187 and 0.159% of the diet, respectively); NC2 = negative control 2 (the PC reduced in Ca and avP by 0.231 and 0.275% of the diet, respectively); and NC1+BSP and NC2+BSP = the NC diets.

Table 3.6.8: Economic analysis of dietary Ca, available P levels and phytase on egg-laying hens across 68 to 78 wk of age[1]

	Total feed intake (kg/hen)[2]	Feed cost ($ CAD[3]/ton)[4]	Overall feed cost ($ CAD[3]/hen)[5]	Total egg produced (number/hen)[6]	Total egg income ($ CAD[3]/hen)[7]	Profit ($ CAD[3]/hen)[8]
PC[9]	7.45[ab]	280	2.09[a]	68[a]	10.10[a]	8.01[a]
NC1[9]	7.25[bc]	275	1.99[bc]	66[a]	9.73[a]	7.73[a]
NC2[9]	7.14[c]	274	1.96[c]	58[b]	8.51[b]	6.56[b]
NC1+BSP[9]	7.41[abc]	276	2.05[ab]	67[a]	9.91[a]	7.87[a]
NC2+BSP[9]	7.55[a]	275	2.08[a]	67[a]	9.88[a]	7.80[a]
Pooled SEM	0.10	-	0.03	1.88	0.28	0.27
Source of variation	----------*P*-value----------					
Diet	0.044	-	0.006	0.001	0.001	0.002

[a-c]Treatment means with no common superscript within each column differ significantly ($P \leq 0.05$).
[1]Treatment means for each column were calculated with 20 replicates.
[2]Total feed intake throughout the ten-week study.
[3]Canadian dollar
[4]Price of each diet per ton using current ingredient prices at the time of the experiment.
[5]Total feed intake multiplied by the cost of total feed intake throughout the 10-week study.
[6]Total number of eggs laid per hen throughout the ten-week study.
[7]Calculated based on the price of $1.77 CAD per dozen eggs at the time of the experiment.
[8]Calculated by subtracting overall feed cost from total egg income.
[9]PC = positive control (nutrient-adequate diet with 3.5% Ca and 0.35% avP), NC1 = negative control 1 (the PC reduced in Ca and avP by 0.187 and 0.159% of the diet, respectively); NC2 = negative control 2 (the PC reduced in Ca and avP by 0.231 and 0.275% of the diet, respectively); and NC1+BSP and NC2+BSP = the NC diets supplemented with *Buttiauxella sp*. phytase at 600 FTU/kg.

4. PHYTASE EFFECTS ON BONE MINERALIZATION OF WHITE EGG-LAYING HENS: ASSESSMENT WITH MICRO COMPUTED TOMOGRAPHY

ABSTRACT

Bone supports the body and stores mineral for eggshell formation in laying hens. The effects of dietary available P (avP) and Ca levels, and phytase supplementation on bone micro-architecture in femurs of White Leghorn hens were studied. In trial I, 72 hens (4 per cage) were fed one of three diets: (1) a positive control (PC; nutrient-adequate diet based on the primary breeder recommendation), (2) a negative control (NC; the PC with avP and Ca levels marginally reduced by 0.146 and 0.134% of the diet, respectively); or (3) the NC supplemented with 300 FTU/kg phytase (NC+PHY). In trial 2, 100 hens were fed one of 5 diets from 68 to 78 woa. The diets were (1) a PC with 0.35% avP and 3.5% Ca, (2) NC1; the PC diet with avP and Ca moderately reduced (by 0.187 and 0.159% of the diet, respectively), and (3) NC2; the PC diet severely reduced in avP and Ca (by 0.231 and 0.275% of the diet, respectively). Other diets for the trial 2 were the NC1 and NC2 supplemented with 600 FTU/kg phytase (4) NC1+PHY and (5) NC2+PHY, respectively. At the end of each trial, the right femur of each hen was scanned ex vivo using micro-computed tomography and analyzed for cortical, trabecular, and medullary bone tissues for 2- and 3-dimensional (2D and 3D) micro-architectural traits. In trial I, the NC and NC+ECP hens had 12±0.4% higher 2D cortical separation, 15±0.4% higher 3D trabecular bone volume to non-bone, and 25% lower medullary bone mineral density than PC hens. While diet did not affect cortical bone, the trabecular bone tissue was detrimentally affected by the PC diet, possibly because of the adverse effects of the high Ca level in the primary breeder recommendation based-diet. In trial II, the 3D cortical bone mineral density was 1.6% higher in PC hens than in NC2 hens, and the 3D medullary bone volume to non-bone and bone mineral density tended to be lower (P<0.068) in the NC2 hens than in hens fed the other diets. The

low avP and Ca levels in the NC2 decreased bone quality, but phytase supplementation alleviated the adverse effects of the deficiencies on bone micro-architecture. Overall, high and deficient dietary AvP and Ca levels lowered quality of bone tissues; however, phytase supplementation in the Ca- and avP-deficient diet maintained fortified bone in egg-laying hens.

Keywords: laying hen, phytase, micro-architecture, structural bone, medullary bone,

4.1 INTRODUCTION

Bones such as the femur and tibia in laying hens are comprised of cortical, trabecular, and medullary tissues (Whitehead and Fleming, 2000). Anatomically, the cortical and medullary bone tissues are mainly concentrated around the mid-diaphysis (Shahnazari et al., 2006), whereas the trabecular bone tissue is primarily found towards the proximal and distal ends of long bones (Barak et al., 2010). The cortical and trabecular bones are strength-providing structural bone, and although medullary bone provides minimal resistance to bone fracture, it is primarily a non-structural labile mineral reserve for eggshell formation (Fleming et al., 1998). Cortical and trabecular bones are not formed in actively laying hens (Fleming et al., 1998), however, structural bone tissues are resorbed even in hens fed nutrient-adequate diets (Wilson and Thorp, 1998; Cransberg et al., 2001). However, medullary bone is formed and resorbed in laying hens as long as egg production continues (Dacke et al., 1993). An eggshell takes about 20 h to form in laying hens, throughout which a supply of Ca is required as calcium carbonate (Hodges, 1970; Kerschnitzki et al., 2014). The influx of Ca into the eggshell gland comes directly from the diet when hens are feeding, and from the bone in the absence of dietary Ca supply (Kerschnitzki et al., 2014). When eggshell is not being formed, and hens are feeding, the Ca and P intake are primarily utilized for mineralization of the medullary bone (Van De Velde et al., 1985; Kerschnitzki et al., 2014). Adequate metabolism of medullary bone is essential for eggshell formation.

Diets deficient in Ca and P decreased volumes of structural and medullary bones in laying hens (Wilson and Duff, 1991; Boling et al., 2000). On the other hand, excess dietary Ca decreased gastrointestinal tract (**GIT**) availability of P and Ca (Pelicia et al., 2009; Beulter, 2009) in laying hens. Hence, both excess and deficient levels of dietary Ca and P may adversely affect each of cortical, trabecular, and medullary bone tissues in laying hens. Bone metabolism is dependent on blood Ca and P levels, which in turn depend on GIT availability and absorption. Therefore, the availability of P and Ca absorption from the GIT is vital to bone metabolism. Approximately 60 to 75% of P in plant-based ingredients used in poultry diets is unabsorbed because of the presence of phytate (Tahir et al., 2012). Therefore, inorganic P is supplemented in diets to meet the requirements for available P (**avP**). However, the inorganic P sources are expensive, so their usage increases feed cost (Ponnuvel et al., 2014) and P excretion into the environment to increase pollution (Lim et al., 2003). Calcium, a divalent cation, has a high binding affinity with the negatively-charged phytate-bound phosphate to form an insoluble and high-pH precipitate, which reduces the availability of Ca and P (Hurwitz and Bar, 1971). In fact, increases in laying hen dietary Ca levels linearly increased the Ca-phytate complex formation in the GIT, decreasing the availability and increasing excretion into the environment (Van der Klis et al., 1997; Beulter, 2009). Currently, exogenous phytase is commonly supplemented in poultry diets to increase the availability of phytate-bound P and decrease the formation of the Ca-phytate complexes, thus increasing Ca and P availability. Dietary phytase supplementation allows for a reduction in the use of inorganic P to lower feed cost, and dietary Ca level to increase the efficiency of P and Ca absorption (Van der Klis et al., 1997) and bone mineralization (Hughes et al., 2009) in laying hens. Various commercial phytases of different microbial sources are available. Only limited information on the influence of phytase supplementation in Ca- and avP-reduced diets on cortical, trabecular, and medullary bone tissues in laying hens is available.

Imaging technologies have been used to assess changes in the bones of laying hens. These include dual energy X-ray absorptiometry (Shahnazari et al., 2006), digitized fluoroscopy and ultrasound (Fleming et al., 2004), quantitative computed tomography (Saunders-Blades et al., 2009), and micro-computed tomography (micro-CT; Shahnazari et al., 2006; Kerschnitzki et al., 2014; Regmi et al., 2017). Of these, only micro-CT can provide specific information about each of cortical, trabecular, and medullary bone tissues in laying hens (Shahnazari et al., 2006; Kerschnitzki et al., 2014; Martinez-Cummer et al., 2006). Information on each bone tissue provided by micro-CT includes thickness, sizes, arrangements, porosity, separation spaces and distribution of bone tissues, and mineral density among other parameters (Bouxsein et al., 2010). It was hypothesized that supplementation of phytase in diets moderately or severely deficient in avP and Ca would alleviate the adverse effects of the mineral deficiency on bone micro-architecture. Hence, the objective of this study was to assess the effects of phytase supplementation to Ca- and avP-reduced diets on the change in the micro-architecture of laying hen femur cortical, trabecular, and medullary bone tissues at 70 and 78 weeks of age (**woa**).

4.2 MATERIALS AND METHODS

The protocols for the studies were approved by the Animal Care and Use Committee: Livestock of the University of Alberta and the procedures followed the guidelines of the Canadian Council on Animal Care (CCAC, 2009). Standard management recommended by the primary breeder companies was followed in Trial I (Lohmann Tierzucht, 2011) and II (H&N International, 2016).

4.2.1 Trial I

At 30 woa, 72 healthy Lohmann LSL Lite egg-laying hens were housed with four hens in each of 18 cages in a double-tier cage system in an environmentally-controlled facility and assigned to one of three diets (Chapter 2). Phase I (30 to 48 woa) and phase II (48 to 70 woa) diets were fed

to meet the age-related changes in nutrient requirement due to performance and feed intake of the hens as recommended by the primary breeder management guide (Lohmann Tierzucht, 2011). The diets were a positive control (0.38% avP and 3.73% Ca in phase I and 0.37% avP and 3.64% Ca in phase II). The other diets were the PC marginally reduced in levels of avP and Ca by 0.146 and 0.134% of the diet, respectively, (NC), and the NC supplemented with 300 FTU/kg of *Escherichia coli* phytase (NC+ECP). The ECP used (Phyzyme® XP, Danisco Animal Nutrition, DuPont Industrial Biosciences, Marlborough, UK) was sourced from *Escherichia coli* and expressed in *Saccharomyces pombe*. The reduction of avP and Ca in the NC diets compared to the PC diets was based on the expected liberation of these minerals from phytate with 300 FTU phytase/kg feed. The phytase activity in each of the PC, NC, and NC+ECP was analyzed using method 30024 of ISO (2009). The dietary ingredient composition, calculated nutrient composition, and analyzed Ca and total P levels, and phytase activities are presented in Chapter 2.

4.2.2 Trial II

Forty hens were housed individually in a double-tier cage system located in an environmentally-controlled facility (Chapter 3). Each of five diets was fed to eight laying hens from 68 to 78 woa. The diets included a PC diet containing 0.35% avP and 3.5% Ca and the PC diet with avP and Ca levels moderately reduced by 0.187 and 0.159% of the diet, respectively (NC1) or severely reduced by 0.231 and 0.275% of the diet, respectively (NC2). Other diets were each of the NC diets supplemented with 600 FTU *Buttiauxella sp*. phytase (BSP)/kg feed (NC1+BSP and NC2+BSP; Table 4.6.1). The avP and Ca reductions in the NC1 diets were based on the expected liberation of these minerals due to 600 FTU BSP phytase/kg feed. The severe avP and Ca reductions in the NC2 diets were to ensure the birds were negatively affected, and thus provide an opportunity to evaluate the efficacy of BSP at 600 FTU/kg on bone metabolism of laying hens. The BSP phytase used (Axtra® PHY, Danisco Animal Nutrition, DuPont Industrial Biosciences, Marlborough, UK)

was sourced from *Buttiauxella sp*. and expressed in *Trichoderma reesei*. The phytase activity in each of the diets was analyzed using Method 30024 of ISO (2009), as described for trial 1. The dietary ingredient composition, calculated nutrient composition, and analyzed Ca and total P levels, and phytase activities are presented in Chapter 3.

4.2.3 Sample collection and micro-CT imaging

At the end of each study, the right femur of each of the hens in the trial I (n=72) and II (n=40) was excised following euthanasia by cervical dislocation. Each femur was wrapped in a 0.9% saline solution-dampened paper towel, placed in a 50 ml conical screw cap centrifuge tube, and preserved at -20°C. Subsequently, each femur was thawed at room temperature for 8 h before scanning. A 2 cm region at the mid-diaphysis (MD; Figure 4.7.1A) and proximal metaphysis (PM; Figure 4.7.2A) was scanned using an 11 Mp X-ray camera- and high-resolution micro-CT scanner (SkyScan 1176; Bruker-Micro-CT, Kontich, Belgium). Before each scan, background noise was corrected with a 1 mm aluminum filter, and the camera reflectance was tared with automation of the camera flat-field correction. The MD and PM of each femur were scanned at 17.8 μm^3 voxel size, 70 kV X-ray voltage, and 280 µA X-ray current. Subsequently, CT reconstruction software (Nrecon 1.6.1.5; Bruker-Micro-CT, Kontich, Belgium) was used to convert each scan into re-constructed stack of 1,142-slices in transverse view for the MD (Figure 4.7.1B) and PM (Figure 4.7.2B), from which 150 contiguous slices were respectively segmented out for further analyses.

4.2.4 Quantitative data generation

Skyscan CTAn software (1.11.6.0 Bruker-Micro-CT, Kontich, Belgium) was used to select a 150-slice segment from each of the reconstructed MD at the mid-point and PM at a region from the growth-plate downward as the respective region of interest (ROI). As illustrated in Figure 4.7.1B, the outer whitish ring-like structure and the grayish inner structure of the MD-ROI was delineated as the volume of interests (VOI) of the cortical (Figure 4.7.1C) and medullary (Figure 4.7.1D) bone

tissues, respectively. Also as illustrated in Figure 4.7.2B, the grayish inner structure of the PM-ROI was delineated as the trabecular bone tissue VOI (Figure 4.7.2C). The threshold tool of the CTAn software was used to separate "bone" from "non-bone" and "structural bone" from "non-structural bone" with a greyscale of 40–119 for the medullary bone VOI and 120–255 for each of the cortical and trabecular bones VOI. The greyscale thresholds used to distinguish between each of the bone tissues was based on the presence or absence, and the age-dependent amount of cortical, trabecular, and medullary bone tissues in femurs at 6 (Figure 4.7.3A), 18 (Figure 4.7.3B) 42 (Figure 4.7.3C), and 74 woa (Figure 4.7.3D). The age-related changes in the amount of cortical, trabecular, and medullary bone tissues present were used to establish 40-119 and 120-255 as the greyscale thresholds at which medullary bone tissue as a non-structural bone, and cortical and trabecular bone tissues as structural bones, respectively, least overlapped within the 0 ~ 255 greyscale. For each of the cortical, trabecular, and medullary bone tissues VOI, two- and three- dimensional (2D and 3D) morphometry parameters were calculated based on the slice-by-slice cross-sectional images and volume of the delineated VOI, respectively. Parameters measured in 2D and 3D were bone thickness (BTh), separation (BSp), cross-sectional area (BCSA), and fractal dimension (BFD). Parameters measured only in 2D were bone maximum moment of inertia (BMMI) and those measured in 3D only were bone volume relative to total volume (BVTV), volume (BVol), mineral density (BMD), and BFD. A majority of the parameters were assessed for each of cortical, trabecular, and medullary bone, however, BMMI was only evaluated for cortical bone, and BFD was only assessed for trabecular bone. Bone thickness, BMD, BVol, and BVTV each provide information on bone mass and mineralization; BSp and BPo measure sponginess within bone tissue; and BCSA quantifies the volume of space covered by bone tissue. Also, BMMI provides information on the mechanical resistance to rotational stress on a slice by slice basis of cortical bone of long bones (Martin and Burr, 1984). The MMI assessment is not relevant to trabecular or medullary bone because they are

not longitudinal along the long bone as is cortical bone. Bone fractal dimension considers the distance between two trabeculae and the proportional levels of pores within each trabecula to aggregate bone matrix and pore fillings within trabecular bone (Chappard et al., 2001), hence, BFD is irrelevant to cortical and medullary bone. The 3D BMD in each of the three bone tissues was determined based on an attenuation coefficient calibrated with a pair of hydroxyapatite phantoms with 0.25 and 0.75 g/cm^3 BMD (Computerized Imaging Reference Systems Inc., Norfolk, VA, USA). Additional detail on the procedures followed in the micro-CT bone scanning, image reconstruction with the Nrecon software, and further image processing for the 2D and 3D morphometric parameters calculation with the CTAn software was as described by Bouxsein et al. (2010) and Wu et al. (2015).

4.2.5 Experimental design and statistical analysis

The cage, with four hens in Trial I and with an individual hen in Trial II was the experimental unit. A completely randomized experimental design was used for assignment of diets to cages in each trials. Each of the 2D and 3D morphometric parameters was analyzed for diet effects, with individual hen body weight as covariate. The MIXED procedure of SAS 9.3 (SAS Institute, 2013) was used for all data analysis. Diet effects were considered significant when $P \leq 0.05$ and means were subsequently separated using the LSMEANS procedure. For Trial 2, no comparison was made between NC1 and NC2+BSP diets or between NC2 and NC1+BSP diets because of the differences in avP and Ca levels in the NC1 and NC2 and the different effects expected in the NC1+BSP and NC2+BSP.

4.3 RESULTS

4.3.1 Trial I

The 2D cortical BSp was lower (P=0.002) in the PC hens than in the NC and NC+ECP hens (Table 4.6.1). The 3D cortical BPo tended to be lower (P = 0.076) in the PC hens than in the

NC+ECP hens. There was no effect of diet on any other cortical bone parameter. The 2D trabecular BCSA and BSp, and 3D trabecular BPo and BFD were higher ($P \leq 0.004$) in the PC hens than in the NC and NC+ECP hens. The 3D trabecular BVTV was lower ($P=0.001$) in the PC hens than in the NC and NC+ECP hens. The 3D trabecular BSp was higher ($P=0.025$) in the PC hens than in the NC+ECP hens and intermediate in the NC hens. There was no effect of diet on any other trabecular bone parameter. The 3D medullary BMD was higher ($P=0.018$) in the PC hens than in the NC and NC+ECP hens. There was no effect of diet on any of the other medullary bone parameter.

4.3.2 Trial II

The 2D cortical BCSA and 3D cortical BVol were not affected by NC1 or NC2, but tended to be higher ($P = 0.074$) in the NC1+BSP hens than in NC2+BSP hens (Table 4.6.2). The 3D cortical BMD was not different between the PC, NC1, and NC1+BSP hens, but was lower ($P=0.050$) in the NC2 hens than in PC hens and intermediate in NC2+BSP hens. The 2D cortical BTh was not different between the PC, NC1, and NC1+BSP hens, but tended to be higher ($P=0.058$) in PC hens than in the NC2 and NC2+BSP hens, and also tended to be higher in the NC1+BSP hens than in NC2+BSP hens. There was no effect of diet on any other cortical bone parameter or any of the trabecular bone parameters. Neither 2D nor 3D medullary BTh was different between the PC, NC1, and NC1+BSP hens, but was lower ($P \leq 0.036$) in the NC2 hens than in PC hens and intermediate in NC2+BSP hens. The 3D medullary BMD was not different between the PC, NC1, and NC1+BSP hens, but tended to be lower ($P=0.060$) in the NC2 hens than in each of PC and NC2+BSP hens. The nearly significant ($P=0.068$) treatment effect for 3D medullary BVTV was similar to the effect for 3D medullary BMD. The 3D medullary BPo was not different between the PC, NC1, and NC1+BSP hens, but was higher ($P=0.032$) in the NC2 hens than in PC hens and intermediate in NC2+BSP hens. There was no effect of diet on any other medullary bone parameter.

4.4 DISCUSSION

4.4.1 Trial I

Egg production and eggshell quality of the NC hens from 30 to 70 woa was not different than that of the PC hens (Chapter 2), indicating that the avP and Ca levels in the NC diet were not deficient, and also that the mineral levels in the PC diet were above the minimum requirement.

The effects on 2D cortical BSp and 3D cortical BPo (Table 4.6.2) indicated smaller spaces within the cortical bone of the PC hens relative to those in the NC and NC+ECP hens. The lack of diet effect on the 2D cortical BTh and BCSA and 3D cortical BVol, BMD, and BTh (Table 4.6.2) indicated that the diet effects on 2D cortical BSp and the 3D cortical BPo were not related to cortical bone mass and mineralization. A high degree of bone separation and porosity in cortical bone tissue may be because structural bone is primarily resorbed on the endosteal surface (Schlesinger et al., 1997). The increased BSp and nearly significantly increased BPo in the NC and NC+ECP hens relative to the PC hens may have been related to the larger size or greater number of canaliculi. Canaliculi serve as a communication channel between osteocytes, which are trapped and mineralized osteoblasts (Dong et al., 2014). However, because cortical bone is not formed in actively laying hens (Dacke et al., 1993), the increased BSp and almost increased BPo in the NC and NC+ECP hens relative to the PC hens were not related to differences in canaliculi formation in cortical bone. While the increased cortical BSp and the nearly significantly increased cortical BPo in the NC and NC+ECP hens relative to the PC hens were apparently not related to changes in the size or number of canaliculi, the mechanisms involved in these diet effects are not clear. As the reasons and mechanisms for the diet effects on cortical BSp and BPo are unknown, the implication of the findings on bone health of hens are not clear, and hence, requires further research.

Overall, the lack of adverse effect on any of the cortical, trabecular, and medullary bone micro-architectural properties of the NC hens demonstrated that the avP and Ca levels in the NC

diets were adequate to maintain bone quality in addition to sustaining egg production and eggshell quality (Chapter 2). The simultaneous decrease in trabecular bone and increase in medullary bone at 70 woa in the PC hens relative to the other treatments is puzzling. The hens' requirements for Ca and P change gradually, but dietary phase changes are made abruptly. Increased dietary Ca levels cause a linear increase in Ca-phytate complex formation in the GIT of laying hens, thus reducing P and Ca availability (Van der Klis et al., 1997; Beulter, 2009). The higher Ca level in the PC diet than in the NC and NC+ECP in the current study may had led to a greater formation of Ca-phytate complexes which may have resulted in decrease in metabolic availability at least one of P or Ca in the PC hens. As medullary bone is formed and resorbed while structural bone is only resorbed and not formed in actively laying hens, the higher dietary Ca in the PC diet may have resulted in the decreased medullary bone formation, and increased exposure of the trabecular bone to osteoclast activity. The irreversible loss of the trabecular bone in the PC hens relative to hens of other diets was observed at 70 woa. As described below, a deficiency in dietary Ca can lead to a decrease, followed by an increase in medullary bone formation. If this happened in the current study, it could explain the simultaneous decrease in trabecular bone and increase in medullary bone at 70 woa.

On a more explicit note, the diet effects on femur 2D trabecular BSp, and BCSA and 3D trabecular BVTV, BSp, BPo, and BFD (Table 4.6.2) indicated that trabecular bone micro-architecture was poor in the PC hens relative to the NC and NC+ECP hens. This poor trabecular bone micro-architecture caused by the PC diet was because of a reduction in bone volume and greater porosity within the bone tissue, and are indicative of irreversible loss of the structural bone in the actively laying PC hens. Loss of trabecular bone volume in laying hens decreases the weight-bearing integrity of long bones, and hence, render the bones susceptible to fracture (Passi and Gefen, 2005; Reich and Gefen, 2006). Bone cavity formation partly indicates the presence of lacuna, resorption pits, which was positively correlated to BSp within the trabecular bone (Chappard et al.,

2001). Cortical bone only has its endocortical surface exposed to the high osteoclastic activity within the endosteal space, whereas trabecular bone, which is entirely situated within the endosteal space, is more exposed to osteoclasts, and hence, trabecular bone is susceptible to greater loss (Kim and Park, 2013). The decreased trabecular bone reserves in the PC hens indicated a greater resorption of structural bone relative to those in the NC and NC+ECP hens. The trabecular bone loss in the PC hens after 40 wk on the diet may have been the result of the cumulative effects from 30 to 70 woa of a periodic inadequate deposition of medullary bone when hens were eating and not forming eggshell. A higher Ca-phytate complex formation with the marginally higher dietary Ca in the PC relative to NC diet may have resulted in a lower supply of at least one of P or Ca for formation of medullary bone during the 40 wk laying cycle, thus increasing the amount of structural bone surface exposure to osteoclasts. However, the 70 woa medullary BMD of the PC hens was increased relative to those of the NC and NC+ECP hens, and there was no effect of diet on any of the other parameters measured. Medullary bone is formed in actively laying hens when they are absorbing dietary Ca but not forming an eggshell, and increases even as structural bone reserves decrease with hen age, particularly in laying hens fed avP- and Ca-reduced diet (Dacke et al., 1993, Knott et al., 1995). The cascade of events that resulted in the loss of the 70 woa trabecular bone loss in the PC hens may have involved intermittent shortages of at least of one of P or Ca metabolic availability to cause time to time inadequate depostion of medullary bone within the 30 to 70 woa laying cycle. The lower Ca level in the NC diet with the usage of ECP in the NC+ECP diet might have decreased Ca-phytate complex formation and associated adverse effects, resulting in prevention of the trabecular bone loss in the NC and NC+BSP hens as observed in the PC hens. The greater trabecular bone loss in the PC hens relative to the other treatment groups was due to the resorption but not formation of structural bone during the laying cycle (Dacke et al., 1993), and because trabecular bone is more exposed to the higher osteoclastic activities within the endosteal

space than cortical bone (Reich and Gefen, 2006). Japanese quail fed a severely Ca-deficient diet for 23 d had grossly decreased medullary bone reserves with unaffected structural bone by 16 d, but at the end of the study, medullary bone was being replenished with decreasing structural bone volume (Dacke et al., 1993). The current study assessed bone fraction microarchitecture at the end-of-study (70 woa) only. Because a reduced metaboolic supply of Ca results in a loss and subsequent replenishment of medullary bone (Dacke et al., 1993), the increased medullary BMD in the PC hens relative to the NC and NC+ECP hens at 70 woa was not likely linear. A periodic reduction in metabolic availability of at least one of P or Ca that culminated in the increased loss of trabecular bone at 70 woa may be related to an adverse effect of the high Ca levels in the PC diet on the availability of at least one of P or Ca. Increasing dietary Ca from 2.5 to 5.5% decreased availability of P and Ca linearly, with an increase in Ca-phytate complex formation in the gastrointestinal tract of laying hens (Beutler, 2009). Hence, the decrease in trabecular bone micro-architectural quality of the 70 woa PC hens relative to the hens of other diets may have resulted from a cascade of events that started with adverse effect of high Ca-phytate complex formation on availability of at least one of P or Ca in the intestine.

Bone breaking strength and ash content are commonly used to assess bone quality of egg-laying hens (Boling et al., 2000; de Lima et al., 2010), as they are easy, fast, and cost effective to measure. Bone breaking strength provides limited information on morphological changes in trabecular bone tissue because it measures bone resistance to fracture at the mid-diaphysis and not at the proximal or distal metaphyses and epiphyses. These regions contain a greater proportion of trabecular bone, which is spongy and less compact relative to cortical bone, and hence more suceptible to bone breakage (Reich and Gefen, 2006). Similarly, bone ash measures cortical, trabecular and medullary tissues, and does not distinguish between the different fractions. Therefore, increased medullary bone with hen age (Dacke et al., 1993) can mask important changes

in structural bone mineralization. The primary breeder recommendations for dietary avP and Ca are based on optimal productivity and welfare of laying hens (Lohmann Tierzucht, 2011; H&N International, 2016), levels which are adequate to support egg production, eggshell quality, and bone breaking strength and ash (Boling et al., 2000; de Lima et al., 2010). The current study shows that diets containing the primary breeder-recommended levels of avP and Ca maintained egg production, eggshell quality, and bone breaking strength and ash. However, these recommended levels also increased trabecular bone porosity and separation, resulting in the decreased structural bone thickness and volume compared to a diet marginally reduced in avP and Ca by 0.146 and 0.134% of the diet, respectively. A linear decrease in trabecular bone volume in laying hens fed avP- and Ca-deficient diets was strongly correlated to a linear increase in the incidence of osteoporosis (Knott et al., 1995). Hence, the high Ca levels in commercial laying hen diets can detrimentally affect trabecular bone micro-architecture and therefore increase the susceptibility of hens to osteoporosis and fracture, particularly around the proximal and distal metaphyses.

The PC hens had greater 70 woa 3D medullary BMD than the NC and NC1+ECP hens, indicating greater end-of-study bone mineralization (Table 4.6.2). The lack of a diet effect on the other 3D and any of the 2D medullary bone micro-architectural traits (Table 4.6.2) indicated that there was no adverse effect of diet on medullary bone. Medullary bone is continuously resorbed and formed in activity laying hens, and increases with age, even in those fed avP- and Ca-deficient diets (Dacke et al., 1993; Knott et al., 1995). Considering the adverse effects of the PC diet on trabecular bone, the lack of adverse effects on medullary bone of the PC hens indicated the avP and Ca levels in the diet maintained the medullary bone at the expense of trabecular bone across the 40 wk trial. A linear increase in medullary bone volume and a linear decrease in trabecular bone volume in the hens fed Ca- and avP-deficient diets was strongly correlated to a linear increase in the severity of osteoporosis (Knott et al., 1995). Because medullary bone provides minimal resistance to bone

fracture (Fleming et al., 1998), the increased medullary BMD in the PC hens would not compensate for the decreased structural strength indicated by the loss of trabecular bone micro-architecture. Hence, use of bone ash to assess skeletal quality in laying hens would not be a good measure of fracture resistance because an increase in medullary bone mineralization may mask any treatment effect on the primary strength-providing bone tissues.

It is worth mentioning that neither of P nor Ca digestibility of these same PC and NC diets was different at any of 32, 48, or 70 woa (Chapter 2) and while the phytase evaluated in Chapter 2 was BSP, ECP was used in the current trial. However, the lack of significant differences between the PC and NC diets on P or Ca digestibility may be related to the the ability of hens to physiologically increase absorption of the minerals in response to decrease in Ca and P metabolic availability (Elaroussi et al., 1994) and the marginal reduction of 0.134% unit in dietary Ca level between the PC and NC diets. Decreasing dietary Ca from 5.5% to 2.5% linearly decreased digestibility of P and Ca with increase in Ca-phytate complex formation in the gastrointestinal tract of laying hens (Beutler, 2009). Therefore, the marginally higher dietary Ca level in the PC relative to the NC may at the least numerically increased Ca-phytate complex to cause periodic shortage of P and Ca metabolic availability, which medullary bone deposition in actively laying hen rely on. The trabecular bone loss in the PC hens from 30 to 70 woa relative to the other treatment groups may have resulted from a cascade of events that started with adverse effect of a high Ca-phytate complex formation on availability of at least one of P or Ca. While it is important to have adequate dietary Ca in commercial laying hen diets to maintain productivity, performance, eggshell quality, bone breaking strength and ash content, it is also vital to avoid excess, which can detrimentally affect micro-architecture bone tissues. Therefore, a re-evaluation of the recommended levels of avP and Ca levels in commercial laying hen diets fed to is necessary. The lack of adverse effects of the NC diet on any of the cortical, trabecular, and medullary bone micro-architectural traits denied an

opportunity to observe the ECP effects on the bone quality. On the other hand, phytase was not supplemented in the PC diet. The efficient use of phytase in laying hen diets should involve reductions in avP and Ca levels, allowing the phytase to liberate sufficient, but not excessive amounts of these minerals from phytate in the dietary ingredients. This would reduce Ca-phytate complex formation and the associated adverse effects on Ca and P supply to the hen's metabolism. Therefore, a reduction in commercial laying hen dietary Ca may benefit long-term bone health.

4.4.2 Trial II

The avP and Ca levels in the NC1 diet did not adversely affect performance or eggshell quality, but decreased BW of hens from 68 to 78 woa by 4.3% relative to the PC diet (Chapter 3). The avP and Ca levels in the NC2 diet decreased performance and eggshell quality, and hen BW from 68 to 78 woa by 7.9% relative to the PC diet (Chapter 3). Hence, the NC1 was considered as a moderately deficient diet, while the NC2 was held as a severely deficient diet in the discussion of the current study.

The diet effects on 2D cortical BCSA and 3D cortical BVol, BMD, and BTh (Table 4.6.3) indicated that the avP and Ca levels in the NC1 diet maintained the bone micro-architectural traits after the 11 wk feeding period. Apparently, laying hens maintained cortical bone micro-architecture when fed the diet moderately deficient in avP and Ca diet from 68 to 78 woa. Bone mineralization in laying hens fed a diet with a similar avP level to that in the NC1 of the current study was also maintained after 22 wk, but was decreased after 50 wk on the diet (Boling et al., 2000). Another diet with similar avP level also maintained cortical BMD in white and brown egg-laying hens after 25 wk on the diet (Silversides et al., 2006). Hence, the length of time on the diet was also an essential factor to consider on the effects of Ca and avP reductions on cortical bone micro-architecture. However, the NC2 diet decreased 3D cortical BMD and nearly significantly decreased 2D cortical BCSA and 3D cortical BVol and BTh relative to the PC diet. The 12% decrease in egg production of

the NC2 hens was explained by the fact that 20% of the NC2 laying hens completely stopped egg production towards the end the study, and the remaining NC2 hens laying at a lower rate (Chapter 3). The effect of the NC2 diet on cortical bone micro-architectural traits may be discussed in the context of the actively laying and the out of lay NC2 hens. For the actively laying NC2 hens, the overall diet effects of the NC2 diet on the bone parameters during the trial indicate a progressive decrease in the cortical bone micro-architecture of the hens. A 10-d forced molting of 88 woa laying hens increased femur and tibiotarsus cortical bone thickness by 19 and 33%, respectively (Yildiz and Alpay, 2008) as structural bone was replenished. The out-of-lay NC2 hens were molting and therefore recovering from the avP and Ca deficiency-induced decrease in cortical bone quality. Although the avP and Ca deficiency adversely affected cortical bone micro-architecture in laying hens, the extent of the negative effect depended on the severity and duration of the deficiency.

There was no diet effect on any of the 2D or 3D trabecular bone micro-architectural traits (Table 4.6.3). It was not surprising that the trabecular bone in the NC1 hens was not adversely affected relative to the PC and NC1+BSP hens, as the hens were able to compensate for the moderate reduction of dietary avP and Ca levels. A diet severely deficient in Ca resulted in a gradual loss of trabecular bone in egg-laying hens (Wilson et al., 1992). Hence, it is logical that the NC2 hens that persisted in laying would eventually lose trabecular bone. The lack of diet effects on trabecular bone micro-architecture may also be linked to the diet effects on the medullary bone. The NC1 hens maintained medullary bone micro-architecture (3D medullary BVTV, BMD, BTh, and BPo; Table 4.6.3). The NC2 hens had decreased medullary bone volume and mineral density, with increased resorption pits relative to the PC hens. These effects implied greater osteoclast than osteoblast activity within the medullary bone of the NC2 hens. Although medullary bone volume increases with age in hens fed diets with adequate to marginally-reduced Ca levels (Dacke et al., 1993; Knott et al., 1995), the loss of medullary bone by the NC2 hens was because of the severe

dietary avP and Ca deficiencies. During a molt, decreased serum estrogen results in cessation of egg production and the formation of structural rather than medullary bone tissue (Dacke et al., 1993). In the NC2 group, 20% of the hens had completely stopped egg production towards the end the study (Chapter 3). This molt would have decreased circulating estrogen levels, and the hens would have stopped medullary bone formation and resumed structural bone formation. Bone metabolism in the 20% of NC2 hens that were molting relative to the 80% of NC2 hens that were actively laying would have been dramatically different. Nevertheless, the severe deficiency of avP and Ca in the NC2 diet resulted in decreased medullary and cortical bone tissues, but maintained trabecular bone relative to the PC diet. The lack of an effect on trabecular bone micro-architecture of the NC2 hens may have been due to the accretion of trabecular bone in the molting NC2 hens masking the decrease in the actively laying NC2 hens. While it would have been ideal to separate the molting hens from the actively laying hens for the micro-architectural analysis of each bone tissue, the low number of hens per treatment and the overall experimental design of the study did not allow for separation of the hens by laying status. Overall, structural bone tissue micro-architecture was not adversely affected by the severe avP and Ca deficiencies of the NC2 diet as expected, however, the treatment group included both molting and actively laying hens, each of which were undergoing different stages of bone metabolism.

The fact that the moderate reduction of avP and Ca in the NC1 diet maintained the cortical, trabecular, and medullary bone micro-architecture limited the opportunity to observe phytase effects on bone tissues. Phytase supplementation in the NC2 diet only partially alleviated the decreased cortical BMD, with no alleviating effect on the cortical BCSA and BVol. Since cortical bone is not formed in actively laying hens (Whitehead and Fleming, 2000), the mechanism for supplemental phytase would be to prevent loss of the structural bones in the first place. Hence, the phytase in the NC2+BSP diet was not sufficient to completely prevent the detrimental effects of the severe avP and

Ca reduction on some cortical bone micro-architecture traits. It is possible that higher dietary phytase activity might further increase P and Ca availabilities from the NC diet to increase supply of the minerals to bone and decrease the gradual loss of cortical, trabecular, and medullary bones to support eggshell formation caused by the deficiencies. While the severe avP and Ca reductions in the NC2 diet did not adversely affect the trabecular bone, cortical bone micro-architecture tended to be decreased. The lack of effects of reduced Ca and avP on trabecular bone micro-architectural traits prevented the opportunity for supplemental phytase to affect structural bone micro-architecture. On the other hand, the inability of phytase supplementation in the NC2 diet to prevent the adverse effects of the severe avP and Ca deficiencies on cortical bone micro-architecture may indicate the insufficiency of the liberated P and increased Ca availability, in respect to the phytase dose usage and the calculated amount of phytate-P in the NC2 diet. However, the supplemental BSP tended ($P<0.068$) to alleviate the adverse impact on medullary BMD and BVTV in the NC2+BSP hens. Also, supplementation of 600 FTU/kg of BSP in the NC2 diet prevented the 12% decrease in egg production caused by the NC2 diet without phytase (Chapter 3). These results indicate the potential of the supplemental phytase to support maintenance of medullary bone volume and mineralization to support eggshell formation for sustained egg production. It is possible that phytase inclusion beyond 600 FTU/kg to the NC2 diet could reduce the adverse effects of severe Ca and avP reductions on cortical bone micro-architecture by liberating additional Ca and P from the diet. Also, another possibility is that the substrate (phytate) level in the NC2 diet was not sufficient to allow liberation of sufficient P to alleviate the effect of completely removing dietary inorganic P. The NC2 diet was calculated to contain approximately 1.2% phytate and 0.24% phytate P (Chapter 3), and the NC2 was reduced in avP by 0.231% of the diet relative to the PC diet, a reduction which was only attainable with zero usage of inorganic P sources. While the 0.231% avP reduction of the NC2 diet would not be fully liberated from the calculated dietary level of 0.24% phytate P, use of BSP in diets

with high-phytate ingredients could allow for further increased P availability. Ensuring adequate structural and medullary bone to maintain bone health and egg production, respectively, requires a balance between the avP and Ca reduction levels and the activity of phytase supplemented in the diet. This would maintain adequate medullary bone remodeling and limit the irreversible loss of structural bone in actively egg-laying hens.

Long-term feeding of the primary breeder-recommended levels of Ca and avP (the PC diet) in Trial I detrimentally affected micro-architecture of structural bone tissues but maintained overall medullary bone quality and even increased medullary BMD relative to the NC diet. However, long-term feeding of the marginally reduced avP and Ca levels in the NC diet of Trial I had no adverse effect on cortical, trabecular, or medullary bone tissue micro-architectural traits. Similarly, short-term feeding of the moderately reduced avP and Ca levels in NC1 diet of Trial II also did not adversely affect the bone tissues. Lastly, the short-term feeding of the severely reduced avP and Ca levels in the NC2 diet of Trial II had no effect on trabecular bone micro-architecture, but adversely affected or tended to negatively affect cortical and medullary bone micro-architecture. The lack of adverse effects of marginal or moderate reductions in dietary avP and Ca on bone tissues limited the possibility to evaluate the efficacy of phytase supplementation. However, the supplementation of phytase in the severely deficient diet partially prevented the detrimental effects on cortical bone micro-architectural properties, and tended to alleviate adverse effects on medullary bone volume and mineral density. This implies the need for reduction in avP and Ca levels in the commercial layer diets to ensure the bone health, volume, mineralization, and micro-architecture in egg-laying hens.

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4.6 TABLES

Table 4.6.1: Effects of dietary Ca, available P, phytase on micro-architectural traits of cortical, trabecular, and medullary bone tissue in femurs of 70 weeks of age white egg laying hens: Trial I[1]

Parameters[2]	2-dimensional				3-dimensional						
	BCSA (mm^2)	BMMI (mm^4)	BTh (mm)	BSp (mm)	BMD (g/cm^3)	Bvol (mm^3)	BVTV (mm^3)	BFD	BTh (mm)	BSp (mm)	BPo (%)
Cortical bone tissue											
PC	13.2	74.8	0.51	0.37[b]	1.28	35.5	-	-	0.62	2.17	66.3
NC	13.1	75.9	0.54	0.41[a]	1.28	35.1	-	-	0.64	2.29	67.1
NC+ECP	12.8	78.3	0.52	0.43[a]	1.28	34.7	-	-	0.62	2.37	68.5
Pooled SEM	0.27	2.04	0.02	0.01	0.01	0.70	-	-	0.01	0.08	0.69
Source of variation	----------*P*-value----------										
Diet	0.671	0.485	0.288	0.002	0.874	0.750	-	-	0.517	0.204	0.076
Co-variate: BW	<0.001	<0.001	0.018	0.049	0.231	<0.001	-	-	0.001	0.836	0.018
Trabecular bone tissue											
PC	10.3[a]	-	0.026	0.034[a]	0.93	-	43.2[b]	2.45[a]	0.061	0.085[a]	56.9[a]
NC	8.06[b]	-	0.028	0.026[b]	0.92	-	50.4[a]	2.35[b]	0.064	0.081[ab]	49.6[b]
NC+ECP	7.90[b]	-	0.026	0.024[b]	0.92	-	50.8[a]	2.35[b]	0.062	0.070[b]	49.2[b]
Pooled SEM	0.53	-	0.001	0.002	0.01	-	1.39	0.018	0.001	0.006	1.45
Source of variation	----------*P*-value----------										
Diet effect	0.004	-	0.449	0.018	0.651	-	<0.001	<0.001	0.412	0.025	<0.001
Co-variate: BW	0.073	-	0.323	0.876	0.004	-	<0.001	0.122	0.976	0.008	0.040
Medullary bone tissue											
PC	21.4	-	0.18	0.030	0.16[a]	-	83.0	-	0.20	0.12	17.0
NC	20.5	-	0.15	0.036	0.12[b]	-	79.0	-	0.19	0.14	21.0
NC+ECP	22.0	-	0.17	0.033	0.12[b]	-	82.5	-	0.20	0.15	17.5
Pooled SEM	0.61	-	0.01	0.003	0.01	-	2.17	-	0.01	0.02	2.17
Source of variation	----------*P*-value----------										
Diet	0.124	-	0.405	0.388	0.018	-	0.281	-	0.969	0.390	0.281
Co-variate: BW	0.068	-	0.341	0.070	0.034	-	<0.001	-	0.139	0.245	0.009

[a-b]Treatment means in each column, within each of Trial I and II with no common superscript differ significantly ($P \leq 0.05$).

[1]Each diet mean was calculated from 24 hens (n = 72) fed experimental diets from 30 to 70 week of age.
[2]BCSA = bone cross-sectional area, BMMI = bone maximum moment of inertia, BTh = bone thickness, BSp = bone separation, BMD = bone mineral density, BVol = bone volume, BVTV = bone volume relative to total volume, BFD = bone fractal dimension, and BPo = bone porosity.
[3]PC = positive control (0.38% avP and 3.73% Ca in phase I and 0.37% avP and 3.64% Ca in phase II, based on the primary breeder recommendation), NC= negative control; diet with the PC reduced in avP and Ca levels by 0.146 and 0.134% of the diet, respectively, and NC+ECP= NC supplemented with 300 FTU/kg diet of *Escherichia coli* phytase.

Table 4.6.2: Effects of dietary Ca, available P, phytase on micro-architectural properties of cortical, trabecular, and medullary bone tissue in femurs of 78 weeks of age white egg laying hens: Trial II[1]

Parameters[2]	2-dimensional				3-dimensional						
	BCSA (mm^2)	BMMI (mm4)	BTh (mm)	BSp (mm)	BMD (g/cm^3)	BVol (mm^3)	BVTV (mm^3)	BFD	BTh (mm)	BSp (mm)	BPo (%)
Cortical bone tissue											
PC	11.4	61.6	0.46	0.37	1.22[a]	30.4	-	-	0.56	1.71	69.0
NC1	11.2	60.7	0.46	0.40	1.21[ab]	30.1	-	-	0.55	1.63	69.2
NC2	10.7	56.6	0.43	0.40	1.20[b]	28.6	-	-	0.51	1.63	69.3
NC1+BSP	11.8	65.4	0.46	0.38	1.22[a]	31.6	-	-	0.56	1.67	66.2
NC2+BSP	10.8	58.3	0.43	0.40	1.21[ab]	29.0	-	-	0.51	1.62	70.4
Pooled SEM	0.31	2.76	0.02	0.02	<0.01	0.84	-	-	0.02	0.04	2.37
Source of variation	----------*P*-value----------										
Diet	0.074	0.966	0.105	0.306	0.050	0.074	-	-	0.058	0.106	0.231
Co-variate: BW	0.006	0.001	0.059	0.691	0.415	0.005	-	-	0.043	0.342	0.005
Trabecular bone tissue											
PC	8.74	-	0.019	0.013	1.02	-	52.7	1.62	0.082	0.039	47.3
NC1	9.68	-	0.019	0.013	1.05	-	55.1	1.60	0.057	0.039	44.9
NC2	10.2	-	0.020	0.012	1.11	-	58.4	1.48	0.061	0.038	41.6
NC1+BSP	9.75	-	0.019	0.014	1.02	-	53.6	1.65	0.058	0.039	46.5
NC2+BSP	8.12	-	0.023	0.013	1.13	-	59.6	1.70	0.077	0.041	40.4
Pooled SEM	0.74	-	0.002	0.001	0.06	-	3.18	0.08	0.017	<0.001	3.32
Source of variation	----------*P*-value----------										
Diet	0.617	-	0.174	0.835	0.659	-	0.467	0.121	0.762	0.201	0.467
Co-variate: BW	0.001	-	0.057	0.669	0.588	-	0.401	0.008	0.494	0.114	0.401
Medullary bone tissue											
PC	22.2	-	0.40[a]	0.011	1.85	-	96.5	-	0.34[a]	0.08	3.16[b]
NC1	20.8	-	0.27[ab]	0.020	1.65	-	87.0	-	0.27[a]	0.10	10.8[ab]
NC2	17.9	-	0.09[b]	0.023	1.34	-	72.9	-	0.12[b]	0.12	19.6[a]
NC1+BSP	21.3	-	0.34[a]	0.019	1.74	-	91.1	-	0.34[a]	0.14	8.13[b]
NC2+BSP	22.9	-	0.25[ab]	0.017	1.69	-	89.4	-	0.22[ab]	0.06	10.1[ab]
Pooled SEM	1.53	-	0.06	0.003	0.1	-	4.73	-	0.05	0.03	3.51

Source of variation	----------*P*-value----------									
Diet	0.225	-	0.036	0.406	0.060	-	0.068	-	0.025	0.144	0.032
Co-variate: BW	0.776	-	0.736	0.877	0.713	-	0.748	-	0.799	0.227	<0.001

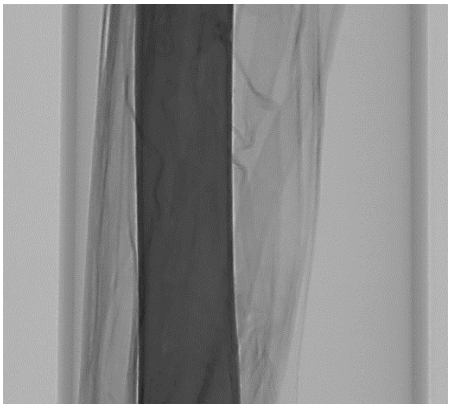
[a-b]Treatment means in each column, within each of Trial I and II with no common superscript differ significantly ($P \leq 0.05$).

[1]Each diet mean was calculated from 8 hens (n = 40) fed experimental diets from 68 to 78 week of age.

[2]BCSA = bone cross-sectional area, BMMI = bone maximum moment of inertia, BTh = bone thickness, BSp = bone separation, BMD = bone mineral density, BVol = bone volume, BVTV = bone volume relative to total volume, BFD = bone fractal dimension, and BPo = bone porosity.

[3]PC = positive control diet (a 0.35% avP and 3.5% Ca, NC1 = negative control 1 (a 0.16% avP and 3.34% Ca), NC2 = negative control 2 (0.12% avP and 3.23% Ca, NC1+BSP and NC2+BSP = NC1 or NC2 plus 600 FTU/kg diet of *Buttiauxella sp.* phytase.

4.7 FIGURES



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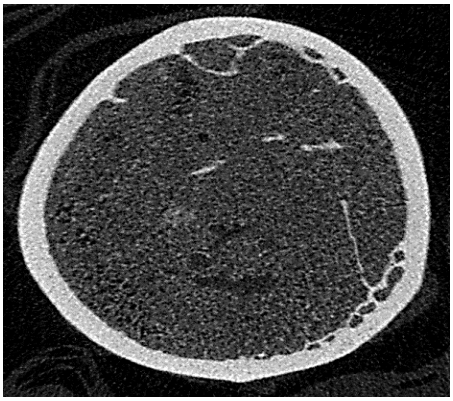

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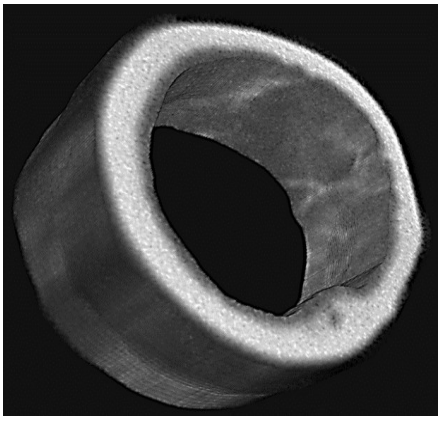

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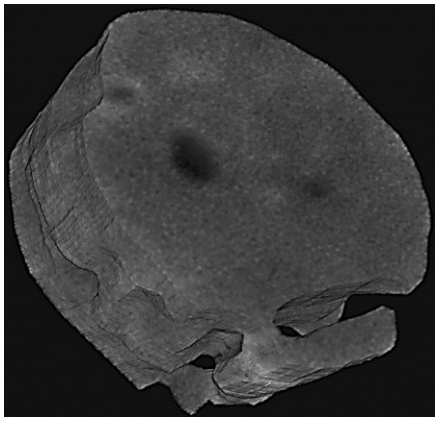
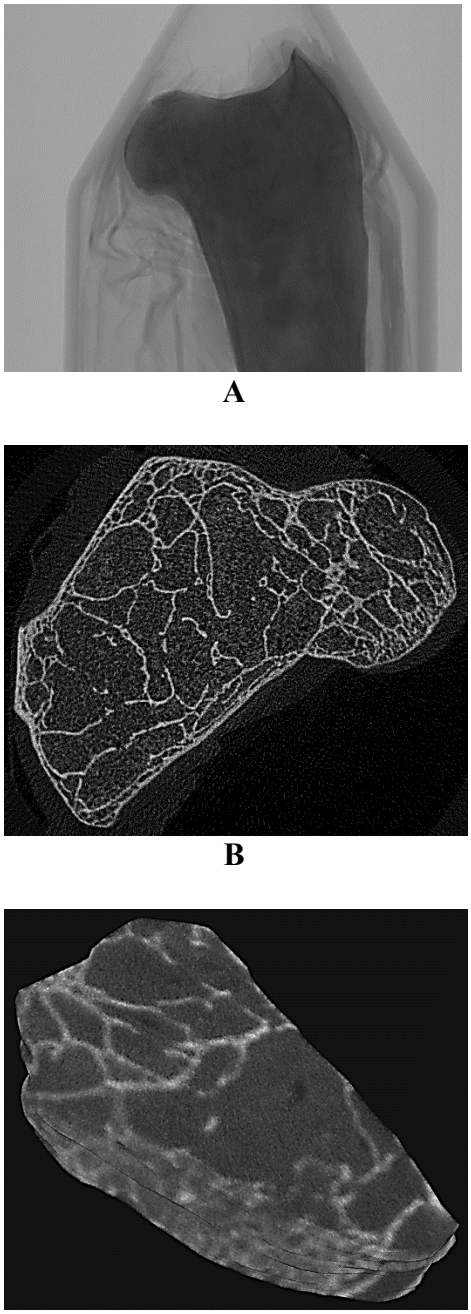


D

Figure 4.7.1: Laying hen femur mid-diaphysis scan conducted with a Skyscan micro-computed tomography unit to generate (A) a 20 mm long bone image, which was reconstructed into (B) 1,142-slice cross-sectional images, and segmented and delineated 3D (C) cortical and (D) medullary bone tissues.



A

B

C

Figure 4.7.2: Laying hen femur proximal metaphysis scan conducted with a Skyscan micro-computed tomography unit to generate (A) a 20 mm long bone image, which was reconstructed into (B) 1,142-slice cross-sectional images, and (C) segmented and delineated 3D trabecular bone tissues.

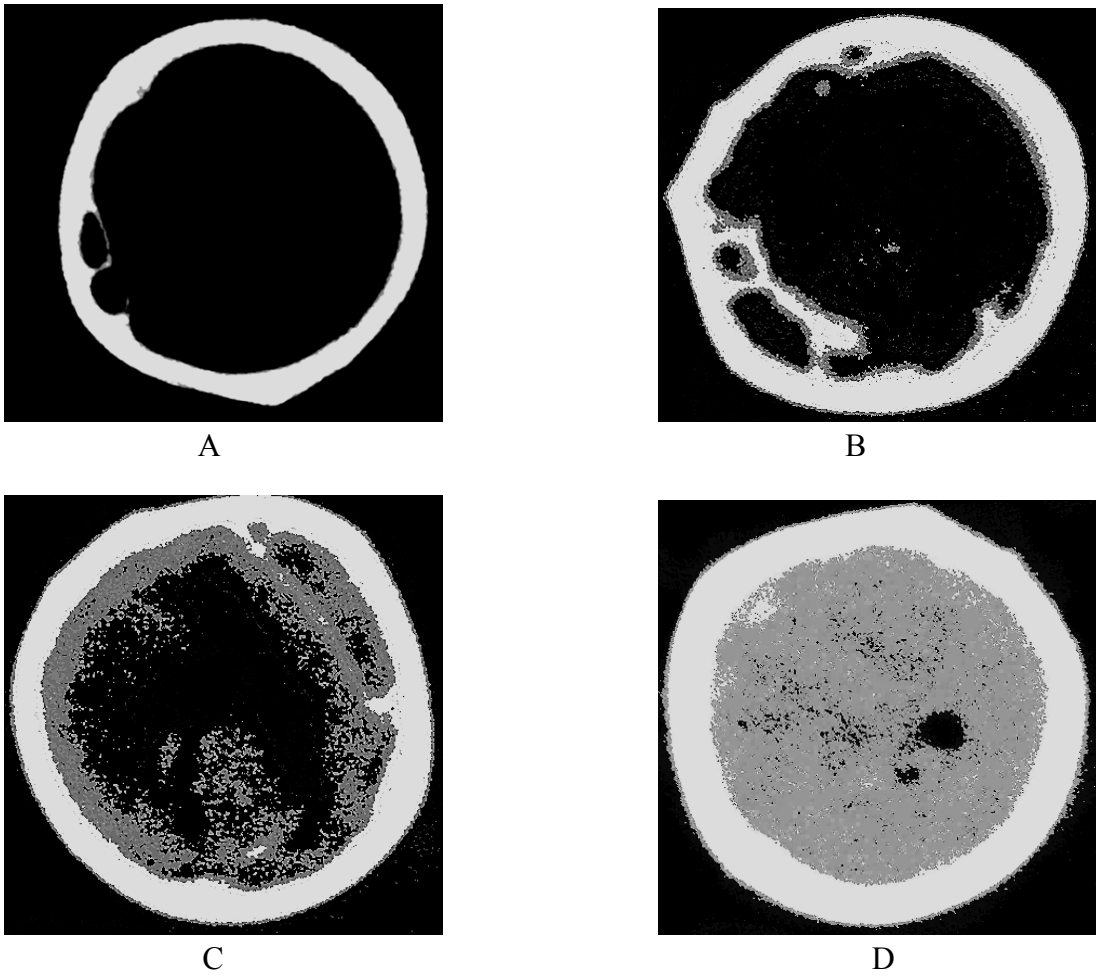


Figure 4.7.3: Cross-sectional images of cortical, trabecular, and medullary bone tissues in the femur mid-diaphysis of pullets at 6 (A) and 18 (B) wk of age, and of laying hens at 42 (C) and 74 (D) wk of age, which were used for the determination of greyscale threshold at which non-bone, non-structural bone, and structural bone were separated for subsequent morphometric analysis of each of the three bone tissues.

5. THE EFFICACY OF TWO PHYTASES ON INOSITOL PHOSPHATE DEGRADATION IN DIFFERENT SEGMENTS OF THE GASTROINTESTINAL TRACT, CALCIUM AND PHOSPHORUS DIGESTIBILITY, AND BONE QUALITY OF BROILERS

ABSTRACT

The anti-nutritional effects of dietary inositol phosphates (IP_6 through IP_3) have been recognized, however, inositol hexaphosphate (IP_6) is more potent than the lower IP esters. The efficacies of two commercial phytases (PHYA and PHYB) at 500 and 1,000 FTU/kg were studied on IP_{6-3} concentrations in the crop, proventriculus + gizzard, and distal ileum digesta, and ileal IP_6 disappearance in broilers at d 22. Apparent ileal P and Ca digestibility and bone quality at d 22 and 33 were also measured. Female Ross 308 broilers (n=1,890; 30 birds×7 diets×9 replicates) were fed corn-soy-based mash diets. The seven diets included a primary breeder recommendation-based positive control diet (PC); the PC marginally reduced in available P by 0.146% and Ca by 0.134% of the diet, (NC1) or moderately reduced by 0.174% and 0.159% of the diet, respectively (NC2). Other diets were the NC1 + PHYA or PHYB at 500 FTU/kg (NC1+500PHYA and NC1+500PHYB); and the NC2 + PHY A or PHYB at 1,000 FTU/kg (NC2+1000PHYA and NC2+1000PHYB). The distal ileum IP_6 disappearance from NC1 and NC2 was not different from that of PC for any of IP_6 through IP_3, but each was lower than the PC for P digestibility and the majority of bone quality parameters measured. The crop, proventriculus + gizzard, and ileal IP_{6-3} levels were decreased by 18.8 and 12.9% for NC1+500PHYA and NC1+500PHYB, respectively, relative to NC1 and by 38.7 and 26.3% for NC2+1000PHYA and NC2+1000PHYB, respectively, relative to NC2 (P<0.001), with a similar effect for distal ileum IP_6 disappearance. Overall, phytase in the NC diets alleviated the

decrease in P digestibility and femur breaking strength and cortical bone mineral density at d 22 and 33. Overall, each of the phytases, at each dose degraded IP_{6-3} across the gastrointestinal tract segments to increase P digestibility and the P and Ca utilization in bone. However, dietary PHY A at 1,000 FTU/kg was most effective. Supplemental PHY degrades phytate to decrease the anti-nutritional effects in a dose- and phytase-dependent manner.

Keywords: broiler chicken, gastrointestinal tract, inositol phosphate, phytase, bone

5.1 INTRODUCTION

The ability of commercial phytases to increase nutrient digestibility, performance, and bone ash content of broilers has been well studied (Simons et al., 1990 and Powell et al., 2011). However, the effects of supplemental phytase in available P- (**avP**) and Ca-deficient diets depends on the ability of the enzyme to degrade phytate (inositol hexaphosphate; **IP_6**) in the gastrointestinal tract (**GIT**). The efficacy of phytase to degrade phytate has been validated in *in vitro* (Menezes-Blackburn et al., 2015; Sommerfeld et al., 2017) and *in vivo* (Walk et al., 2014; Li et al., 2016; Beeson et al., 2017; Sommerfeld et al., 2018). Phytase degrades **IP_6** into inositol penta-, tetra-, tri-, di-, and mono-phosphate (IP_5 through IP_1), releasing one phosphate molecule at a time in a stepwise manner (Greiner and Konietzny, 2011), however IP_{6-3} are of more importance because of their higher anti-nutritional effects. The presence of IP_{6-3} in the GIT decreases availability of nutrient absorption in monogastric animals (Persson et al., 1998). Commercially-available phytases are from various bacterial or fungal sources. *In vitro* assessment of seven commercial bacterial or fungal phytases showed large variations in biochemical and catalytic properties (Menezes-Blackburn et al., 2015). Substrate level, pH, temperature, and moisture content vary in each GIT segment of the chicken. Hence, the activity of a phytase in the different conditions of each GIT segment may contribute to its phytate-degrading efficacy. Increasing phytase dose increased *in vivo* IP_6 degradation in the

gizzard and ileum (Li et al., 2016; Beeson et al., 2017). Overall, only limited information is available on phytase source and dose effects on *in vivo* IP degradation in each GIT segment of broilers.

Phytase supplementation in avP- and Ca-reduced diets increased tibia breaking strength, weight, and ash content in broilers (Powell et al., 2011). Broiler bones contain both cortical and trabecular bone tissues. Cortical bone is compact and dense and trabecular is spongy and cancellous, and are both important for load bearing and fracture resistance (Reich and Gefen, 2006). Cortical and trabecular bone densitometry (bone mineral density, cross-sectional area, and mineral content) can be individually analyzed using quantitative computed tomography (**QCT**; Korver et al., 2004). The efficacy of phytase supplementation in avP- and Ca-reduced diets to prevent bone loss to maintain bone ash, breaking strength, and mineral density across trabecular and cortical bone tissues is clear (Chung et al., 2012; Shang et al., 2015). However, information available on the effects of phytase source and dose on of broiler cortical and trabecular bone densitometry is limited. In addition, there is no information on how source and dose of dietary phytase affect IP degradation in each segment of the GIT to affect cortical and trabecular bone densitometry in broilers. It was hypothesized that effect of phytase source would differ and higher phytase dose would further degrade IP_{6-3} to increase cortical and trabecular bone densitometry in broilers. Therefore, the objective of this study was to assess the effects of two phytases at 500 and 1,000 FTU/kg on IP_{6-3} degradation in different GIT segments; P and Ca digestibility; and bone strength and ash and cortical and trabecular bone densitometry in broilers.

5.2 MATERIALS AND METHODS

The protocol for this trial was approved by the Animal Care and Use Committee: Livestock of the University of Alberta and followed the Canadian Council on Animal Care

guidelines (CCAC, 2009). Female Ross 308 broilers (n=1,890) were obtained from a commercial hatchery at d 0 and housed with 30 chicks in each of 63 wire-floored pullet cages (59 × 53 × 44 cm for width, depth, and height, respectively; Specht Canada Inc., Stony Plain, AB, Canada) in an environmentally-controlled facility. Seven dietary treatments were each assigned to nine replicate cages and were fed to the birds as starter (d 0 to 8), grower (d 9 to 22), and finisher (d 23 to 33) mash diets. Birds were provided feed and water *ad libitum* throughout the trial. Standard broiler rearing management specified by the primary breeder (Aviagen, 2014) was maintained throughout the trial.

5.2.1 Treatments

The dietary treatments were a nutritionally adequate positive control diet **(PC)**; the PC diet with avP and Ca marginally reduced by 0.146 and 0.134% of the diet, respectively, **(NC1)** or moderately reduced by 0.174 and 0.159% of the diet, respectively **(NC2)**. The remaining diets were phytase A (PHYA) or B (PHYB) added at 500 FTU/kg to the NC1 diets (**NC1+500PHYA** and **NC1+500PHYB**, respectively) and at 1,000 FTU/kg to the NC2 diets **NC2+1000PHYA** and **NC2+1000PHYB**. Celite® was included in all diets at 2% as an indigestible marker. Also, the Na level in the PC diet was reduced in the NC1 diet by 0.030% of the diet and in the NC2 diet by 0.041% of the diet across the three phases. The ME level in the NC1 diet was reduced by 2.50, 2.32, and 2.19% and in the NC2 diet by 2.73, 2.55, and 2.38% relative to the PC diet in the starter, grower, and finisher phases, respectively. In addition, the minimum specification for the eight most limiting digestible amino acids (Met, Lys, Met plus Cys, Thr, Val, Ile, Arg, and Trp) in the PC diets were reduced in the NC1 diets by 0.116, 0.083, and 0.070% of the diet and in NC2 by 0.223, 0.160, and 0.138% of the diet in the starter, grower, and finisher phases, respectively. The levels of digestible amino acids in each of PC, NC1, and NC2 were formulated to meet or exceed the primary breeder

recommendation (Aviagen, 2014; Table 5.6.1). The reductions in Ca, avP, Na, ME, and amino acids in the NC1 and NC2 diets compared to the PC diet were based on the expected nutrient release by PHYA or PHYB at 500 or 1,000 FTU/kg in the respective diets. Dietary ingredient and calculated nutrient composition are shown in Table 5.6.1. Also, the analyzed Ca and total P levels, and phytase activities in each diet at each phase are shown in Table 5.6.2. Dietary Ca and total P levels in the diets were determined in triplicate using modified (see Chapter 2 for details) AOAC methods 964.06 and 935.13, respectively (AOAC, 1990; Table 5.6.2). Details of the P and Ca analyses was as described in Chapter 2. The identity of the two phytases was withheld from the authors to prevent bias. The seven dietary treatments were randomly and evenly distributed across the 63 cages. The analyzed phytase activities in the phytase-supplemented starter, grower and finisher diets were at 124 to 244%, 104 to 124%, and 92 to 154%, respectively, of the expected 500 or 1,000 FTU/kg.

5.2.2 Sample collection

At 8, 22 and 33 d of age, 14, 11 and 5 birds, respectively, from each cage were euthanized by cervical dislocation. The right femurs of four birds from each cage were excised and stored at -20°C. The digesta from the distal half of the ileum were collected and pooled within the cage (n=63). At d 22 only, crop and proventriculus + gizzard digesta were collected and individually pooled within the cage (n=189). All samples were freeze-dried and ground (<1 mm) using a centrifugal mill (model ZM200; Retsch GmbH, Haan, Germany).

5.2.3 Bone quality analysis

At each of 8, 22 and 33 d, two femurs per cage (n=378: 2 birds × 63 cages × 3 ages) were thawed and analyzed for bone breaking strength (**BBS**) using an Instron Universal Testing Instrument (Table Model 4411, Instron Corp., Canton, MA) with a 200 N load cell (Riczu et al., 2004). All fragments of the broken bones were oven-dried at 105°C for 7 days

and ashed at 500°C for 48 h using a muffle furnace (Table Model 30400; Barnstead Thermolyne Corp., Dubuque, IA) for determination of bone ash content (Chapter 2). At each of 22 and 33 d, two femurs per cage (n=252: 2 birds × 63 cages × 2 ages) were also thawed and analyzed for bone densitometry at the mid-diaphysis (50% mid-point of bone length) and proximal metaphysis (30% of bone length from the proximal end) using QCT (Saunders-Blades et al., 2009). Bone cross-sectional areas (**BCSA** in mm^2) and mineral density (**BMD** in mg/mm^3) of total, cortical, and trabecular bone tissues were measured. Bone mineral content (**BMC** in mg/mm bone length) in each of total, cortical, and trabecular bone tissues was calculated by multiplying the respective BCSA by BMD to yield the amount of mineral (mg) contained in a volume of each of the bone tissues (Chapter 2).

5.2.4 Digestibility assays

Samples of starter, grower, and finisher diets, and distal ileum digesta collected at the end of each dietary phase were analyzed for ileal P and Ca levels (Chapter 2). The diets and the distal ileum samples were analyzed for acid insoluble ash (**AIA**, Scott and Boldaji, 1997). Apparent ileal digestibility of P (**AIDP**) and Ca (**AIDCa**) were calculated with the formula: AIDP or AIDCa = 100 - [100 × (% AIA in diet/% AIA in digesta) × (% mineral in digesta/% mineral in diet)].

5.2.5 Inositol phosphate degradation assays

The seven grower diets and 189 digesta samples (63 cages × 3 GIT segments) were analyzed for IP concentrations using high-performance liquid chromatography anion exchange with a post-column reagent for detection (Newkirk and Classen, 1998). The concentrations of IP_{6-3} in each diet are shown in Table 5.6.8. The analyzed IP_6 concentration in the diet and distal ileum samples and the analyzed diet and distal ileum AIA were used to calculate ileal IP_6 disappearance as follows:

100 - [100 × (% AIA in diet/% AIA in ileal digesta) × (% ileal IP_6/% diet IP_6)].

5.2.6 Statistical Analysis

The cage was the experimental unit, with nine cages assigned to each of the seven dietary treatments. A completely randomized design was used in the placement of chicks in cages and in the assignment of cages to diets. Crop, proventriculus + gizzard, and ileal $IP_{6\text{-}3}$ concentrations and ileal IP_6 disappearance at d 22, and BBS, bone ash content, and total, cortical, and trabecular BCSA, BMD, and BMC at d 22 and 33 were each analyzed for diet effect using Proc. Mixed of SAS 9.3 (SAS Institute, 2013). Apparent ileal digestibility of P and AIDCa were each analyzed for diet × age interaction also with Proc Mixed of SAS 9.3 (SAS Institute, 2013). For the BBS and bone ash content, and total, cortical, and trabecular BCSA, BMD, and BMC analyses, BW was used as a covariate. Differences between diet means were separated using the Least Significant Difference test when $P \leq 0.05$.

5.3 RESULTS

5.3.1 Bone quality traits

The d 22 the NC1 and NC2 diets decreased mid-diaphysis and proximal-metaphysis cortical BMD relative to the PC group. At 500 FTU/kg, only PHYA alleviated the adverse effects of NC1, but at 1,000 FTU/kg, both PHYA and PHYB alleviated the adverse effects of NC2 ($P \leq 0.001$; Table 5.6.3). Diet effects on each of d22 mid-diaphysis total and cortical BMC were similar to the diet effects on d 22 mid-diaphysis cortical BMD ($P \leq 0.001$). The d 22 mid-diaphysis total and trabecular BSCA each tended to be decreased ($P = 0.055$ and 0.092, respectively) by NC1 and NC2 relative to PC; PHYA and PHYB at 500 and 1,000 FTU/kg tended to alleviate the adverse effect of the NC1 and NC2 diets, respectively. Relative to the PC, the d 22 proximal metaphysis trabecular BMD and BMC were each maintained by the NC1 diet but were decreased by the NC2 diet and at 1,000 FTU/kg, only PHYA alleviated the

adverse effect of NC2 (P=0.010). In comparison to PC, the d 22 proximal metaphysis total BMC was decreased by both NC1 and NC2, and were alleviated by PHYA at 500 and 1,000 FTU/kg, respectively (P<0.001). Also relative to the PC, the d 22 proximal metaphysis cortical BMC was decreased by both NC1 and NC2, and supplementation of either phytase alleviated the adverse effect (P=0.011).

The d 33 mid-diaphysis cortical BMD was decreased by NC1 and NC2 relative to PC, and each phytase alleviated the adverse effect of the NC1 and NC2 diets (P=0.005; Table 5.6.4). Relative to the PC, the d 33 mid-diaphysis total BMC was maintained by NC1 but tended to be decreased by NC2, which tended to be alleviated by both PHYA and PHYB at 1,000 FTU/kg (P=0.073). Also, the d 33 mid-diaphysis cortical BMC was maintained by NC1 and NC2 relative to PC and was increased only by NC2+1000PHYA compared to PC, NC2, and NC1+PHYA (P=0.014). Proximal metaphysis cortical BMD was nearly significantly decreased (P=0.096) by NC2 relative to the PC, but was restored by supplementation of either phytase at 1,000 FTU/kg feed. The d 33 proximal metaphysis cortical BMC was maintained by NC1 and NC2 relative to PC, but each phytase at 1,000 FTU/kg increased cortical BMC compared to NC2 (P=0.037). None of the other d 33 bone densitometry parameters was affected by diet.

Relative to the PC, the d 8 BBS was maintained by the NC1 diet but decreased by the NC2 diet, and the use of PHYA or PHYB at 1,000 FTU/kg alleviated the adverse effect (P<0.001 Table 5.6.5). The d 22 BBS was decreased by the NC1 and NC2 diets relative to PC. At 500 FTU/kg, only PHYA alleviated the adverse effects of NC1, while at 1,000 FTU/kg, both PHYA and PHYB alleviated the adverse effects of NC2 (P<0.001). The d 33 BBS was decreased in NC1 and NC2 birds relative to PC. Both PHYA and PHYB at 500 and 1,000

FTU/kg alleviated the adverse effect of the NC1 and NC2 diets, respectively, however the d 33 BBS was higher in the NC2+1000PHYA birds than in the NC2+1000PHYB birds (P<0.001). Relative to the PC, the d 22 bone ash was decreased by the NC1 diet and maintained by NC1+500PHYA and NC1+500PHYB, with a similar effect on NC2 and the respective phytase-supplemented diets (P<0.001). There was no diet effect on d 8 or d 33 bone ash.

5.3.2 Apparent ileal digestibility of P and Ca

The d 8 AIDP was maintained by NC1 but decreased by NC2 relative to PC, and was increased by NC1+500PHYA and NC1+500PHYB relative to NCI and PC (P<0.001; Table 5.6.6). Relative to NC2, each phytase alleviated the reduction in AIDP. The d 22 and 33 AIDP were each decreased by NC1 and NC2 relative to PC, and were alleviated by supplemented phytase regardless of type and dose. However, at d 22 and 33 AIDP were each higher with PHYA relative to PHYB, regardless of dose (P<0.001). The d 22 AIDCa was increased by NC1, maintained by NC2 compared to PC, and was increased by PHYA and PHYB at 1,000 FTU/kg. There were no treatment effects at d 8 and 33 (P<0.001).

5.3.3 IP degradation across the gastrointestinal tract and IP6 disappearance in the distal ileum

In each section of the GIT, concentrations of each IP_{6-3} ester were maintained by NC1 and NC2 relative to PC (P≤0.006), with the exception of proventriculus + gizzard IP_5, which was lower in NC1 than in PC and NC2 (P<0.001; Table 5.6.8). Relative to PC and NC1, each of crop IP_{6-3} concentrations was not different for NC1+500PHYA, but both IP_6 and IP_5 concentrations were decreased, and each of IP_4 and IP_3 concentrations was increased by NC1+500PHYB. Relative to the PC and NC2, crop IP_6 concentration was decreased by each of NC2+1000PHYA and NC2+1000PHYB, the IP_5 concentration was only decreased by NC2+1000PHYB, and each of the IP_{4-3} concentrations was increased by each of the two

phytases except for NC2+1000PHYA, which had similar crop IP_3 to the controls. Overall, concentrations of each IP ester in the crop were decreased, and both IP_4 and IP_3 concentrations were increased by the PHYB relative to the PHYA at each dose. Relative to PC and NC1, proventriculus + gizzard IP_6 concentration was decreased by each of NC1+500PHYA and NC1+500PHYB. The IP_5 concentration was decreased by NC1+500PHYA, the IP_4 concentration was increased by NC1+500PHYB, and the IP_3 concentration was increased by each phytase at 500 FTU/kg. Relative to PC and NC2, proventriculus + gizzard IP_6 and IP_5 concentrations were decreased by NC2+1000PHYA and NC2+1000PHYB each, and IP_4 and IP_3 concentrations were maintained by NC2+1000PHYA, but increased by NC2+1000PHYB. The proventriculus + gizzard IP_3 concentration was not affected by phytase type at 500 FTU/kg, but was decreased by PHYA relative to PHYB at 1,000 FTU/kg; the IP_{6-4} concentrations were also decreased by PHYA relative to the PHYB at both doses. Also, the proventriculus + gizzard IP_4 and IP_3 concentrations were lower in the NC2+1000PHYA birds than in the NC1+500PHYA birds. Relative to PC and NC1, distal ileum IP_6 concentration was decreased, the IP_5 concentration was maintained, and the IP_{4-3} concentrations were each increased by each of NC1+500PHYA and NC1+500PHYB. Relative to PC and NC2, the distal ileum IP_6 concentration was decreased by NC2+1000PHYA and NC2+1000PHYB, the IP_5 concentration was decreased only by NC2+1000PHYA, and the IP_4 and IP_3 concentrations were increased by each phytase. Overall, distal ileum IP_{6-5} concentrations were decreased, and the IP_{4-3} concentrations were increased by the PHYA relative to the PHYB at both doses and by NC2+1000PHYA relative to NC1+500PHYA. Also, the distal ileum IP_6 disappearance was maintained in the NC1 and NC2 diets relative to the PC and was increased by each respective phytase supplementation (Figure 1). Relative to the control diets, the distal ileum IP_6

disappearance was also further increased by PHYA relative to PHYB across doses and by 1,000 FTU/kg relative to 500 FTU/kg across sources.

5.4 DISCUSSION

The analyzed phytase activity in the finisher PC diet tended to be high relative to the expected level (Table 5.6.2). The analyzed phytase activities in the 500 and 1,000 FTU/kg PHYA-supplemented diets were 172 and 124%, 124 and 108%, and 121 and 92% during the starter, grower, and finisher dietary phases, respectively. The analyzed phytase activities in the 500 and 1,000 FTU/kg PHYB-supplemented diets were each 244 and 176%, 104 and 114%, and 154 and 133% during the starter, grower, and finisher dietary phases, respectively. Because the phytase activity in the finisher PC diet was well below levels recovered in the phytase-supplemented diets, and the analyzed enzyme activity in the 500 FTU/kg phytase-supplemented diets were substantially lower than in the 1,000 FTU/kg phytase-supplemented diets, the variances in analyzed phytase activity in each of the diets does not compromise the objectives of the study.

5.4.1 Bone quality traits

The avP and Ca reductions in the NC1 and NC2 diets relative to the PC decreased bone mass, mineralization and strength of broilers. Reduced dietary Ca and avP in broilers decreased bone mineralization and strength (Powell et al., 2011; Mello et al., 2012; Amerah et al., 2014). The relative reductions in avP and Ca levels in the NC1 and NC2 diets also increased the Ca-avP ratio relative to that in the nutrient-adequate PC diet, which may have resulted in a mineral imbalance. The Ca:avP ratios in the grower PC, NC1 and NC2 diets of the current study were 1.98, 2.55, and 2.73, respectively. Hence, the decreased femur bone mass, ash and strength of the NC1 and NC2 birds could be related to reduced availability, and utilization of dietary P and Ca. Cortical and trabecular bone densitometry, as well as BBS and

bone ash were decreased by the avP and Ca reductions of the NC1 and NC2 diets relative to the PC diets at d 22 and 33. Similarly, a decrease in dietary Ca from 9.1 to 4.5 g/kg and in total P from 7.2 to 3.7 g/kg linearly decreased dual energy x-ray absorptiometry-measured total BMD and BMC (Onyango et al., 2003). As bone tissues are actively remodeled in broilers, the decreased femur cortical and trabecular BMD and BMC in the NC1 and NC2 chickens at d 22 and 33 indicated higher osteoclastic activity relative to osteoblastic activity in the bone of the birds (Nakagi et al., 2013).

Regardless of source and dose, dietary phytase supplementation alleviated the decrease in the bone quality of NC1 and NC2 birds. Powell et al. (2011) also affirmed that phytase use in a diet reduced in avP and Ca alleviated the decrease in BBS and bone ash in d 21 broilers. Overall, while no difference was observed for phytase source and dose on BBS, bone ash, and total and cortical densitometry; the proximal metaphysis trabecular BMD and BMC were each increased to a greater extent by NC2+1000PHYA than by NC2+1000PHYB. Trabecular bone tissue is concentrated towards the proximal and distal ends of the long bones of broilers (Aguado et al., 2015), which explains the proportionally higher trabecular BMD, BCSA, and BMC at the proximal metaphysis than at the mid-diaphysis at d 22 and 33. The higher trabecular densitometry values at the proximal metaphysis than at the mid-diaphysis enabled the opportunity to better understand the diet effects on overall structural bone densitometry. There was no diet effect on mid-diaphysis trabecular densitometry, while proximal metaphysis trabecular BMD and BMC were each higher in NC2+1000PHYA birds than those of the NC2+1000PHYB at d 22. Also, relative to the PC, the d 22 trabecular BMD and BMC were each maintained by NC1 but decreased by the NC2, which allowed the chance to assess phytase effects on the severe avP and Ca deficiency. The diet effects showed that the cortical

bone densitometry was generally not affected by phytase source or dose. Hence, the increase in d 22 trabecular BMD and BMC by NC2+1000PHYA relative to NC2+1000PHYB resulted in the higher d 33 BBS in the NC2+1000PHYA birds than in NC2+1000PHYB birds. The high avP and Ca reduction in the NC2 diet was completely alleviated by the PHYA at 1,000 FTU/kg but not by PHYB at the same dose, which resulted in decreased trabecular mass and mineralization at d 22 and decreased BBS at d 33 in the NC2+1000PHYB birds. Not only does phytase supplementation increase P and Ca digestibility to subsequently increase mineral availability and utilization, (Bedford et al., 2015), effective phytase usage in avP- and Ca-deficient diets also maintains overall bone health (Powell et al., 2011). In the current study, phytase supplementation in avP- and Ca-deficient diets increased AIDP at each age and d 22 AIDCa to alleviate the adverse effects on bone mineralization and strength. Overall, usage of the highly efficient PHYA at 1,000 FTU/kg in broiler diets deficient in avP and Ca fortified trabecular bone densitometry traits to increase load-bearing integrity of bone.

5.4.2 IP degradation across the gastrointestinal tract, IP_6 disappearance in the distal ileum, and P and Ca digestibility

Phytase liberates phosphate molecules through a stepwise dephosphorylation pathway to degrade IP_6 to IP_{3-1} (Greiner and Konietzny, 2011; Menezes-Blackburn et al., 2015). The effects of diets on P and Ca digestibility (Table 5.6.6), IP_{6-3} in the crop, proventriculus + gizzard, and distal ileum (Table 5.6.8), IP_6 distal ileum disappearance (Figure 1) indicated that phytase degraded phytate in a stepwise manner to increase P and Ca availability. This in vivo phytate-degrading efficacy of phytase was consistent with findings of previous studies (Walk et al., 2014; Li et al., 2016; Beeson et al., 2017; Sommerfeld et al., 2018). However, the magnitude of increase in P and Ca digestibilities; crop, proventriculus + gizzard, and distal

ileum IP_{6-3} degradations; and distal ileum IP_6 disappearance was greater in for PHYA than PHYB and for 1,000 FTU/kg than the 500 FTU/kg.

Overall, IP_6 was the most abundant IP ester in the diet (Table 5.6.7); this has the greatest anti-nutritional effects relative to other IP esters (Li et al., 2016). The IP_6 concentrations in each of the crop, proventriculus + gizzard, and distal ileum were decreased relative to the NC2 birds by 27.4, 92.6, and 73.5%, respectively, with PHYA at 1,000 FTU/kg and 38.1, 74.1, and 50.8%, respectively, with PHYB at same dose Hence, the majority of IP degradation by phytase occurred in the proventriculus + gizzard, showing the importance of low pH on phytase activity. The pH in the crop, proventriculus + gizzard, and ileum in broilers are 4.5, 2.8, and 5.8, respectively, indicating high variability in pH across the GIT of broilers (Amerah et al., 2014). Compared to overall IP_{6-3} concentration across NC1 and NC2, the overall IP_{6-3} degradation in the crop, proventriculus + gizzard, and distal ileum were 2.6, 73.0, and 33.2%, respectively for PHYA diets and 10.8, 37.9, and 18.6%, respectively, for PHYB diets. Average optimal activity for 7 commercial phytases declined from 105% at pH 3 to 3% at pH 7 (Menezes-Blackburn et al., 2015), indicating that phytases are generally more active around the pH 3 of the proventriculus + gizzard than in relatively higher pH of other GIT segments. The higher IP_{6-3} degradation by PHYB than PHYA in the crop suggested that the relatively high pH of the crop was more favorable for PHYB. The ileal pH was likely higher than the crop pH (Amerah et al., 2014), so the PHYB would be expected to maintain a higher activity in the distal ileum than PHYA. However, the fact that IP_{6-3} degradation was not higher for PHYB than PHYA in the distal ileum, as was observed in the crop, indicates a considerable loss of PHYB activity in the low pH of the proventriculus + gizzard. The activity of a commercial phytase with an optimal activity at pH 5 was decreased in the proventriculus +

gizzard by 98% relative to the optimal enzyme activity (Onyango et al., 2005). This indicates that the high acidity of the proventriculus + gizzard may reduce efficiency of phytases with a relatively higher pH of optimal activity. On the other hand, the higher IP_{6-3} in the proventriculus + gizzard for PHYA than PHYB showed that PHYA exhibited the highest activity at the low pH of the proventriculus + gizzard. Based on the IP_{6-3} degradation efficacy, PHYA exhibited a lower efficacy at the ~4.5 pH of the crop, but significantly higher efficacy at the ~2.8 pH of proventriculus + gizzard and the ~5.8 pH of the distal ileum than PHYB. Therefore, PHYA may have had a broader pH spectrum of efficacy relative to PHYB in the low pH environment of the proventriculus + gizzard and in the higher pH environment of the distal ileum. Overall, this finding suggests that PHYA degraded IP_{6-3} across the crop, proventriculus + gizzard, and distal ileum to increase disappearance of distal ileum IP_6 and apparent digestibility of ileal P and Ca to subsequently increase broiler bone mass and strength relative to the PHYB. Hence, these findings imply that the IP ester-degrading efficacy and nutrient digestibility and utilization of phytases are dependent on the enzyme source.

Relative to 500 FTU/kg of PHYA, the 1,000 FTU/kg dose exhibited higher degradation of IP_6 and IP_5 across the GIT, although effects of each dose were similar in the proventriculus + gizzard. The 1,000 FTU/kg dose of PHYA also showed higher degradation of IP_{4-3} in the proventriculus + gizzard but resulted in an increase of IP_4 in the crop and IP_3 in the distal ileum, compared to 500 FTU/kg. This likely resulted from the higher degradation of IP_{6-5} across the GIT by the higher dose. Because of the linear decrease in the number of chelated phosphate groups from IP_6 to IP_1 (Greiner and Konietzny, 2011), IP_{4-3} exhibit significantly lower affinity to complex with cations such as Ca relative to IP_6 (Persson et al., 1998). Hence, the decreased IP_{6-5} and increased IP_{4-3} across the GIT of birds with phytase, particularly PHYA

at 1,000 FTU/kg relative to 500 FTU/kg, suggest that the higher phytase dose would reduce the cumulative anti-nutritional effects of phytate in the diet. Effective phytase usage requires an enzyme with high efficiency, at dose sufficient to optimally decrease IP esters, particularly IP_6 which is the most potent (Li et al 2016), to increase P availability. The 1,000 FTU/kg does of PHYB degraded IP_{6-3} in each GIT segment to a similar extent as 500 FTU/kg, except that the higher dose further degraded distal ileum IP_6. Particularly with PHYA, 1,000 FTU/kg increased degradation of IP_{6-5}, with a smaller increase of IP_{4-3} across the GIT segments to increase P availability compared to 500 FTU/kg. Hence, 1,000 FTU/kg of PHYA had higher efficacy to reduce the anti-nutritional effects of phytate and its metabolites and increase P and Ca availability for absorption than the 500 FTU/kg in the GIT of broilers. The effects of 500, 1,000 or 1,500 FTU/kg of dietary phytase on gizzard IP_{6-4} concentrations were similar, but 1,000 and 1,500 FTU/kg each decreased gizzard IP_3 concentration relative to 500 FTU/kg (Walk et al., 2014). Similarly, 1,000 FTU/kg phytase decreased crop and proventriculus + gizzard IP_6 concentration relative to 500 FTU/kg but observed no difference between the two doses on distal ileum IP_6 disappearance (Li et al., 2016). Also, 1,500 and 500 FTU/kg phytase had similar effects on gizzard IP_{6-5} degradation, but the lower dose increased IP_4 concentration, while no difference was observed for distal ileum IP_6 disappearance between the two doses (Beeson et al., 2017). The level of dietary phytate and inorganic P usage also influence the efficacy of phytase on the substrate degradation. Unlike the aforementioned studies (Walk et al., 2014; Beeson., 2017), in the current study greater reductions in P and Ca were used for the higher phytase dose, which may have contributed to the higher GIT IP_{6-3} degradation for the 1,000 FTU/kg dose of PHYA relative to the 500 FTU/kg dose. Overall, 1,000 FTU/kg degraded IP to a greater extent across the GIT than 500 FTU/kg, possibly because the higher

phytase dose may have exhibited a faster rate of reaction with the IP substrate. Phytate was completely solubilized within 6 mins by a 0.3 FTU/ml phytase dose, within 8.5 mins by a 0.2 FTU/ml dose, and in 14 mins by a 0.1 FTU/ml dose in an *in vitro* assessment (Tran et al., 2011). Also, the higher IP-degrading efficacy of phytase at 1,000 FTU/kg than at 500 FTU/kg could be related to a higher residual activity at each subsequent GIT segment of broilers with higher phytase doses (Nyannor et al., 2013). Residual phytase activity declines across the GIT (Nyannor et al., 2013), hence, the 1,000 FTU/kg phytase dose used in the current study may also have retained a higher residual enzyme activity in each GIT segment than the 500 FTU/kg dose. Dietary phytase diet at 1,000 FTU/kg was more effective at degrading IP than 500 FTU/kg, particularly with PHYA.

To efficiently degrade and decrease the anti-nutritional effects of IP on nutrient digestibility and utilization, the source and dose of dietary phytase are essential factors to consider. Both PHYA and PHYB at 500 and 1,000 FTU/kg each decreased IP_6 across the crop, proventriculus + gizzard, and distal ileum to increase P availability, and bone quality traits relative to the non-phytase containing diets. However, PHYA at 1,000 FTU/kg had the greatest effect on $IP_{6\text{-}4}$ degradation across the digestive tract segments and increased P availability and bone quality. The poultry industry can effectively use phytase to reduce inorganic P supplementation, which could decrease feed cost, and P excretion, without reducing bone quality.

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5.6 TABLES

Table 5.6.1: Ingredient and calculated nutrient composition of basal diets fed during starter, grower, and finisher phases[1,2,3]

	Starter phase (d 0-8)			Grower phase (d 8-22)			Finisher phase (d 22-33)		
	PC	NC1	NC2	PC	NC1	NC2	PC	NC1	NC2
Ingredient (%)									
Yellow corn	50.0	52.7	52.9	54.5	56.9	56.9	58.4	60.7	60.6
Soybean meal	32.8	31.9	31.2	28.0	27.2	26.6	23.5	22.7	22.3
Canola meal	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50
Canola oil	3.41	1.50	1.19	4.19	2.36	2.07	5.05	3.26	3.00
Calcium carbonate	1.13	1.20	1.22	1.02	1.09	1.11	0.93	1.00	1.01
Dicalcium phosphate	1.72	0.92	0.77	1.52	0.73	0.58	1.35	0.56	0.41
Salt	0.32	0.25	0.22	0.33	0.25	0.22	0.33	0.25	0.23
L-lysine	0.16	0.16	0.15	0.16	0.16	0.16	0.17	0.17	0.16
DL-methionine	0.32	0.31	0.29	0.29	0.28	0.27	0.27	0.25	0.24
L-threonine	0.02	0.01	-	-	-	-	-	-	-
Phytase carrier: -/+[4,5]	-	1.00	2.00	-	1.00	2.00	-	1.00	2.00
Nutrient values (as fed)									
M. E., MJ/kg	12.6	12.3	12.2	13.0	12.9	12.7	13.4	13.1	13.1
Crude protein, %	23.6	23.4	23.2	21.5	21.4	21.2	19.6	19.4	19.3
Calcium, %	0.960	0.826	0.801	0.870	0.736	0.711	0.790	0.656	0.631
Total phosphorus, %	0.806	0.661	0.631	0.743	0.598	0.569	0.686	0.541	0.512
Available phosphorus, %	0.480	0.334	0.306	0.435	0.289	0.261	0.395	0.249	0.221
Phytate phosphorus, %	0.295	0.298	0.297	0.283	0.286	0.285	0.271	0.274	0.274
Dig. Lys, %[6]	1.28	1.26	1.24	1.15	1.13	1.12	1.03	1.01	1.00
Dig. Met, %	0.66	0.65	0.63	0.61	0.60	0.58	0.56	0.55	0.54
Dig. Met and Cys, %	0.95	0.93	0.91	0.87	0.85	0.84	0.80	0.79	0.77
Dig. Thr, %	0.86	0.84	0.83	0.77	0.76	0.76	0.70	0.70	0.69
Dig. Val, %	1.00	0.99	0.98	0.91	0.90	0.90	0.83	0.82	0.81
Dig. Ile, %	1.05	1.05	1.04	0.96	0.96	0.95	0.88	0.87	0.87
Dig. Arg, %	1.37	1.35	1.34	1.23	1.22	1.20	1.10	1.09	1.08
Dig. Trp, %	0.26	0.26	0.26	0.23	0.23	0.23	0.21	0.21	0.20

[1]Vitamin-mineral premix used at 0.50% in each diet to provide 1.65 mg I, 0.3 mg Se, 10,000 IU vitamin A, 4,000 IU vitamin D_3, 50 IU vitamin E, 4 mg vitamin K, 4 mg thiamin, 10 mg riboflavin, 65 mg niacin, 15 mg pantothenic acid, 5 mg pyridoxine, 2 mg folic acid, 0.02 mg cobalamins, 0.2 mg biotin, and 2.64 mg choline per kg.
[2]Monensin, Coban 90 (Elanco Animal Health, Indianapolis, IN) used in each diet at 0.05%.
[3]Celite (Celite Corp., Lompoc, CA) used per diet at 2%.
[4]Finely ground yellow corn added without phytase in the negative controls 1 (NC1) and 2 (NC2) diets; with phytase A (PHYA) in the NC1 + PHYA at 500 FTU/kg (NC1+500PHYA) and NC2 + PHYA at 1,000 FTU/kg (NC2+1000PHYA) diets; and with phytase B (PHYB) in the NC1 + PHYB at 500 FTU/kg (NC1+500PHYB) and NC2 + PHYB at 1,000 FTU/kg (NC2+1000PHYB) diets.
[5]Information on identity of PHYA and PHYB were withheld from authors.
[6]Digestible amino acid.

Table 5.6.2: Analyzed dietary calcium, total phosphorus, and phytase activities[1]

	PC[2]	NC1[2]	NC1+500 PHYA[2,3]	NC1+ 500PHYB[2,3]	NC2[2]	NC2+ 1000PHYA[2,3]	NC2+ 1000PHYB[2,3]
Calcium %							
Starter	0.750	0.823	0.950	0.895	0.794	0.983	0.798
Grower	0.977	0.840	0.834	0.753	0.825	0.801	0.766
Finisher	0.642	0.783	0.738	0.718	0.680	0.692	0.660
Total Phosphorus %							
Starter	0.738	0.728	0.720	0.697	0.630	0.699	0.578
Grower	0.844	0.629	0.601	0.572	0.630	0.559	0.526
Finisher	0.637	0.606	0.557	0.548	0.492	0.513	0.493
Phytase activity FTU/kg							
Starter	<100	<100	858	1,221	<100	1,237	1,763
Grower	<100	<100	619	519	<100	1,078	1,140
Finisher	267	<100	606	771	<100	915	1,327

[1]Starter phase was from d 0 to 8, Grower phase was from d 8 to 22, and Finisher phase was from d 22 to 33

[2]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively, (NC1) or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (PHYA) or phytase B (PHYB) at 500 FTU/kg (NC1+500PHYA and NC1+500PHYB); and the NC2 + PHYA or PHYB at 1,000 FTU/kg (NC2+1000PHYA and NC2+1000PHYB).

[3]Information on identity of PHYA and PHYB were withheld from authors.

Table 5.6.3: Effect of dietary Ca, available P and phytase source and dose on broiler femur densitometry at 22d of age[1]

	Bone mineral density (mg/cm^3)			Cross-sectional area (mm^2)			Bone mineral content (mg/mm)^2		
Diets[3]	Total	Trabecular	Cortical	Total	Trabecular	Cortical	Total	Trabecular	Cortical
Mid-diaphysis[5]									
PC	539	61.4	840^{a}	31.2	10.6	18.1	16.8^{ab}	0.63	15.2^{ab}
NC1	525	52.9	803^{b}	28.7	9.59	17.1	15.0^{cd}	0.49	13.6^{de}
NC1+500PHYA[4]	551	58.7	838^{a}	32.2	10.4	19.2	17.7^{a}	0.58	16.1^{a}
NC1+500PHYB[4]	513	49.7	820^{ab}	30.4	11.2	16.9	15.4^{bcd}	0.62	13.9^{de}
NC2	514	46.4	767^{c}	28.5	8.57	17.1	14.6^{d}	0.39	13.2^{e}
NC2+1000PHYA[4]	551	63.9	836^{a}	30.4	9.76	18.1	16.7^{ab}	0.61	15.1^{abc}
NC2+1000PHYB[4]	543	61.1	837^{a}	29.5	9.85	17.4	16.0^{b}	0.59	14.5^{bcd}
Pooled SEM	14.94	9.51	10.69	0.89	0.61	0.66	0.56	0.10	0.53
Source of variation	----------*P*-value----------								
Diet	0.227	0.820	<0.001	0.055	0.086	0.107	0.001	0.668	<0.001
Co-variate (BW)	0.774	0.820	0.739	0.620	0.981	0.534	0.761	0.706	0.612
Proximal metaphysis[6]									
PC	365	99.8^{ab}	747^{ab}	38.4	21.3	13.7	13.9^{a}	2.15^{ab}	10.2^{a}
NC1	347	93.6^{abc}	733^{b}	35.9	20.2	12.5	12.4^{c}	1.87^{abc}	9.19^{bc}
NC1+500PHYA[4]	359	96.8^{ab}	754^{a}	38.6	21.6	13.4	13.7^{ab}	2.14^{ab}	10.1^{ab}
NC1+500PHYB[4]	357	85.5^{bc}	749^{ab}	36.7	20.4	13.1	12.9^{bc}	1.71^{bc}	9.81^{ab}
NC2	325	76.6^{c}	707^{c}	35.7	20.0	12.1	11.5^{d}	1.55^{c}	8.53^{c}
NC2+1000PHYA[4]	358	107.3^{a}	739^{ab}	38.3	21.4	13.4	13.7^{ab}	2.25^{a}	9.93^{ab}
NC2+1000PHYB[4]	343	78.3^{c}	748^{ab}	36.6	20.8	12.8	12.5^{c}	1.64^{c}	9.61^{ab}
Pooled SEM	10.36	6.63	7.51	1.16	0.97	0.65	0.36	0.18	0.34
Source of variation	----------*P*-value----------								
Diet	0.153	0.005	0.001	0.315	0.852	0.075	<0.001	0.020	0.011
Co-variate (BW)	0.087	0.245	0.021	0.111	0.075	0.408	0.954	0.042	0.145

[a-e]Treatment means with no common superscript within a column differ ($P \leq 0.05$).

[1]Each treatment mean was calculated from right femurs of 2 birds in each of 9 cage replicates (n=126) on d 22.

[2]Bone mineral content was calculated by multiplying the respective bone cross-sectional area by bone mineral density to yield the amount of mineral (mg) contained in one mm-thick x-ray scan for each of bone fraction.

[3]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively, (NC1) or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (PHYA) or phytase B (PHYB) at 500 FTU/kg (NC1+500PHYA and NC1+500PHYB); and the NC2 + PHYA or PHYB at 1,000 FTU/kg (NC2+1000PHYA and NC2+1000PHYB).
[4]Information on identity of PHYA and PHYB were withheld from authors.
[5]mid-point of femur length.
[6]30% point from the proximal end of femur.

Table 5.6.4: Effect of dietary Ca, available P and phytase source and dose on broiler femur densitometry traits at 33 d of age[1]

	Bone mineral density (mg/cm^3)			Cross-sectional area (mm^2)			Bone mineral content (mg/mm)^2		
Diets[3]	Total	Trabecular	Cortical	Total	Trabecular	Cortical	Total	Trabecular	Cortical
Mid-diaphysis[5]									
PC	466	50.6	891^{ab}	49.2	23.3	23.4	22.8	1.17	20.8^{b}
NC1	453	60.0	860^{c}	50.9	24.3	24.0	23.0	1.47	20.6^{b}
NC1+500PHYA[4]	468	48.6	885^{ab}	49.4	23.0	23.9	23.0	1.13	21.1^{b}
NC1+500PHYB[4]	465	49.3	884^{ab}	48.8	23.2	23.1	22.4	1.11	20.4^{b}
NC2	466	51.4	871^{bc}	47.4	22.2	23.1	22.0	1.23	20.1^{b}
NC2+1000PHYA[4]	483	48.0	897^{a}	50.7	23.2	25.0	24.4	1.10	22.4^{a}
NC2+1000PHYB[4]	484	47.5	901^{a}	48.1	22.5	23.6	23.2	1.07	21.2^{ab}
Pooled SEM	9.31	6.0	8.56	1.19	0.8	0.54	0.54	0.15	0.45
Source of variation	----------*P*-value----------								
Diet	0.238	0.811	0.005	0.347	0.644	0.174	0.073	0.523	0.014
Co-variate (BW)	0.069	0.739	<0.001	<0.001	<0.001	<0.001	<0.001	0.108	<0.001
Proximal metaphysis[7]									
PC	325	74.1	784	60.9	37.4	19.2	19.7	2.77	15.0^{abc}
NC1	319	65.9	789	58.9	38.9	18.7	18.8	2.44	14.8^{bc}
NC1+500PHYA[4]	334	74.1	797	58.5	35.6	19.1	19.5	2.63	15.1^{ab}
NC1+500PHYB[4]	330	81.6	784	59.0	36.4	18.5	19.3	2.96	14.5^{bc}
NC2	310	73.6	777	60.1	37.8	18.0	18.5	2.8	14.0^{c}
NC2+1000PHYA[4]	338	71.4	805	59.6	36.2	19.8	20.1	2.59	15.9^{a}
NC2+1000PHYB[4]	330	65.9	793	59.7	36.3	19.6	19.5	2.38	15.5^{ab}
Pooled SEM	8.48	4.03	6.94	1.41	1.59	0.48	0.51	0.18	0.41
Source of variation	----------*P*-value----------								
Diet	0.286	0.117	0.096	0.921	0.793	0.191	0.225	0.292	0.037
Co-variate (BW)	0.605	0.671	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	<0.001

[a-c]Treatment means with no common superscript within a column differ ($P \leq 0.05$).

[1]Each treatment mean was calculated from right femurs of 2 birds in each of 9 cage replicates (n=126) on d 33.

[2]Bone mineral content was calculated by multiplying the respective bone cross-sectional area by bone mineral density to yield the amount of mineral (mg) contained in one mm-thick x-ray scan for each of bone fraction.

[3]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively, (NC1) or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (PHYA) or phytase B (PHYB) at 500 FTU/kg (NC1+500PHYA and NC1+500PHYB); and the NC2 + PHYA or PHYB at 1,000 FTU/kg (NC2+1000PHYA and NC2+1000PHYB).
[4]Information on identity of PHYA and PHYB were withheld from authors.
[5]mid-point of femur length.
[6]30% point from the proximal end of femur.

Table 5.6.5: Effect of dietary Ca, available P and phytase source and dose on femur traits of broilers[1]

	Bone breaking strength (kgF)			Bone ash content (%)		
Diets[2]	D 8	D 22	D 33	D 8	D 22	D 33
PC	3.18[a]	14.7[a]	20.2[bc]	33.4	36.1[ab]	31.5
NC1	3.06[a]	13.0[bc]	18.7[cd]	30.4	33.5[d]	31.8
NC1+500PHYA[3]	3.22[a]	14.3[a]	21.3[ab]	30.2	35.2[abc]	33.3
NC1+500PHYB[3]	3.17[a]	13.7[ab]	20.9[b]	30.1	35.1[bc]	31.8
NC2	2.70[b]	12.4[c]	17.9[d]	29.9	34.2[cd]	31.3
NC2+1000PHYA[3]	3.24[a]	14.5[a]	22.7[a]	30.0	36.3[a]	33.4
NC2+1000PHYB[3]	3.22[a]	14.4[a]	20.5[b]	32.2	36.3[a]	31.5
Pooled SEM	0.09	0.39	0.58	1.63	0.46	0.77
Source of variation	----------*P*-value----------					
Diet	<0.001	<0.001	<0.001	NS	<0.001	NS
Co-variate (BW)	<0.001	<0.001	<0.001	NS	NS	0.01

[a-d]Treatment means with no common superscript within a column differ ($P \leq 0.05$).

[1]Treatment means were calculated for each of bone breaking strength and ash content from right femurs of 2 birds in each of 9 cage replicates per treatment (n=126) on each of d 22 and 33.

[2]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively, (NC1) or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (PHYA) or phytase B (PHYB) at 500 FTU/kg (NC1+500PHYA and NC1+500PHYB); and the NC2 + PHYA or PHYB at 1,000 FTU/kg (NC2+1000PHYA and NC2+1000PHYB).

[3]Information on identity of PHYA and PHYB were withheld from authors.

Table 5.6.6: Effect of dietary Ca, available P and phytase source and dose on apparent ileal digestibility of phosphorus (AIDP) and calcium (AIDCa) in broilers[1]

	AIDP%			AIDCa%		
Diets[2]	D 8	D 22	D 33	D 8	D 22	D 33
PC	45.5^{de}	48.3^{c}	44.6^{c}	40.5	41.2^{bc}	32.6
NC1	42.8^{ef}	39.6^{d}	36.8^{d}	41.4	50.1^{a}	33.5
NC1+500PHYA[3]	56.3^{b}	58.9^{b}	56.7^{a}	46.9	50.7^{a}	32.2
NC1+500PHYB[3]	54.5^{bc}	48.2^{c}	51.5^{b}	43.8	40.9^{bc}	34.9
NC2	39.5^{f}	42.6^{d}	34.6^{d}	35.7	37.6^{c}	38.4
NC2+1000PHYA[3]	63.3^{a}	66.9^{a}	61.4^{a}	50.9	53.2^{a}	28.5
NC2+1000PHYB[3]	49.3^{d}	50.1^{c}	54.7^{b}	39.7	46.8^{ab}	29.0
Pooled SEM		2.21			3.09	
Source of variation	----------*P*-value----------					
Diet × Age	<0.001			<0.001		

[a-d]Treatment means with no common superscript for each parameter differ (P ≤ 0.05).
[1]Treatment means were calculated for each of AIDP, and AIDCa from 9 cage replicates (n=63) each pooled from 11 and 5 birds for d 22 and 33, respectively.
[2]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively, (NC1) or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (PHYA) or phytase B (PHYB) at 500 FTU/kg (NC1+500PHYA and NC1+500PHYB); and the NC2 + PHYA or PHYB at 1,000 FTU/kg (NC2+1000PHYA and NC2+1000PHYB).
[3]Information on identity of PHYA and PHYB were withheld from authors.

Table 5.6.7 Concentrations of IP_{6-3} as a percentage of grower broiler diets (dry matter basis)[1]

	IP_6	IP_5	IP_4	IP_3
Diets[2]				
PC	1.02	0.11	0.02	0.06
NC1	1.07	0.13	0.04	0.06
NC1+500PHYA[3]	1.00	0.16	0.05	0.06
NC1+500PHYB[3]	1.05	0.12	0.04	0.06
NC2	1.07	0.14	0.04	0.06
NC2+1000PHYA[3]	1.00	0.17	0.06	0.06
NC2+1000PHYB[3]	1.02	0.13	0.04	0.06

[1]IP_{6-3} (IP_6 = myo-inositol hexa-phosphates, IP_5 = myo-inositol penta-phosphates, IP_4 = myo-inositol tetra-phosphates, and IP_3 = myo-inositol di-phosphates) means calculated from triplicate analyses of each diet.
[2]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively, (NC1) or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (PHYA) or phytase B (PHYB) at 500 FTU/kg (NC1+500PHYA and NC1+500PHYB); and the NC2 + PHYA or PHYB at 1,000 FTU/kg (NC2+1000PHYA and NC2+1000PHYB).
[3]Information on identity of PHYA and PHYB were withheld from authors.

Table 5.6.8: Effect of dietary Ca, available P and phytase source and dose on digesta IP_{6-3} concentrations in the crop, proventriculus + gizzard, and distal ileum of broilers at day 22[1]

	Crop, % of DM				Proventriculus + gizzard, % of DM				Distal ileum, % of DM			
Diets[2]	IP_6	IP_5	IP_4	IP_3	IP_6	IP_5	IP_4	IP_3	IP_6	IP_5	IP_4	IP_3
PC	0.85^{a}	0.10^{ab}	0.08^{c}	0.06^{c}	0.26^{a}	0.04^{a}	0.03^{bc}	0.04^{b}	1.69^{a}	0.23^{a}	0.13^{d}	0.09^{d}
NC1	0.83^{a}	0.09^{bc}	0.05^{c}	0.05^{c}	0.30^{a}	0.02^{b}	0.02^{bc}	0.03^{b}	1.83^{a}	0.19^{ab}	0.09^{d}	0.11^{cd}
NC1+500PHYA[3]	0.80^{a}	0.11^{ab}	0.11^{c}	0.05^{c}	0.03^{c}	0.00^{c}	0.04^{b}	0.06^{a}	0.88^{c}	0.27^{a}	0.43^{a}	0.17^{b}
NC1+500PHYB[3]	0.58^{b}	0.06^{d}	0.23^{ab}	0.08^{ab}	0.10^{b}	0.02^{b}	0.07^{a}	0.06^{a}	1.23^{b}	0.28^{a}	0.28^{c}	0.15^{b}
NC2	0.84^{a}	0.10^{ab}	0.08^{c}	0.05^{c}	0.27^{a}	0.03^{a}	0.03^{bc}	0.04^{b}	1.81^{a}	0.21^{a}	0.11^{d}	0.12^{cd}
NC2+1000PHYA[3]	0.61^{b}	0.13^{a}	0.18^{b}	0.06^{c}	0.02^{c}	0.00^{c}	0.02^{c}	0.04^{b}	0.48^{d}	0.13^{b}	0.38^{ab}	0.21^{a}
NC2+1000PHYB[3]	0.52^{b}	0.05^{d}	0.28^{a}	0.09^{a}	0.07^{b}	0.01^{b}	0.06^{a}	0.06^{a}	0.89^{c}	0.30^{a}	0.31^{bc}	0.16^{b}
Pooled SEM	0.04	0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.07	0.03	0.04	0.01
Source of variation	----------*P*-value----------											
Diet	<0.001	<0.001	<0.001	0.004	<0.001	<0.001	<0.001	0.004	<0.001	0.006	<0.001	<0.001

[a-d]Treatment means with no common superscript within a column differ ($P \leq 0.05$).

[1]IP_{6-3} (IP_6 = myo-inositol hexa-phosphates, IP_5 = myo-inositol penta-phosphates, IP_4 = myo-inositol tetra-phosphates, and IP_3 = myo-inositol di-phosphates) means were calculated for each of crop, proventriculus + gizzard, and distal ileum from 9 cage replicates (n=189) each pooled from 11 birds.

[2]Diets were a nutritionally adequate positive control diet (PC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively, (NC1) or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (PHYA) or phytase B (PHYB) at 500 FTU/kg (NC1+500PHYA and NC1+500PHYB); and the NC2 + PHYA or PHYB at 1,000 FTU/kg (NC2+1000PHYA and NC2+1000PHYB).

[3]Information on identity of PHYA and PHYB were withheld from authors.

5.7 FIGURE

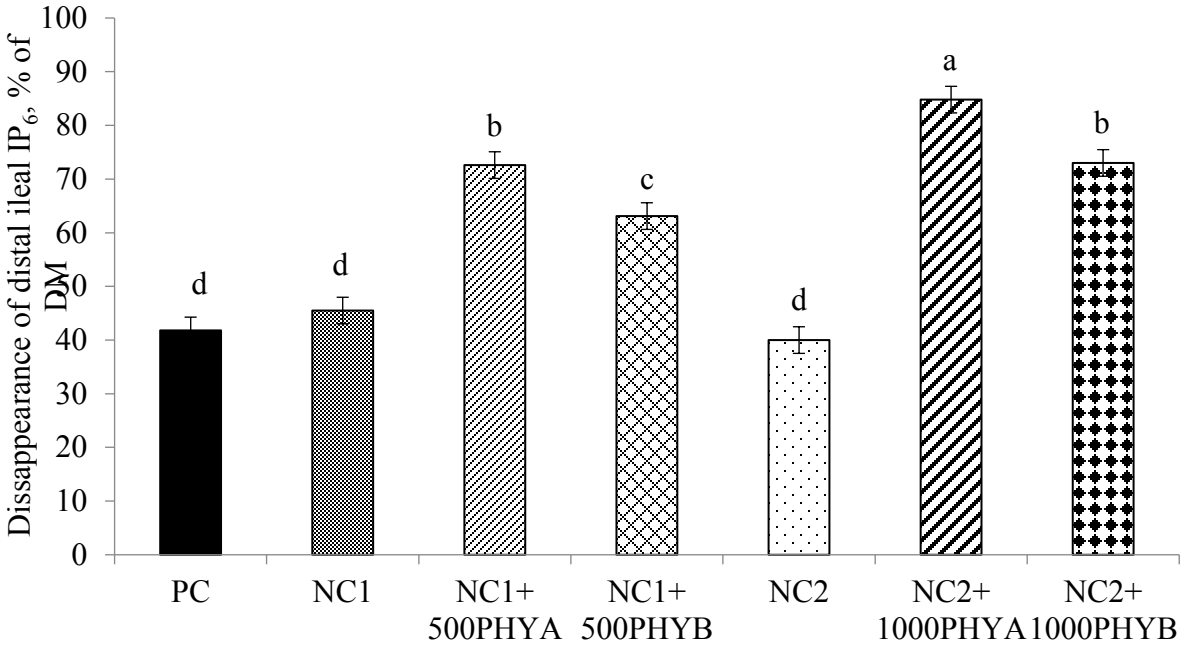

Figure 5.7.1: Effect of dietary Ca, available P and phytase source and dose on the disappearance of inositol hexa-phosphate (IP_6) in the distal ileum of broilers at day 22[1]
[a-d]Treatment means with no common superscript differ ($P \leq 0.05$). [1]Each treatment mean for distal ileum IP_6 concentration was calculated from 9 replicates each pooled from 11 birds at 22 d of age (n=63).[2]Diets were a nutritionally adequate positive control diet (P
µC); the PC with available P and Ca reduced by 0.146 and 0.134% of the diet respectively, (NC1) or by 0.174 and 0.159% of the diet respectively (NC2); the NC1 + phytase A (PHYA) or phytase B (PHYB) at 500 FTU/kg (NC1+500PHYA and NC1+500PHYB); and the NC2 + PHYA or PHYB at 1,000 FTU/kg (NC2+1000PHYA and NC2+1000PHYB).
[3]Information on identity of PHYA and PHYB were withheld from authors.

6. RESEARCH SYNTHESIS

6.1 OVERVIEW

Approximately 55 to 75% of P in plant-based feed ingredients is bound to phytate and is unavailable for absorption in the gastrointestinal tract (**GIT**) of birds (Tahir et al., 2012). Hence, limiting and expensive inorganic P sources such as monocalcium phosphate and dicalcium phosphate are supplemented in diets to meet the requirement for available P (**avP;** Neset et al., 2012). Dietary supplementation of inorganic P increases feed cost (Ponnuvel et al., 2013) and also increases P excretion into the environment resulting in P pollution (Lim et al., 2003). In addition, calcium is low in most plant-based feed ingredients. Hence, ingredients such as calcium carbonate, oyster shell, and bone meal are added in laying hens diet to meet the high dietary requirement for Ca (Hughes et al., 2009; Saunders-Blades et al., 2009). However, a considerable fraction of the dietary Ca is bound to phytate to form a Ca-phytate complex, which decreases P and Ca availability for absorption in monogastrics, and increases P and Ca excretion. Adequate availability of P and Ca for absorption is essential to ensure long-term egg production with high shell quality and bone traits in laying hens. Overall, while P and Ca are essential for performance and bone health, high use of inorganic P and Ca source to meet the dietary requirement is also detrimental to mineral availability and subsequent performance and bone quality of the hens. However, supplementation of phytase in diets allows for considerable reduction in dietary P and Ca with no adverse effects on the productivity and bone health of laying hens. Phytase use in diets reduced in avP and Ca alleviated the decrease in bone breaking strength and ash in laying hens (Boling et al., 2000; de Lima et al., 2010). Because bone ash only provides information on mineralization across strength-providing cortical and trabecular

bone and non-structural medullary bone, the measurement provides no specific information for bone strength. Also, bone breaking strength is not sufficient to evaluate the strength-providing potential of cortical and trabecular bone tissues. Bone breaking strength is assessed specifically on the mid-diaphysis of bone, which is the region that houses primarily cortical and medullary bone (de Lima et al., 2010; Regmi et al., 2015). However, the bone at the proximal and distal ends of long bones are spongier with higher concentration of the trabecular bone tissue at the site (Reich and Gefen, 2006). Therefore, the distal and proximal epiphyses are more susceptible to fracture than the bone of the mid-diaphysis (Reich and Gefen, 2006). Bone ash, P, Ca and breaking strength provide information on bone mineralization and resistance to fracture and are fast, easy, and cost-effective. Hence, the relevance of bone strength and resistance is limited to determining the risk of osteoporosis and fracture in laying hens.

Understanding the mechanisms by which phytase supplementation can alleviate the adverse effects of marginal, moderate, and severe reductions of dietary avP and Ca on bone quality of laying hens requires details beyond breaking strength and ash content. Bone densitometry and micro-architecture, and serum bone biomarkers are essential tools understand the mechanisms by which supplemental phytase alleviates adverse effects of reduced dietary avP and Ca in laying hens. The ability of bone densitometry and micro-architecture to provide information for each of cortical, trabecular, and medullary bone tissues is vital for a more definite comprehension of avP and Ca reduction and phytase supplementation effects on bone quality. The densitometry and micro-architecture of each bone tissue were measured using quantitative computed tomography (**QCT**) and micro-CT imaging technology (**micro-CT**), respectively. Serum levels of osteocalcin and pyridinoline are measurable as biomarkers of bone formation or resorption, respectively. Hence, the primary objective of this Ph.D. thesis was to

evaluate effects of phytase supplementation in Ca- and avP-reduced diets on bone biology, laying performance and eggshell quality of laying hens. The secondary objective was to validate the relationship between phytate degradation by two phytases at two doses on digestibility of Ca and P, and bone quality in broilers.

6.1.1 Review of thesis hypotheses

Four hypotheses were tested in this Ph.D. research work:

Hypothesis 1: The inclusion of *Buttiauxella sp* phytase (**BSP**) in laying hen diets from 30 to 70 wk of age would alleviate the adverse effects of reduced dietary Ca and avP.

This hypothesis was rejected because the negative control diet used in this long-term study was not deficient to allow any alleviating effect from the supplemental phytase. The fact that the efficacy of the supplemental BSP could not be validated by this hypothesis does not mean the phytase was not effective. As the NC diet used in the trial supported performance, eggshell quality, and the majority of evaluated bone trait parameters to a similar level as the PC diet, the supplemented BSP did not have the opportunity to prevent an adverse effect of avP and Ca reduction.

Hypothesis 2: Phytase supplementation would maintain the micro-architecture of femur cortical, trabecular, and medullary bone tissues in laying hens fed Ca- and avP-reduced diets (NC) at similar levels as hens fed Ca- and avP-adequate diets (PC).

This hypothesis was accepted as the supplementation of BSP in diets moderately or severely reduced in avP and Ca alleviated the decrease in volume, thickness, and mineralization, and increased the porosity of the medullary bone to maintain the labile mineral reserve for eggshell formation. Also, the acceptance of this hypothesis based on the result of the micro-architectural analysis of the laying hen femurs was also supported by the result of densitometry analysis of the bone.

Hypothesis 3: Phytase supplementation in Ca- and avP-deficient diets would maintain osteoblast and osteoclast activities, as indicated by serum levels of osteocalcin, pyridinoline, PTH, Ca, and P, at similar levels as Ca- and avP-adequate diet in 78-wk-old laying hens.

This hypothesis was accepted as the supplementation of BSP in a diet severely reduced in avP and Ca alleviated the increase of bone resorption as indicated by a decreased serum pyridinoline concentration. Osteocalcin, PTH, Ca, and P were not affected by the avP and Ca deficiency in the first place, hence the limited opportunity for the supplemental BSP to influence the serum bone biomarkers.

Hypothesis 4: Degradation of phytate in each of the crop, proventriculus + gizzard, and distal ileum would differ by phytase source, be higher with an increase in phytase dose, and would be directly linked to P and Ca digestibility and bone quality in broilers.

This hypothesis was accepted as the supplementation of either of two phytases at 500 or 1,000 FTU/kg in avP- and Ca-reduced diets fed to broilers increased degradation of hexa-, penta-, tetra-, and tri-phosphate across the digestive tract segments to increase P digestibility and bone mineralization. Also, phytase A exhibited higher phytate-degrading efficacy relative to phytase B, and the 1,000 FTU/kg usage was also more effective than the 500 FTU/kg.

6.2 FINDINGS, ANALYSES AND IMPLICATIONS

The marginal reductions of avP and Ca in the NC diet (Chapter 2 and Trial I of Chapter 4) did not decrease egg production, eggshell quality, and the majority of bone breaking strength, ash, and densitometry measures, but tended to decrease BW relative to other diets across 30 to 70 woa. The moderate reductions in avP and Ca in the NC1 diet (Chapter 3 and Trial II of Chapter 4) did not adversely affect egg production and eggshell quality, but decreased BW of hens by 4.3% relative to the PC diet from 68 to 78 woa. The severe reductions in avP and Ca in

the NC2 diet (Chapter 3 and Trial II of Chapter 4) decreased performance and eggshell quality, and also decreased hen BW by 7.9% relative to the PC diet from 68 to 78 woa.

Phytase is supplemented in diets reduced in avP and Ca levels to degrade phytate and alleviate adverse effects on the availability of these minerals, performance, and bone quality of laying hens. Commercially, phytase is used in diets fed to laying hens at 300 or 600 FTU/kg. The marginal reduction in dietary avP and Ca levels, as in Chapter 2 does not automatically translate to deficiency of the minerals in the diet. Modern egg-laying hens have high efficiency to maintain productivity diets marginally or moderately reduced in avP and Ca. Depending on the degree of dietary avP and Ca restriction, laying hens also maintain egg production and eggshell quality at the expense of BW and bone reserves. The sufficiency of the avP and Ca levels in the negative control diets indicated that the mineral levels in the PC were in excess of the requirements. The NRC nutrient specifications for poultry was last updated in 1994 (NRC, 1994). However, productivity in modern egg-laying hens is higher than it was the 1990s (Bain et al., 2016), so the NRC-recommended nutrient composition may be less than the requirements needed to maintain performance in modern egg-laying hens. For example, the specification of NRC, 1994 for Ca was 3.25 g/kg of non-phytase supplemented diet, which is evidently less than the mineral requirement in modern egg-laying hens (Cransberg et al., 2001; Chapter 3). Currently, avP and Ca levels in commercial-type diets are commonly based on the recommendation from primary breeders. However, the dietary avP and Ca levels recommended by the primary breeders have considerable safety margins. This thesis shows that the primary breeder recommended avP and Ca levels do not allow the opportunity to evaluate phytase effects.

Furthermore, the general phenomenon is that feeding a diet with high avP and Ca levels as recommended by the primary breeders helps to ensure optimal performance, BW, and bone integrity in egg-laying hens. Adequate dietary avP and Ca are essential to support bone metabolism and eggshell formation (Dacke et al., 1993). However, high dietary Ca also decreases the availability of P and Ca for absorption from the GIT by forming an insoluble and high pH Ca-phytate complex in laying hens (Beutler, 2009). The higher the dietary Ca level relative to the requirement, the greater the adverse effect on the decreased availability of P and Ca. Chapter 4 showed that relative to a diet marginally reduced in avP and Ca, feeding a diet with high avP and Ca decreased trabecular bone volume and mineral density and increased porosity and separation within the structural bone. Loss of structural bone in egg-laying hens fed long-term avP- and Ca-adequate diets has been demonstrated (Cransberg et al., 2001). The finding of the current study made it clear that the structural bone loss in laying hens over long-term laying cycle may be in part explained by the anti-nutritional effects of high dietary Ca in commercial laying hen diets. The laying hens fed the primary breeder recommended levels of avP and Ca over a long-term laying cycle had an irreversible loss trabecular of bone, which may be partially due to the adverse effects of higher than required Ca in the PC diet. While phytase was not included in the primary breeder recommended based-PC diet, it is not clear on how the enzyme usage would have influenced trabecular bone micro-architecture in laying hens. Phytase usage prevents the decrease in performance of hens on avP- and Ca-deficient diets (Lui et al., 2007; Beutler, 2009). The use of phytase in diets fed to laying hens would also allow for a reduction in dietary Ca level to decrease the level of Ca-phytate complex formation in the GIT of hens, and the adverse effects on minerals digestibility and gradual loss of structural bone.

The effects of marginal, moderate, or severe reductions of avP and Ca in the diet on performance and eggshell quality of laying hens are dependent on the length of the period for which the birds were on either of the diets. Also, the mechanism by which dietary avP and Ca deficiency decrease performance of egg-laying hens requires a clear understanding of the diet effects on bone metabolism. Decrease in bone mass and mineralization in part resulted in loss of BW in hens (Boling et al., 2000; Cransberg et al., 2001). Also, the degree and duration of dietary avP and Ca deficiencies determine the effects on cortical, trabecular, and medullary bone. Fundamentally, the responses of hens fed avP- and Ca-adequate diet on performance, eggshell quality, and bone properties are important to understanding the effect of the diets reduced in the minerals and the phytase supplementation in the diet. The primary breeder recommendation-based PC utilized in Chapter 2 maintained medullary bone mineral density and volume, had inconsistent effects on cortical bone, and increased porosity with decreased volume of trabecular bone. The PC utilized in Chapter 3 maintained each of the three bone tissues over time. The marginal dietary reduction in avP and Ca in NC in Chapter 2 also maintained each of the three bone tissues. For the moderate reduction of avP and Ca in the NC1 of Chapters 3 and 4, the micro-CT-measured micro-architectural quality of each of the three bone tissues was maintained while the QCT-measured trabecular space mineral density and content and BW were each decreased. The supplementation of phytase in the moderately reduced diet alleviated the adverse effects on the trabecular space densitometry and BW. The severe reduction in avP and Ca of the NC2 diet decreased the QCT-measured trabecular space bone mineral density and content (Chapter 3) and also the micro-CT-measured mineral density in cortical and volume with increased bone porosity (Chapter 4). Phytase use in the NC2 diet was not able to completely alleviate the adverse effects on the bone densitometry and micro-architecture traits.

When laying hens are fed diets with adequate or only marginally reduced avP and Ca levels, phytase has little to no opportunity to increase nutrient availability. Based on this Ph. D. research, phytase usage in commercial layer diet could allow for further reductions in avP and Ca levels, which would increase the efficiency of phytase usage in commercial practice. The efficient use of phytase inclusion in diet fed to egg-laying hens would further lower dietary Ca to limit the adverse on bone metabolism and feed cost.

The efficacy of two phytases at the two commercial doses (500 and 1,000 FTU/kg) were evaluated in broilers because broilers share the same GIT as laying hens and the short duration of a broiler production cycle. The GIT in egg-laying hens is more mature relative to those in broilers, and conditions such as pH considerably differ in the each of crop, proventriculus, gizzard, duodenum, jejunum, and ileum of the two poultry types (Beutler, 2009; Angel et al., 2010). In comparison, phytase usage in the diet of laying hens increased P availability by approximately 45 to 60% in laying hens (Beutler, 2009; Chapter 2) and by about 60 to 80% in broilers (Amerah et al., 2014; Bikker et al., 2016; Chapter 5). The differences in effects of phytases in digestibility of P in laying hens and broilers can also be related to the relatively higher maturity, and much high Ca level in the diet of laying hens. The higher maturity of the GIT in laying hens in part entails less favorable conditions such as higher pH for nutrient absorption and enzyme activity, which may explain the relatively lower P digestibility in laying hens. However, comparison of the results of Chapter 5 to that of a previous study that evaluated the effect of a 500 FTU/kg phytase on phytate degradation in the GIT of laying hens (Gao et al., 2013) showed similar responses on the enzyme in the two poultry types. While the variation in the effect of phytase supplementation on P and Ca digestibility in laying hens and broilers are considered, the efficacy of phytase to degrade phytate across the crop, proventriculus + gizzard,

and ileum of broilers (Chapter 5) can also be related to the phytate-degrading efficacy in laying hens. Also, the efficacy of 2 phytase types in broiler diets differed based on the phytate-degrading activity of each phytase. Hence, high phytate-degrading efficiency in the low pH environment of the upper GIT in laying hens is essential for the efficacy of a commercial phytase. The ability of dietary phytase alleviate adverse effects on bone metabolism depends on the degradation of phytate to increase P availability. In this study, the phytase with the most phytate-degrading efficacy also supported the greatest bone mass, mineralization, and resistance to fracture.

The use of phytase in diets fed to laying hens has now become a common practice in the poultry industry. Commercial phytase usage has allowed for increased efficiency in the industry with a decrease in feed cost and P and Ca excretion into the environment. However, the findings of this thesis show the need for a more efficient way of using dietary phytase in order to optimize the enzyme efficacy on performance, bone health, profitability, and sustainability in egg layers industry. Maintenance of cortical and trabecular bone tissues are essential for bone health, and adequate metabolism of medullary bone is crucial for eggshell formation in actively laying hens. Supplementation of phytase in diet moderately reduced in avP and Ca maintains the metabolism of the three bone tissues in laying hens.

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