Effects of phytase in laying hen diets reduced in available phosphorus and calcium on productivity, eggshell quality, and bone mineralization of white egg layers

by

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ABSTRACT

Phosphorus and Ca are essential for maintaining egg production and bone health of laying hens. The effects of marginal or moderate reductions in available P (aP) and Ca in layer diets, with or without phytase supplementation on performance, egg production, apparent ileal digestibility (AID) and retention of P and Ca, and eggshell and bone quality of pullets and laying hens were investigated. In Trial 1, 84 hens were fed one of seven dietary treatments. Treatments were: a positive control (PC) diet with 0.45% aP, 3.70% Ca and 0.16% Na from 25 to 28 wk, and 0.38% aP, 3.73% Ca and 0.15% Na from 29 to 37 wk; a negative control (NC) diet similar to the PC diet, but with 0.22% aP, 3.00% Ca and 0.13% Na from 25 to 28 wk, and 0.19% aP, 3.02% Ca and 0.13% Na from 29 to 37 wk; the NC diets supplemented with phytase at 150 (NC + 150), 300 (NC + 300), 600 (NC + 600), or 1,200 (NC + 1,200) phytase unit (FTU)/kg, respectively; and the PC diet supplemented with phytase at 1,200 (PC + 1,200) FTU/kg. Egg production, eggshell quality, bone traits, and AID of P and Ca were measured. The reduced aP and Ca in the NC diet did not decrease egg production, but the NC hens had lower cortical ($P < 0.001$) and trabecular + medullary bone mineral density (BMD; $P = 0.004$), and total bone mineral content (BMC; $P < 0.001$) than the PC hens. The NC + 600 and NC + 1,200 increased AID of P ($P = 0.024$). In Trial 2, one-d-old White Leghorn pullets (n = 480) were randomly allocated to six dietary treatments: a PC diet with a sequence of 0.48-0.45-0.37-0.45% aP, 1.05-1.00-0.90-2.00% Ca, and 0.18-0.17-0.16-0.16% Na for Starter-Grower-Developer-Pre-lay phases, respectively; a NC diet with marginal reduction, similar to the PC but reduced in aP, Ca, and Na by 0.15%, 0.16%, and 0.035% of the diet in each phase, respectively; the NC diet supplemented with phytase at 300 (NC + 300), 600 (NC + 600), 1,200 (NC + 1,200) or 2,400 (NC + 2,400) FTU/kg. Pullet performance, bone characteristics, and retention of P and Ca were determined. Reduced

dietary aP, Ca, and Na did not decrease pullet performance, but decreased bone breaking strength at 6 wk. The NC + 2,400 birds had greater P retention than the NC + 600 and PC birds at 6 wk. In Trial 3, hens (n = 256) were maintained on the first five respective dietary treatments previously fed from hatch to 19 wk in Trial 2. Hen productivity, retention of P and Ca, eggshell quality, and bone mineralization were determined. Hen BW in the NC was lower than the PC ($P < 0.001$), but the NC + 1,200 restored BW. At 74 wk of age, the NC + 600 hens had higher ($P < 0.001$) P retention than NC + 300 hens. The NC + 600 hens had greater distal femur ash than the NC hens ($P = 0.013$). The NC + 600 and NC + 1,200 hens had increased total BMD of the proximal ($P = 0.001$) and distal ($P = 0.002$) femurs relative to the NC hens. Laying hens fed the NC + 600 diet had increased proximal ($P = 0.002$) and mid-bone ($P = 0.008$) total femur BMC relative to the NC hens. Hens at 74 wk had greater total BMD and BMC than at 42 wk, likely due to an increase of medullary bone. Overall, moderate (Trial 1) and marginal (Trials 2 and 3) reductions in dietary aP and Ca did not decrease hen performance, egg production, nor eggshell quality. The NC hens were able to compensate for the reductions in dietary aP and Ca by increasing the absorption of these minerals in the short- and long-term. However, the marginal reduction of dietary aP and Ca in the long-term caused a subtle decrease in hen BW. Marginally and moderately reduced aP and Ca in the NC diets decreased bone quality, and phytase supplementation at 600 and 1,200 FTU/kg restored bone quality in laying hens fed reduced dietary aP and Ca.

PREFACE

This thesis is an original work by Koonphol Pongmanee. Koonphol conducted experiments, data collection and analysis, laboratory work, and thesis writing. The research protocols of this thesis were approved by the University of Alberta Animal Care and Use Committee for Livestock and followed principles established by the Canadian Council on Animal Care guidelines and policies (Canadian Council on Animal Care, 2009). The protocol for a series of research experiments was AUP00000161; the effect of phytase on phosphorus digestibility, production and bone traits in laying hens. Data from the short-term trial were presented in Chapter 2 and data from the long-term experiment were presented in Chapters 3, 4, and 5. Part of the information from this thesis had been presented and published as abstracts in the proceedings of 2014, 2015, 2016, and 2018 Poultry Science Association Annual Meetings.

Chapter 2 of this thesis has been published as Pongmanee, K., I. Kühn, and D. R. Korver, "Effects of phytase supplementation on eggshell and bone quality, and phosphorus and calcium digestibility in laying hens from 25 to 37 wk of age," *Poultry Science*, vol. 99, issue 5, 2595-2607. Koonphol was responsible for the data collection and analysis as well as the manuscript preparation. I. Kühn contributed to manuscript edits. D. R. Korver was the supervisory author and was involved with the concept formation and manuscript edits.

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ABBREVIATIONS

µCT	micro-computed tomography
$1,25(OH)_2D_3$	1,25-dihydroxycholecalciferol
Ad-SoS	amplitude-dependent speed of sound
AIA	acid insoluble ash
AID	apparent ileal digestibility
aP	available phosphorus
BBS	bone breaking strength
BMC	bone mineral content
BMD	bone mineral density
BW	body weight
BWG	body weight gain
Ca	calcium
cm	centimetre
d	day
D	depth
DEXA	dual-energy X-ray absorptiometry
DF	digitized fluoroscopy
FCR	feed conversion ratio
FI	feed intake
FTU	phytase unit
g	gram
h	hour

H	height
HDEP	hen-day egg production
IP_6	*myo*-inositol hexakisphosphate
kg	kilogram
kgf	kilogram of force
L	length, litre
LSM	least squares means
mg	milligram
mL	millilitre
mm	millimetre
Na	sodium
NC	negative control
nm	nanometre
NPP	non-phytate phosphorus
P	phosphorus
PC	positive control
PTH	parathyroid hormone
QCT	quantitative computed tomography
QUS	quantitative ultrasound
SG	specific gravity
U	unit
vs.	versus
W	width

wk	week

1. LITERATURE REVIEW

1.1 INTRODUCTION

Bone metabolism in egg-type chickens is different from meat-type chickens and other animals because of the large demand for Ca for almost daily eggshell formation during the laying period. Bone growth and development of pullets during the rearing phase can affect hen bone quality in the production period (Regmi et al., 2015). Although the economic impact is less clear during pullet rearing, managing the pullet properly is essential to maintain a healthy and productive layer flock and allows a high peak rate of lay and a long laying cycle with good persistence of egg production. Laying hens are capable of producing 500 eggs/hen in a long cycle production to 100 wk of age (Bain et al., 2016). In Canada, laying hens produce, on average, 340 eggs/hen per year (Agriculture and Agri-Food Canada, 2019). Over the years, genetic selection has increased skeletal health and resistance to structural bone loss (Bishop et al., 2000; Fleming et al., 2006) while increasing egg production and reducing mortality (Stratmann et al., 2016). Laying hens deposit 2.2 to 2.5 g Ca (about 10% of the total body Ca) for daily eggshell formation (Bar et al., 1996; Rodriguez-Navarro et al., 2018). Calcium demand is extremely high during eggshell calcification, especially during the latter stages of the dark period, when hen is not consuming Ca (Kerschnitzki et al., 2014). Hens therefore mobilize Ca from bone reserves (Taylor and Moore, 1954; Fleming et al., 2006). Although medullary bone serves as a labile source of Ca for eggshell formation, structural bone can also be mobilized but cannot be re-formed in actively laying hens (Fleming et al., 1998a; Whitehead, 2004). Structural bone loss over time can lead to bone weakness, fractures and osteoporosis, especially in older laying hens (Fleming et al., 1998b; Whitehead and Fleming, 2000; Fleming et al., 2006). The incidence of bone fractures was 10% at 36 wk and approximately 40% at 60 wk of age, and bone

strength and eggshell thickness decreased in aged commercial Lohmann LSL hens (Stratmann et al., 2016). Osteoporosis and trabecular bone loss were also observed in aged laying hens fed inadequate Ca diets (Cransberg et al., 2001; Jiang et al., 2019; Bello et al., 2020). Therefore, it is important to ensure that dietary Ca and other related nutrients such as P and vitamin D are sufficient to maximize hen productivity and maintain skeletal health and eggshell quality.

1.2 CALCIUM

Calcium is the most abundant mineral in the bone of chickens. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) of the bone accounts for approximately 99% of body Ca (Dacke et al., 1993; Proszkowiec-Weglarz and Angel, 2013). Intracellular Ca accounts for about 0.9% and the remaining Ca is extracellular (< 0.1% of Ca in the body) as ionized Ca, Ca bound to proteins (albumin, vitellogenin), and Ca bound to interstitial ions such as lactate, citrate, and bicarbonate (Taylor and Dacke, 1984; Dacke et al., 1993; de Matos, 2008). Total blood Ca refers to all forms of Ca (ionized form, Ca bound to proteins and Ca bound to anions), whereas blood ionized Ca is the physiologically active form and plays an important role in blood coagulation, muscle and nerve conduction, hormone secretions, eggshell calcification and bone homeostasis (de Matos, 2008). Ionized Ca is transported through the blood to support eggshell calcification in hens (Dacke et al., 1993). Under normal physiological status, the body keeps a very tight control on plasma ionized Ca concentrations (Li et al., 2017). Plasma total Ca in layers during pullet rearing (70 to 80 mg/L) is lower than during the laying period (158 mg/L), because Ca levels in pullet diets are lower than in hen diets (Gloux et al., 2019). Blood Ca concentration is regulated by many factors such as parathyroid hormone (**PTH**), calcitonin, sex hormones, 1,25-dihydroxycholecalciferol (**$1,25(OH)_2D_3$**) and its metabolites, vitamin D receptor, and calbindin

(Bar et al., 1992; Bar et al., 1996; Dimke et al., 2011; Li et al., 2017). Major changes in concentrations of ionized Ca may cause abnormal Ca metabolism (Dacke et al., 1993).

1.3 PHOSPHORUS

Phosphorus is the second most abundant mineral in the bone after Ca (Raina et al., 2012). Approximately 80% of body P is in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), which is located in the bone (Rath et al., 2000; Raina et al., 2012). The remaining 20% of P is located in cell membranes, cell cytoplasm and contents, blood, and body fluids, in the form of nucleic acids, nucleotides, phospholipids, phosphoproteins (Veum, 2010). Phosphorus has roles in cellular functions and membrane lipid synthesis, energy metabolism, bone mineralization and intracellular signal transduction (Berndt and Kumar, 2009; Sabbagh et al., 2009; Bergwitz and Jüppner, 2010; Lin et al., 2017). Phosphorus is also important in acid-base balance, as P is used to decrease blood acidosis by combining with excess hydrogen ions to maintain the concentrations of bicarbonate during eggshell formation (Pelicia et al., 2009b; Neijat et al., 2011). However, excessive dietary P causes the formation of insoluble Ca phosphate in the small intestine, which limits Ca absorption and may cause Ca deficiency, and impairs eggshell quality (Huber et al., 2006; Pelicia et al., 2009b; Neijat et al., 2011).

1.4 CALCIUM AND PHOSPHORUS METABOLISM

The absorption of Ca occurs in the small intestine via paracellular and transcellular pathways (Bronner, 1987). Paracellular is a non-saturable or passive absorption, and is characterized by ion channels between cells (intercellular tight junction) such as junctional adhesion molecule, claudin, and occludin (Figure 1.1; Gloux et al., 2019). Paracellular transport occurs throughout the small intestine (Bronner, 1987; Proszkowiec-Weglarz and Angel, 2013). The transcellular pathway is a calbindin-mediated, active and saturable transport process, is

dependent on vitamin D and requires metabolic energy, and takes place primarily in the duodenum and jejunum (Bronner, 1987; Bronner and Pansu, 1999; Gloux et al., 2019). There are at least four groups of proteins involved in transcellular Ca transport: epithelial Ca channels or transient receptor potential cation channels; calbindins; plasma membrane Ca ATPases (PMCAs or Ca^{2+} ATPases); and Na^{+}/Ca^{2+} exchangers (NCXs; Bar, 2009; Dimke et al., 2011; Li et al., 2018). In transcellular Ca absorption in laying hens, Ca enters across the apical brush border membrane by Ca-selective channels such as transient receptor potential cation channels subfamily V members 2 (TRPV2), 5 (TRPV5), 6 (TRPV6), or subfamily M number 7 (TRPM7; Gloux et al., 2019). It binds with calbindin in the cytoplasm and the Ca-calbindin complex then move to the basolateral membrane (Hurwitz, 1964; Bar et al., 1992) and Ca is exported to blood through ATPase plasma membrane Ca transporting 1 (ATP2B1), 2 (ATP2B2), and 4 (ATP2B4; Li et al., 2018; Gloux et al., 2019) as shown in Figure 1.1.

Calbindin is a Ca-binding and buffering protein, found in high concentrations in the intestinal, renal and eggshell gland tissues of layers (Bar et al., 1992; Li et al., 2018). Avian tissues contain primarily calbindin 28kDa protein (calbindin D_{28k}) that buffers excessive intracellular Ca^{2+} concentrations and transports Ca^{2+} to maintain Ca homeostasis (Nemere et al., 1991; Bar et al., 1996; Bar, 2009). Low dietary Ca induces the synthesis of calbindin D_{28k}, which stimulates intestinal Ca absorption via the transcellular pathway (Bronner and Pansu, 1999), whereas Ca absorbed by paracellular route decreases (Bronner, 1987). Calcium binding protein 1 (CALB1) tended to be higher in hens receiving low dietary Ca than in hens receiving high dietary Ca (Sommerfeld et al., 2020). High dietary Ca, on the other hand, decreases the transcellular process but increases paracellular absorption (Bronner, 1987; Christakos et al., 2014). However, both paracellular and transcellular pathways work cooperatively throughout the

length of the small intestine, and a high rate of Ca absorption occurs after sexual maturity in layers (Cohen et al., 1978; Bar, 2009; Gloux et al., 2019).

In addition to the mechanisms of Ca absorption in the small intestine, layers also have adaptive responses to dietary Ca concentrations in the kidney. Low dietary Ca induces PTH secretion, which stimulates the synthesis of renal 25-hydroxycholecalciferol-1-hydroxylase ($25(OH)D_3$-1-hydroxylase) which converts $25(OH)D_3$ to $1,25(OH)_2D_3$ (Elaroussi et al., 1994), increasing reabsorption and reducing Ca excretion by the kidney (Pelicia et al., 2009b). However, the activity of renal $25(OH)D_3$-1-hydroxylase decreases with hen age; older hens, therefore, produce less $1,25(OH)_2D_3$ than younger hens (Elaroussi et al., 1994), thereby reducing Ca absorption and leading eggshell problems in older laying hens.

In laying hens, the small intestine, kidney and bone are the main sites involved in P homeostasis (Li et al., 2017). There are two P absorption pathways in the small intestine; paracellular (passive) and transcellular (active) transport processes (Figure 1.2; Sabbagh et al., 2011; Christakos et al., 2014). Paracellular transport predominates when a P-adequate diet is fed (Hu et al., 2018), whereas the transcellular process predominates under low dietary P intake (Sabbagh et al., 2009). The paracellular process allows the movement of P ions along a concentration gradient through the tight junction between cells (Hu et al., 2018). In transcellular transport, P is regulated by sodium-dependent phosphate co-transporter (NaPi co-transporter) across the apical brush border membrane and exits at the basolateral membrane (Murer et al., 2001; Huber et al., 2006; Yan et al., 2007; Jing et al., 2018a). The mechanism of the efflux of P ions at the basolateral membrane is not known and there is very scant information in humans (Christakos et al., 2014) and poultry. In Light Sussex-Rhode Island Red crossbred pullets, phosphate transport across the basolateral membrane is independent of Na^+ ions, possibly

exchange with OH^- ions (Myint and Butterworth, 1989). This assumes that the pattern of P efflux at the basolateral membrane of hens during laying period would be the same as in pullet rearing period.

There are three different NaPi co-transporters: (i) NaPi type I in the brain and kidney, (ii) NaPi type II in the kidney (types IIa and IIc) and in the intestine (type IIb, known as solute carrier family 34 member 2; SLC34A2), (iii) NaPi type III in the intestine (PiT1, also known as solute carrier family 20 member 1; SLC20A1) and in the kidney (PiT2; Murer et al., 2001; Sabbagh et al., 2009; Huber et al., 2015; Hu et al., 2018; Proszkowiec-Weglarz et al., 2019). The PiT1 serves the basal P uptake – housekeeping function (Murer et al., 2001). In laying hens, NaPi IIb is the major small intestinal P co-transporter and presents highest in the duodenum, moderately in the jejunum, and lowest in the ileum (Huber et al., 2006; Jing et al., 2018a; Li et al., 2018; Nie et al., 2018). However, NaPi type III (PiT1 or SCL20A1) is the main P co-transporter in the ileum of laying hens (Gloux et al., 2019). Either $1,25(OH)_2D_3$ or low dietary P increases NaPi IIb activity in the small intestine (Yan et al., 2007; Sabbagh et al., 2009; Omara et al., 2020). Low dietary P levels also increase renal P reabsorption by NaPi IIa (Li et al., 2018). High dietary P intake decreases plasma Ca concentration, increases plasma P concentration and P excretion in laying hens (Huber et al., 2006; Bergwitz and Jüppner, 2010). Many factors affect P homeostasis, absorption, and retention such as PTH, calcitonin, $1,25(OH)_2D_3$, $25(OH)D_3$-1-hydroxylase, Klotho (a membrane-bound co-receptor protein), and phosphatonin or fibroblast growth factor 23 (FGF-23; Elaroussi et al., 1994; Sabbagh et al., 2009; Bergwitz and Jüppner, 2010; Sabbagh et al., 2011; Bian et al., 2014). High levels of PTH, FGF-23, and Klotho decrease renal reabsorption of P, which in turn increases renal P excretion (Bian et al., 2014; Li et al., 2017).

In laying hens, approximately 30% of aP is not utilized by birds and is excreted in urine (Kebreab et al., 2009). Also, renal P excretion occurs when bone is mobilized to provide Ca during eggshell formation, P is mobilized from bone as well but the majority of P is not required for eggshell formation or maintenance and is subsequently excreted (Kebreab et al., 2009).

1.5 CALCIUM REQUIREMENTS IN LAYERS

Proper Ca nutrition during pullet rearing is important for maximizing structural bone content before the onset of sexual maturity (Whitehead and Fleming, 2000; Khanal et al., 2019). Egg-type chickens require more Ca than meat-type chickens, especially from the onset of egg production throughout the laying period, to support eggshell formation (Wilkinson et al., 2011) and medullary bone remodeling (Kerschnitzki et al., 2014). The NRC (1994) recommendation for dietary Ca in Leghorn pullets is 0.9% during the first 6 wk and 0.8% from 6 to 18 wk of age, increase to 2.0% from 18 wk of age to the first egg. However, the commercial recommendations for dietary Ca for different ages are higher than the NRC (1994) recommendations. Lohmann Tierzucht (2019) recommends dietary Ca at 1.05% during the first 3 wk, 1.0% from 4 to 8 wk, 0.9% from 9 to 16 wk and 2.5% at 17 wk of age. Hy-Line International (2020) recommends 1.0% dietary Ca from 1 to 15 wk and 2.5% Ca from 15 to 17 wk of age. Feeding high Ca (3.63%) in pullet diets from 5 to 9 wk of age caused many issues such as hypercalcemia and hypophosphatemia (Guo et al., 2005), metabolic alkalosis, which may cause the failure of respiratory and renal regulatory systems (Guo et al., 2008), and watery excreta and wet litter (Guo et al., 2008; Bedford and Rousseau, 2017). Increasing dietary Ca from 2.5 to 4.0% during the pre-lay period for 2 wk (16 to 17 wk of age) did not increase bone quality in the early phase of egg production (Khanal et al., 2019). Because 4.0% dietary Ca is higher than the actual

requirement of Ca for pullets at this age, the excess Ca is excreted rather than deposited in bone (Pelicia et al., 2009a).

The recommendation for dietary Ca during the laying period is 3.25% (NRC, 1994), or ranging from 3.80 to 4.75% Ca from the pre-peak to after 86 wk (Lohmann Tierzucht, 2019; Hy-Line International, 2020). Primary breeder management guides include a considerable safety margin of Ca in layer diets. Calcium sources for poultry diets such as limestone and oyster shell, are relatively cheap and not constrained by diet costs (Bedford and Rousseau, 2017). Because Ca is inexpensive, it is commonly over-supplemented in monogastric diets (Walk, 2016). For example, high Ca levels are used in layer diets, ranging from 4.0% to 5.0% during the laying period (Saunders-Blades et al., 2009; Jing et al., 2018a; Molnár et al., 2018; Pereira et al., 2019). Dietary Ca above 4 g/d per hen may require a corresponding increase in P in the diet (Bar et al., 2002) because high Ca decreases P digestibility (Pelicia et al., 2009b). Hen fed available P (**aP**)-sufficient diets containing 2.5 to 5.0% Ca in increments of 0.5% from 21 to 32 wk of age showed a significant linear increase in egg production, plasma Ca, bone breaking strength (**BBS**), and bone mineral density (**BMD**) at 32 wk of age (Roland et al., 1996). However, a step-up Ca in phase feeding program (from 3.5 to 4.0, to 4.5, to 5.0, or 5.5% Ca) with adequate aP in the diet did not provide any beneficial effects on egg production, eggshell and bone quality (Keshavarz and Nakajima, 1993; An et al., 2016), and excessive Ca is excreted through the kidney (Akbari Moghaddam Kakhki et al., 2019a), suggesting that 3.5% Ca is sufficient for maintaining egg production and skeletal health. Inconsistent effects of laying hen dietary Ca perhaps indicate differences of strains, age and duration of the experiments. A more effective approach to maintain bone and eggshell quality in laying hens is to include supplemental Ca as 2/3 large particle (gradual release from the gizzard to support eggshell formation during the night) and 1/3

small particle, rather than oversupplying Ca (Guinotte and Nys, 1991; Saunders-Blades et al., 2009; Eusebio-Balcazar et al., 2018), particularly in the late production cycle (Cufadar et al., 2011). Excess dietary Ca caused enlarged kidneys with microscopic lesions caused by high concentration and quantity of Ca excretion (Guo et al., 2008). High Ca in layer diets decreases trabecular bone volume and increases trabecular bone porosity in aged hens (Bello, 2018). This may potentially increase the risk of proximal and distal metaphyseal fracture of the long bones of laying hens. Feeding high dietary Ca (4.5% or 5.5%) during the laying period elevates gut pH, which decreases amino acids availability (Beutler, 2009), and increases renal Ca accumulation, which may cause kidney dysfunction (Akbari Moghaddam Kakhki et al., 2019a). High dietary Ca also reduces the availability of P and Ca through formation of Ca-phytate or insoluble Ca-phosphate complexes (Beutler, 2009), and subsequently decreases P and Ca utilization (Pelicia et al., 2009b). Although 3.25% dietary Ca is adequate to maintain optimum egg production and eggshell quality in White Leghorn hens from 196 to 336 d of age, 3.50% dietary Ca was recommended to support bone quality (Rama Rao et al., 2003). Modern laying hens were able to maintain performance, eggshell and bone quality through 70 wk of age when fed approximately 3.50% Ca in the diet (Bello and Korver, 2019). Reduction of dietary Ca to 3.0% during peak production was sufficient to maintain performance, egg production, and eggshell quality; however, 3.0% dietary Ca decreased total bone mineral content (**BMC**) in White Leghorn hens (Pongmanee et al., 2020), and decreased tibia mechanical properties in aged hens (Cufadar et al., 2011). Conversely, Akbari Moghaddam Kakhki (2019b) reported that 3.0% Ca did not impair bone quality; however, it decreased eggshell strength and thickness in aged hens (Akbari Moghaddam Kakhki et al., 2019a). Therefore, it is likely that the actual Ca requirement of modern laying hens is probably between 3.0 to 3.5% and the recommended Ca levels of primary

breeder management guides are beyond the actual needs. This suggests that dietary Ca in layer diets can be substantially reduced to maximize eggshell and bone quality and minimize adverse effects of excessive Ca as discussed previously. One important factor contributing to the requirement of Ca in laying hens is the level of dietary aP because Ca and P metabolism is strongly interrelated and involved in many biological functions, for example, P is required for maintaining skeletal integrity and for the synthesis of nucleotides, phospholipids and energy through ATP (Keshavarz and Nakajima, 1993; Bar et al., 2002; Kebreab et al., 2009; Li et al., 2017). Other factors include Ca form and particle sizes (Lichovnikova, 2007; Cufadar et al., 2011), age and strain of birds (Keshavarz and Nakajima, 1993; Keshavarz, 1998a; b; Khanal et al., 2019), environmental condition (Roland et al., 1996), and inclusion of phytase (Bedford and Rousseau, 2017). Therefore, the requirements of Ca for layers during the rearing and laying periods need to be re-evaluated, especially with changing P recommendations.

1.6 PHOSPHORUS REQUIREMENTS IN LAYERS

The P requirements of layers have become more frequently discussed because of an increase in the cost of inorganic P (Ponnuvel et al., 2014; Wealleans et al., 2016) and the environmental concern of P excretion and pollution (Deniz et al., 2013; Wang et al., 2013; Wang et al., 2014; Jing et al., 2018a). Proper P nutrition is important to maintain a healthy and productive layer flock, support a high peak and maintain the persistency of egg production throughout the laying cycle. Non-phytate P (NPP) content of a feedstuff refers to total P minus phytate P, whereas aP refers to P that is absorbed from the diet into the body, which is a biologically available P (Applegate and Angel, 2014). Therefore, it is not appropriate to use the terms NPP and aP interchangeably (Rodehutscord, 2013). However, confusion occurs because the NRC (1984) used aP and the NRC (1994) used NPP, but the values of the requirements for

aP and NPP remain the same between two revisions. Although aP is not equivalent to NPP, it is usually assumed that aP and NPP values are very close to each other in order to make comparison among research studies. Leghorn pullets require 0.40, 0.35, 0.30, and 0.32% NPP from 0 to 6, 6 to 12, 12 to 18, and 18 wk of age to the first egg, respectively (NRC, 1994). However, a step-down of dietary NPP to 0.20, 0.15, and 0.10% for 0 to 6, 6 to 12, and 12 to 18 wk of age, respectively, was sufficient to support pullet performance (Keshavarz, 2000), indicating that the NRC (1994) requirement for aP is excessive. However, feeding a 0.13% aP diet from 1 d to 18 wk of age decreased pullet BW, BMD, and BMC (Punna and Roland, 1997). The last updated version of the NRC nutrient specifications for poultry was published in 1994, and the research upon which the recommendations were based may no longer be suitable for modern layers (Applegate and Angel, 2014; Bain et al., 2016). The aP specifications in current primary breeder management guides are higher than the NRC (1994) recommendations, to provide a considerable safety margin. For example, the Hy-Line International (2020) recommendations for dietary aP are 0.50, 0.49, 0.47, 0.45, and 0.48% for 0 to 3, 3 to 6, 6 to 12, 12 to 15, and 15 to 17 wk, respectively. Lohmann Tierzucht (2019) recommends 0.48, 0.45, 0.37, and 0.45% aP in layer pullet diets for 1 to 3, 4 to 8, 9 to 16, and 17 wk to 5% egg production, respectively. Commercial recommendations provide excess aP in layer diets (Applegate and Angel, 2014). Pullets are able to adapt to moderate reductions of dietary aP, from 0.50, 0.475, and 0.45% to 0.20, 0.175, and 0.15% from 0 to 4, 4 to 8, and 8 to 16 wk of age, respectively, maintaining growth without compromising pullet performance and health. There were no benefits to increasing dietary aP above 0.20% on pullet performance and bone quality (Jing et al., 2018b).

The dietary aP requirement is 250 mg/d per hen for white- (feed intake 100 g/d per hen) and brown-egg-laying hens (feed intake 110 g/d per hen), respectively (NRC, 1994). Phosphorus requirements for laying hens are approximately equal each day for maintainting bone quality and for the synthesis of egg yolk (Kebreab et al., 2009), but only a very small amount of P is required for eggshell formation (Angel, 2007). Evidently, high or low dietary P had negative impact on eggshell quality in laying hens. Dietary aP at 470, 525 or 760 mg/d per hen (only total P was reported; aP was estimated based on the assumption of 1/3 aP relative to total P) in adequate Ca in the diets decreased egg specific gravity (SG), possibly due to high dietary P reducing the amount of Ca that was mobilized from the bone to support eggshell formation (Miles and Harms, 1982; Miles et al., 1983). Approximately 167 mg aP/d per hen increased thin and cracked eggshell compared to hens fed 133 mg aP/d per hen (Vandepopuliere and Lyons, 1992). These authors also showed that 233 mg aP/d per hen decreased egg specific gravity relative to hens fed 133, 167 or 200 mg aP/d. However, some studies showed that consuming 216 mg aP/d per hen maintained egg production, egg mass and egg SG of white-egg-laying hens (Keshavarz and Nakajima, 1993; Keshavarz, 1998b) or even 167 mg aP/d per hen (Punna and Roland, 1999). Hens showed signs of P deficiency and high mortality when fed 76 mg aP/d per hen (Punna and Roland, 1999). However, research in the 2000s demonstrated that in adequate-Ca diets, hen performance, egg production, egg mass, and eggshell and bone quality decreased when hens were fed 162 (Hughes et al., 2008; 2009), 133 (Persia et al., 2003), or approximately 120 mg aP/d per hen (Francesch et al., 2005; Bello et al., 2020). Between 168 to 244 mg aP/d per hen was sufficient to support performance, egg production, and bone quality of modern laying hens (Meyer and Parsons, 2011; Jing et al., 2018a; Bello and Korver, 2019). The aP requirements in modern layers during the laying period (with a phase feeding program) are not clearly defined,

and inconsistent results may involve the strain of birds, age, dietary Ca levels, and duration of the experiment. It is assumed that the actual requirement for dietary aP in laying hens is less than the NRC (1994) recommendation, and is likely between 168 and 244 mg aP/d per hen (Persia et al., 2003; Francesch et al., 2005; Hughes et al., 2008; 2009; Meyer and Parsons, 2011; Jing et al., 2018a; Bello and Korver, 2019; Chowdhury and Koh, 2019; Fernandez et al., 2019; Bello et al., 2020). This also indicates that the aP levels recommended by the management guide are likely overestimated. However, the aP levels tested in laying hens mentioned in the above studies were conducted over portions of the production cycle, and therefore may not reflect the life cycle requirements for aP. Many factors influence the requirements of P such as dietary Ca level (Neijat et al., 2011; Bello and Korver, 2019), Ca to P ratio (Scott et al., 1999; 2000; Bar et al., 2002; Selle et al., 2009), housing type (Fernandez et al., 2019) or phytase (Meyer and Parsons, 2011; Ahmadi and Rodehutscord, 2012; Ekmay et al., 2012; Bougouin et al., 2014). Therefore, further studies are warranted to determine the actual dietary P requirement for modern layers on productivity, eggshell quality, and bone health throughout the entire production cycle from the hatch. Knowing the actual requirement of aP would be beneficial to producers to manage inorganic P and phytase supplementation in layer diets. Phosphorus liberated by phytase makes use of P that are already in feed ingredients, and reduces the need for dietary inorganic P supplementation. This is an effective way to maintain pullet performance and hen productivity, decrease diet costs and P pollution, and also allow phytase to be used most effectively.

1.7 BONE BIOLOGY AND METABOLISM IN LAYERS

Bone is a dynamic tissue complex of organic (collagen, non-collagenous proteins, and lipids) and inorganic (nanocrystalline carbonate apatite) materials (Sanchez-Rodriguez et al., 2019). Bone provides structural support, protects internal organs, and supports eggshell

formation in laying hens. Bone formation and mineralization of chickens begins during the late embryonic stage (Yair et al., 2012; Li et al., 2014; Kerschnitzki et al., 2016), and becomes fully developed during the pullet rearing phase (Fleming et al., 1998b; Whitehead, 2004; de Matos, 2008). Longitudinal bone growth occurs as the pullet grows. The length of the tibia, for example, is approximately 3 cm at d 4 (van der Pol et al., 2015), 5 to 6 cm at 2 wk (Yaissle and Lilburn, 1998), and increases to 12 cm at 18 wk of age (Anderson and Adams, 1994). In the long bones, cartilage cells (i.e. chondrocytes), osteoblasts, and osteoclasts are responsive for bone formation, modeling, and remodeling. Resting chondrocytes start to differentiate into proliferative chondrocytes. Synthesis of the typical extracellular matrix such as type II collagen forms columns of flattish cells, which subsequently differentiate into a hypertrophic stage and become enlarged (Whitehead, 2004). Osteoblasts, bone-forming cells, produce an organic matrix consisting of type I collagen, non-collagenous proteins, and lipids, and also accumulate Ca^{2+} and PO_4^{3-} ions, resulting in the formation of hydroxyapatite crystal (Kerschnitzki et al., 2014). In the meantime, osteoclasts, bone-resorbing cells, resorb bone, and remodeling (osteoclastic bone resorption coupled with osteoblastic bone formation) occurs, resulting in the development of the irregular network of trabecular bone tissues (Pines and Hurwitz, 1991; Whitehead, 2004). A dynamic process of continuing proliferation of chondrocytes of the head of growth plates at the ends of the long bones, followed by hypertrophy, enlargement, mineralization, and resorption of the trabecular network at the rear, allows the bone to elongate (Whitehead, 2004). The long bones also expand in diameter as the pullet grows (Akbari Moghaddam Kakhki et al., 2019c), for example, tibia diameter is about 1.9 mm at 4 d post-hatch (van der Pol et al., 2015) and increases to 6.2 mm at 16 wk of age (Regmi et al., 2015). Osteoblasts produce spicules of bone in the perichondrium and also deposit new bone at the periosteal surface, whereas osteoclasts resorb

bone within trabecular space at the endosteum (Whitehead, 2004), increasing the size of the trabecular cavity (Fleming et al., 2006). Expansion of pullet long bone diameter occurs very quickly, along with the formation of a pneumatized internal cavity from approximately 3 to 7 wk of age (Hogg, 1984; Whitehead, 2004; Harash et al., 2020). Pneumatized bones are an adaptation of birds to minimize the energy required for flight (Dumont, 2010; Sullivan et al., 2017). Bone widening leaves pores as osteoblasts do not have time to fill minerals – the bone is resorbed from these pores, and re-deposited on the periosteal surface (Riddell, 1992; Anderson and Adams, 1994; Yaissle and Lilburn, 1998; Fleming et al., 2006; Regmi et al., 2015; Khanal et al., 2019). At 10 to 14 d prior to the onset of lay, hen long bones rapidly increase in diameter by about 10 to 20% (Hurwitz, 1964; Taylor and Dacke, 1984; Singh et al., 1986; Riddell, 1992). For example, tibia bone cross-sectional area increased by approximately 12 and 25% in Lohmann Brown-Classic and Bovans White, respectively, from 13 to 18 wk of age (Eusebio-Balcazar et al., 2018).

In actively laying hens, there are three different types of bone tissues, including cortical, trabecular, and medullary bone in the long bones, sternum, rib, pubis, and scapula (Taylor and Moore, 1953). However, only cortical and trabecular bone tissues are present during the pullet rearing period (Figure 1.3). Cortical bone is the outer shell of the bones and has a high mineral concentration because minerals in cortical bone tissues are highly organized with high mineral crystallinity and thick and long mineral particles (Kerschnitzki et al., 2014). Trabecular bone is the inner structural component with less BMD and BMC than cortical bone (Casey-Trott et al., 2017b; Bello, 2018). Trabecular bone forms struts within the trabecular space (Whitehead, 2004), and provides structural support within the cortical bone (Reich and Gefen, 2006). As the pullet approaches sexual maturity, circulating estrogen levels increase (Beck and Hansen, 2004), resulting in growth plate closure and the cessation of long bone growth in terms of length (Pines

and Hurwitz, 1991). At this point, structural bone tissues reach their maximum extent (Fleming et al., 1998b). Osteoblasts stop forming structural bone, but begin to produce a woven bone called medullary bone (Whitehead, 2004). Medullary bone is a unique form of labile bone that acts as a reservoir of Ca for the demands of eggshell formation during the active laying period (Dacke et al., 1993; Kerschnitzki et al., 2014). Medullary bone begins to form at 10 to 14 d before the onset of sexual maturity (Hurwitz, 1964; Taylor and Dacke, 1984; Singh et al., 1986). Deng et al. (2010) reported that medullary bone began to be deposited by pre-lay pullets at 16 wk of age, and was abundant by 17 wk of age. The amount of medullary bone builds up rapidly during the early stage of the production cycle (Hurwitz, 1964; Cransberg et al., 2001), and accumulates over time through the end of the production cycle (Cransberg et al., 2001; Whitehead, 2004). Medullary bone appears as spicules on the structural bone surfaces within the medullary cavities of long bones such as humerus, femur, and tibia (Whitehead, 2004; Kerschnitzki et al., 2014), but also in the sternum, rib, pubis, and scapula (Taylor and Moore, 1953). Medullary bone has less organized mineral particles, and lower mineral crystallinity than the cortical bone (Kerschnitzki et al., 2014).

The skeleton plays a vital role in egg production by providing a source of Ca for eggshell formation when dietary Ca is limiting. Bone accretion and resorption occurs continuously during the approximately 24 h oviposition cycle. After oviposition, the ovulated follicle travels from the ovary to the infundibulum, magnum and isthmus as the outer layer of vitelline membrane is formed, the albumen (thick and thin egg white) and the inner and outer eggshell membranes are formed. These processes take approximately 5 to 6 h (Hincke et al., 2012), and calcium demands are very low because an eggshell is not being formed. Medullary bone tissues begin to accrete immediately after oviposition, when there is no eggshell being formed, and build up rapidly

during the early stages of egg formation, typically after the start of the photoperiod. If hens are fed large-particle Ca that is retained in the gizzard, they will also receive dietary Ca through the entire night (Kerschnitzki et al., 2014). By the end of this period, medullary bone has been replenished (Kerschnitzki et al., 2014). Approximately 6 h after oviposition, the forming egg enters to the eggshell gland and eggshell mineralization begins (Nys et al., 2004; Hincke et al., 2012; Marie et al., 2015) and blood ionic Ca reaches a peak (Kerschnitzki et al., 2014). Eggshell calcification occurs progressively, and the entire process of eggshell formation lasts about 17 h (Hincke et al., 2012). In the meantime, medullary bone volume decreases, indicating that hens mobilize Ca from medullary bone and transport to the oviduct to form Ca carbonate eggshell at the later stages of the oviposition cycle (Kerschnitzki et al., 2014).

1.8 ASSESSMENT OF BONE QUALITY IN LAYING HENS

Bone health plays an important role in eggshell formation (Kerschnitzki et al., 2014; Manangi et al., 2018). Laying hens use bone Ca to support eggshell formation when fed low dietary Ca and aP, meaning that eggshell quality may not be a good predictor of bone quality at a specific point in time. Hens maintained good eggshell quality at the expense of bone quality when fed Ca- and aP-reduced diets (Hughes et al., 2009; Cufadar et al., 2011; Bello et al., 2020; Pongmanee et al., 2020). Therefore, assessment of bone quality, along with monitoring egg production and eggshell quality measurement in laying hens will be useful in the long-term. This will also allow researchers to understand the relationship between bone metabolism, skeletal health and eggshell quality.

Assessments of bone mineralization can involve both invasive and non-invasive methods. Bone ash and BBS measurements are simple, fast and less expensive whereas bone quantitative tomography (**QCT**) allows the birds to be kept alive to follow individual hens throughout the

entire production cycle. However, each invasive and non-invasive method has advantages and limitations, and one method cannot replace another. Therefore, assessment of bone quality using invasive methods in conjunction with non-invasive techniques will be useful to provide a more comprehensive picture of bone metabolism and mineralization throughout the production cycle.

1.8.1 Invasive Methods

Invasive methods such as dried bone weight, bone ash content, bone-specific mineral content (such as P and Ca) by chemical analysis, and BBS, have been widely used to determine bone quality in layers (Hughes et al., 2009; Lei et al., 2011; Casey-Trott et al., 2017a; Bello and Korver, 2019; Neijat et al., 2019; Pereira et al., 2019; Robison and Karcher, 2019). Although bone ash and specific mineral content analyses are simple and can be done quickly, those measurements provide little information on changes in bone integrity over time in individual birds and do not provide information on the amount and structure of the cortical or trabecular tissue. This limits the interpretation of bone breaking strength and osteoporosis. Bone breaking strength is a mechanical property and represents the force required to break a long bone. Measurement of BBS is one of the most accurate methods to assess the resistance of bone fracture (Kim et al., 2004). Bone breaking strength can be assessed by either a three-point or four-point bending test. Three-point BBS is tested with a static load cell to apply a force to the mid-point of the long bone placed on two-fixed point supports (Min et al., 2019). A four-point bending test is assessed with an application of the perpendicular force at two points along the bone length and is commonly used to assess bone strength in large animals such as the dog, pig, or sheep (Stürmer et al., 2006). Three-point BBS testing specifically assesses bone fracture resistance at the mid-diaphysis, which is concentrated with cortical and medullary bone tissues (Cufadar et al., 2011), but does not provide information on bone fracture resistance at the

proximal and distal metaphyses, which are more susceptible to fracture compared to at the mid-bone region because at the proximal and distal ends are more concentrated with trabecular bone tissue (Reich and Gefen, 2006). Much of the current knowledge of bone quality in laying hens has been obtained from invasive methods, but with some limitations of each assessment may limit the ability of researchers to interpret the effect of dietary treatment on bone mineralization in laying hens.

1.8.2 Non-invasive Methods

Non-invasive techniques have been used to determine bone quality in poultry such as digitized fluoroscopy (**DF**), quantitative ultrasound (**QUS**), amplitude-dependent speed of sound (**Ad-SoS**), Dual-energy X-ray absorptiometry (**DEXA**), QCT, and micro-computed tomography (**µCT**). Digitized fluoroscopy is a low-cost technique to determine radiographic density in vivo, uses digitized video from an image intensification system in fluoroscopy mode with computerized analysis of the data (Fleming et al., 2000). Digitized fluoroscopy was used to assess bone quality in pullets and laying hens (Fleming et al., 2000; Fleming et al., 2004; Pereira et al., 2019). This equipment allows the operator to digitize and analyze many images in a short period time and needs much less specialized equipment than DEXA or QCT (Fleming et al., 2000). However, there is a risk of X-ray exposure from DF and this equipment is also too bulky which may be inconvenient to use on farms (Fleming et al., 2004). Quantitative ultrasound is a radiation-free, low-cost, and portable technique for the assessment of bone fracture risk and osteoporosis in humans (Gonnelli et al., 2005; Hans and Baim, 2017; Olszynski et al., 2020). Non-invasive ultrasound devices such as DBM Sonic 1200 and Bone Profiler measures the Ad-SoS (in m/s) at the phalanges in humans (Gonnelli et al., 2005). This technique utilizes sound wave (1.25 MHz) with the speed of sound to define the time from sound wave emission to its

detection (Olszynski et al., 2020). In laying hens, the DBM Sonic 1200 provided a signal of sufficient amplitude when applied to the distal region of the first phalanx of the third toe (Fleming et al., 2004). However, in osteoporotic bone, the amplitude is not strong enough to trigger a reading (Fleming et al., 2004), which may confound the results and this technique may not useful compared to DEXA or QCT. Dual-energy X-ray absorptiometry is one of the non-invasive tools to determine bone skeletal integrity of live birds and has proven useful in bone quality assessment in laying hens (Schreiweis et al., 2003; Hester et al., 2004; Schreiweis et al., 2005) and female breeders (Schallier et al., 2019). In DEXA, photons are produced by an X-ray generator at 2 energy levels, the beam is passed through the bone and to generate BMD values (Hester et al., 2004). The QCT and μCT are imaging techniques based on radiation absorption, which is valuable for assessing bone architecture, volumetric bone mineral density and cross-sectional area (Wu et al., 2015; Christiansen, 2016; Donko et al., 2018; Chen and Kim, 2020). The QCT and μCT assessment employs a system in which an x-ray is passed through the bone at multiple angles within a single plane to create a series of 2-dimensional images, post-processed with the imaging software to reconstruct into 3-dimensional models of bone volume and BMD (Korver, 2004; Korver et al., 2004; Bouxsein et al., 2010; Jones et al., 2010; Wu et al., 2015; Christiansen, 2016; Chen and Kim, 2020). The limitations of DF, QUS, Ad-SoS, and DEXA include the inability to distinguish total, cortical, and bone in the trabecular space (i.e. medullary bone vs. trabecular bone). Conversely, total, cortical, and trabecular space BMD and cross-sectional areas of long bones in White Leghorn pre-lay pullets (Regmi et al., 2015), in hens during laying period (Korver et al., 2004; Regmi et al., 2016; Robison and Karcher, 2019), and in turkeys (Van Wyhe et al., 2014) can be measured using QCT. In addition to the assessment of long bones, QCT can be used to determine keel bone damage in laying hens (Casey-Trott et al.,

2015). Due to the limitation of the current QCT to distinguish between trabecular and the medullary bone (Korver et al., 2004), bone tissues in the trabecular space are assumed to include both trabecular and medullary bone during the laying period (Whitehead and Fleming, 2000). In addition to BMD and bone cross-sectional area from the QCT analysis, BMC can also be calculated by BMD multiply by the bone cross-sectional area (Saunders-Blades et al., 2009). Therefore, BMC indicates the amount of bone mineral in mg contained in a 1 mm thick linear section of the scanned region of the bone, taking into account both bone density and area. The μCT provides higher resolution measures of the cortical, trabecular and medullary bone tissues compared to QCT (Wu et al., 2015; Chen and Kim, 2020). The μCT system can determine numerous measures such as average cortical thickness, cortical area fraction, pore number, total pore volume, bone volume fraction, trabecular number, trabecular thickness, or trabecular separation (Bouxsein et al., 2010; Chen and Kim, 2020). Some measures from the μCT can also be used to predict bone stiffness and failure (Wu et al., 2015). With the advantages of μCT to distinguish structural bone from non-structural bone tissue, this technology allows researchers to assess bone mineralization in laying hens. The μCT was used to predict mechanical properties; bending bone stiffness and failure moment of humerus and tibia in aged hens (Vaughan et al., 2016). Rapid changes of degree of mineralization and mineral organization of medullary bone during the daily egg-laying cycle were determined by the μCT (Kerschnitzki et al., 2014). The μCT was also used to investigate femur bone growth during the late embryonic development of chicken (Kerschnitzki et al., 2016). Both QCT and μCT are suitable for in vivo and ex vivo applications in laying hens. Bone quality data determined by QCT and μCT can be used to correlate with productivity, eggshell quality or even invasive measurements such as bone ash and BBS, than invasive methods alone. Non-invasive techniques can be used on live birds, changes

in bone mineralization of the cortical and bone in trabecular space tissues in individual birds can be more closely monitored (Korver, 2004; Korver et al., 2004).

1.9 PHYTATE IN POULTRY FEED INGREDIENTS

1.9.1 Phytate

Phytate is the mixed salt of phytic acid (*myo*-inositol hexaphosphate; IP_6; Figure 1.4A) and minerals (Figure 1.4B; Erdman, 1979; Maga, 1982; Selle and Ravindran, 2007; Humer et al., 2015) or other nutrients (Humer et al., 2015). Phytate is the storage form of P (inositol ring with 6 phosphate groups) in plant-sourced feed ingredients such as corn, soybean meal, wheat, barley, oats, sorghum, sunflower meal, and rice bran (Kasim and Edwards, 1998; Selle and Ravindran, 2007; Sanz-Penella et al., 2012; Hirvonen et al., 2019). For example, corn and soybean contain phytate P as approximately 45 to 78% of total P (NRC, 1994; Viveros et al., 2000) whereas cereals like oats, wheat, barley, triticale, rye, contain phytate P from 63 to 73% of total P (Steiner et al., 2007; Madsen and Brinch-Pedersen, 2019). Phytate is not well utilized by poultry as a source of P because of limited endogenous phytase in the digestive tract (Maenz and Classen, 1998). The pH conditions have a major impact on phytate solubility. At the low pH in the proventriculus and gizzard ($pH < 5$), weak binding between minerals or other molecules and phytate was observed (Reddy et al., 1982). When the pH increases, in particular in the small intestine ($pH > 6$), strong binding occurs, resulting in poor solubility of phytate molecules (Reddy et al., 1982). Due to the chelation of phytate with other minerals and interactions with amino acids as well as carbohydrates, nutrient availability and utilization are reduced in chickens (Selle et al., 2000; Selle et al., 2009). Phytate also increased endogenous Na loss, due to impaired activity of Na-K-ATPase in enterocyte cells of the small intestine, reducing nutrient absorption in chickens (Cowieson et al., 2004; Liu et al., 2008). Although phytate is a heat-stable molecule,

it can be degraded by thermal treatment above 95˚C (Bullock et al., 1993). For example, 9% of phytate was degraded when soybeans were boiled for 1 h (Schlemmer et al., 1995). Extrusion (130 to 140˚C, 6.5 MPa) can reduce total phytate by approximately 8 and 15% in corn and wheat, respectively (Pontoppidan et al., 2007).

1.9.1.1 Ca-phytate Complex

Phytate can chelate with different divalent minerals and form a salt complex (Maenz et al., 1999; Selle et al., 2009; Humer et al., 2015). The mineral-phytate complex can be either soluble or insoluble in the gastrointestinal tract, depending on gut pH. Weak binding of the mineral-phytate complex exists at low pH (< 4) whereas a strong binding complex between mineral and phytate occurs at neutral and basic pH (Maenz et al., 1999). A de novo complex of phytate and cations forms at neutral pH or above, such as in the small intestine; cations form weak chelates within a single phosphate group or strong chelates when form binding with two phosphate groups of the phytate molecule (Erdman, 1979; Humer et al., 2015). Cation binding between two phosphate groups may occur in one or two molecules of phytic acid (Cheryan, 1980). The rank of affinity for divalent or trivalent cations binding with phytate was $Zn^{2+} > Fe^{2+} > Mn^{2+} > Fe^{3+} > Ca^{2+} > Mg^{2+}$ at pH 7 (Maenz et al., 1999). The formation of insoluble mineral-phytate complexes in the small intestine reduces the absorption of minerals. Dietary Ca levels are high in layer diets, and the formation of Ca-phytate complexes along the lower gastrointestinal tract occurs (Tamim et al., 2004; Beutler, 2009; Hamdi et al., 2015; Sommerfeld et al., 2018). Additionally, the limestone commonly used as a source of Ca in poultry diets has a high acid-binding capacity, which increases gut pH. This increases the formation of insoluble Ca-phytate complexes. Fine particle size limestone has high solubility and elevates gut pH quickly, this also decreases Ca digestibility due to formation of Ca-phytate complexes (Kim et al., 2018).

1.9.1.2 Binary and Ternary Phytate Complexes

Binary phytate complexes refer to proteins chelated with phytate molecules (Figure 1.4C). Anionic phytate molecules have the potential to bind a cationic group of amino acids such as lysine, arginine, and histidine (Cheryan, 1980). The binary protein-phytate complexes occur at acidic pH in the proventriculus and gizzard (Selle et al., 2000). A ternary phytate complex is the chelation of phytate, mineral, and protein. A mineral cation (such as Ca^{2+}, Mg^{2+}, or Zn^{2+}) forms a bridge between phytate and protein (Figure 1.4D; Humer et al., 2015). Ternary phytate complexes occur naturally at alkaline pH (Reddy et al., 1982; Humer et al., 2015). The formation of binary and ternary phytate complexes in the gastrointestinal tract changes protein structure and decreases protein solubility, pepsin and proteolytic enzyme activities, which in turn reduces the digestibility of amino acids (Selle et al., 2000).

1.10 PHYTASE

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) is phosphohydrolytic enzyme from four possible sources of phytase, including intrinsic plant phytase, endogenous mucosal phytase, gut microfloral phytase, and exogenous microbial phytase (Selle et al., 2009; Humer et al., 2015). Phytase initiates the stepwise liberation of phosphate from the phytate molecule (Wyss et al., 1999; Yu et al., 2012; Lei et al., 2013). Phytase first hydrolyzes a complete IP_6, which contains six phosphate groups, to penta-esters of inositol (IP_5) before hydrolyzing the latter to tetra-esters of inositol (IP_4), and then sequentially to IP_3, IP_2, and IP_1 (Wyss et al., 1999), yielding one inositol and six phosphates. Phytase activity in feed is expressed in phytase units (FTU); one FTU is the enzyme activity that liberates 1 μmol inorganic orthophosphate per minute from sodium phytate at pH 5.5 and 37˚C (Engelen et al., 1994), and is usually expressed on a per kg diet basis. However, phytase activity in digesta can be defined as a unit (U); one unit

is the enzyme activity that produces 1 nmol of inorganic P per minute at 50˚C, and expressed on a per mg protein of intestinal digesta (Haros et al., 2005; Raghavendra and Halami, 2009).

1.10.1 Intrinsic Plant Phytase

Intrinsic phytase is found in plants and has the ability to degrade phytate. Intrinsic phytase activity varies among plant species. Rye, triticale, wheat and barley contain high intrinsic phytase activity, and cereal grains contain higher phytase activity than legumes (Steiner et al., 2007). Since phytases are located in the aleurone layer and scutellum in cereal grains, rye bran and wheat bran have the highest level of phytase activity among plant feedstuffs (Steiner et al., 2007; Humer et al., 2015). However, intrinsic plant phytase is heat-labile and can be destroyed during high temperatures feed processing such as pelleting (Jongbloed and Kemme, 1990). In corn-soy, wheat-soy or corn-soy-canola-based layer diets, intrinsic plant phytase activity was below 50 FTU/kg (Gao et al., 2013b; Taylor et al., 2018; Pongmanee et al., 2020) or between 52 and 67 FTU/kg (Bello and Korver, 2019). In addition to intrinsic plant phytase, crops have been genetically modified to express high levels of phytase. For example, *Aspergillus niger* phytase has been expressed in corn endosperm (Chen et al., 2008) and *A. japonicus* phytase gene has been expressed in wheat endosperm (Abid et al., 2017). Phytase expressed in transgenic corn was as efficacious as commercial exogenous phytases in laying hen studies (Gao et al., 2013a; Gao et al., 2013b; Wang et al., 2013).

1.10.2 Endogenous Phytase

Endogenous mucosal phytase can be produced by the small intestine brush border membrane of layers (Marounek et al., 2010). The amount and activity of intestinal endogenous phytase increases with hen age (Marounek et al., 2010) due to a greater small intestine mucosal surface area (Morgan et al., 2015). Mucosal phytase activity is highest in the duodenum and

decreases progressively in the jejunum and ileum (Maenz and Classen, 1998). Endogenous mucosal phytases have limited ability to dephosphorylate lower IPs, especially from IP3 and lower (Zeller et al., 2015a). The optimal pH for endogenous phytase is between 5.5 and 6.5, and marginal phytase activity occurs at a pH between 7 and 11 (Maenz and Classen, 1998). Endogenous phytase activity decreases when diets are supplemented with exogenous phytase or when there is a high amount of luminal P (Huber et al., 2015; Zeller et al., 2015b). Mucosal phytase activity did not differ between Hy-Line White and Hy-Line Brown hens (Abudabos, 2012).

1.10.3 Gut Microfloral Phytase

Phytase can be produced by microflora in the crop (Dijkslag et al., 2019), lower ileum (Zeller et al., 2015b), and ceca of poultry (Marounek et al., 2010; Zeller et al., 2015a). Phytase can be produced by *Lactobacillus salivarius* and *L. taiwanensis*, which is often found in the crop and ileum of the chicken (Witzig et al., 2015; Borda-Molina et al., 2018; Künzel et al., 2019). Lactic acid bacteria such as *Pediococcus pentosaceus* isolated from chicken intestine, have phytate-degrading ability, with phytase activity ranging from 89 to 213 U (Haros et al., 2005; Raghavendra and Halami, 2009), which can be exploited as a starter culture in fermented human food to increase mineral bioavailability (Raghavendra and Halami, 2009). *Pseudomonas* spp. in the ileum can produce phytase and degrade phytate (Borda-Molina et al., 2018).

1.10.4 Exogenous Microbial Phytase

The first commercial exogenous phytase product available for livestock was derived from *A. niger* and was introduced in 1991 (Selle and Ravindran, 2007). There are currently two types of commercial exogenous phytase products; firstly, fungal 3-phytases (EC 3.1.3.8; first generation) derived from *A. niger*. Secondly, 6-phytases (EC 3.1.3.26) – bacterial phytases

(introduced beginning in 1999) derived from *Escherichia coli*, *Peniophora lycii*, *Citrobacter braakii*, or *Buttiauxella* spp. (Lei et al., 2013). The 3- and 6-phytases are named based on the site where the phytate hydrolysis is initiated (Selle and Ravindran, 2007). The 3-phytases liberate P beginning at the 3rd carbon position (Selle and Ravindran, 2007), whereas 6-phytases begin at the 6th carbon site of the phytate molecule (Lei et al., 2013). The 6-phytase products tend to be more effective and have a specific affinity for IP_6 and IP_5, which are less soluble because of a strong chelating capacity with zinc and copper (Persson et al., 1998). Additionally, 6-phytases are more resistant to proteolytic enzymes in the digestive tract than 3-phytases (Adeola and Cowieson, 2011). The removal of phosphate groups from phytate molecules by commercial phytases is not complete in chickens (Yu et al., 2012) because the phosphate at the 2nd carbon site of the inositol ring is resistant to hydrolysis by exogenous phytase (Wodzinski and Ullah, 1996). However, more current commercial phytases used in poultry diets can completely degrade some IP_6 to yield six phosphates and one inositol (Walk et al., 2014; Lee and Bedford, 2016; Gautier et al., 2018). Although some IP_6 in feedstuff can be completely dephosphorylated by exogenous phytase, lower isomer of IPs still remaine in the small intestine (Zeller et al., 2015b).

1.11 THE USE OF PHYTASE IN LAYER DIETS

Typical phytase supplementation in broiler chicken diets is 500 to 1,000 FTU/kg (Adeola and Walk, 2013; Bougouin et al., 2014; Walk et al., 2014; Beeson et al., 2017; Walk et al., 2018). However, high doses and super doses (5 to 20 times the standard level) of phytase have been used experimentally in broiler diets to increase the liberation of phosphates, amino acids, proteins and minerals from phytate (Cowieson et al., 2011; Bougouin et al., 2014; Walk et al., 2014; Beeson et al., 2017; Walk et al., 2018; Farhadi et al., 2019; Walk and Olukosi, 2019). Typical phytase inclusion levels in layer diets are lower than broiler diets, ranging from 100 to

600 FTU/kg (Punna and Roland, 1999; Scott et al., 1999; Um and Paik, 1999; Boling et al., 2000a; Boling et al., 2000b; Scott et al., 2000; Lim et al., 2003; Kozłowski and Jeroch, 2011; Abudabos, 2012; Bougouin et al., 2014; Bello and Korver, 2019; Bello et al., 2020) with limited use of super doses (Kim et al., 2017). However, high levels of inclusion of phytase, ranging from 1,200 to 1,500 FTU/kg have been used in some recent studies (Taylor et al., 2018; Pongmanee et al., 2020). Commercially, phytase supplementation levels in layer diets are generally lower (300 FTU/kg) than in the broiler diets. The longer retention time in layers may allow a lower dose of phytase to be used than in broiler diets (Selle and Ravindran, 2007). Gao et al. (2013b) reported that the highest activity of phytase occurs in the crop, followed by the proventriculus and gizzard of laying hens. Anatomically and physiologically, layers have higher relative gizzard weight and lower gizzard pH than broilers (Mtei et al., 2019), which may also promote phytate hydrolysis. Although higher doses of phytase increase phytate dephosphorylation and liberate more P and Ca relative to lower doses of phytase (Fernandez et al., 2019), inorganic P release increases up to a certain point and maintains a plateau with increasing phytase dose (Wealleans et al., 2016). High dietary Ca levels decrease phytase efficacy, and are much greater in layer diets than in broiler diets, which decreases phytate degradation (Bedford and Rousseau, 2017; Sommerfeld et al., 2018), especially in the small intestine. High dietary Ca causes high pH in the gastrointestinal tract (Nelson and Kirby, 1987), which reduced phytase activity (Van der Klis et al., 1997; Sommerfeld et al., 2018) because phytase worked best under acidic conditions, with only marginal activity in neutral and basic conditions (Maenz and Classen, 1998; Selle and Ravindran, 2007). Therefore, increasing gut pH potentially limits phytase efficacy. Also, increasing dietary Ca from 2.5 to 5.5% increased Ca-phytate complex formation in the gastrointestinal tract of laying hens (Beutler, 2009). When P and Ca liberated by phytase in the

upper gastrointestinal tract interact with the high Ca concentration from the diet and move to the higher pH of the small intestine, insoluble salts of calcium phosphate are formed (Tamim et al., 2004; Hamdi et al., 2015; Sommerfeld et al., 2018). This impaired P and Ca availability of laying hens (Bello and Korver, 2019). To evaluate the efficacy of phytase, it is necessary that the experimental diet contains sufficient phytate as a substrate for phytase to degrade, and that the Ca and P liberated by the phytase is needed by the bird. To achieve the latter condition, reductions in aP and Ca well below current commercial recommendations for layers is in the experimental diets is suggested (Bello and Korver, 2019; Pongmanee et al., 2020). In addition to the reduction of dietary aP and Ca, reduction of other nutrient specifications such as energy and protein would be possible (Scott et al., 2001; Ponnuvel et al., 2014).

1.12 IMPACT OF PHYTASE IN LAYING HENS

1.12.1 Impact of Phytase on Performance, Productivity, Eggshell and Bone Quality

The majority of phytase studies in laying hens in the past involved a reduction of only the dietary aP level. In adequate Ca diets, phytase supplementation to aP-reduced diets had positive effects in laying hens. For example, 300 to 500 FTU phytase/kg in the diets containing 0.10 to 0.13% aP increased egg weight during peak production (Boling et al., 2000a; Sari et al., 2012), increased eggshell thickness (Kozłowski and Jeroch, 2011), increased egg production and decreased mortality of hens during early to peak production (Punna and Roland, 1999). Boling et al. (2000b) reported that 100 FTU phytase/kg in diets with 0.1% aP was sufficient to maintain egg production throughout the production cycle. The addition of 500 FTU phytase/kg to a diet with 1.2 g/kg NPP restored BW, egg production, eggshell quality, and BBS relative to a diet with 3.0 g/kg NPP (Panda et al., 2005). In a long-term study, 200 FTU phytase/kg supplementation to a diet containing 0.15% aP was not sufficient to restore hen BW and egg production, but 600

FTU/kg was (Hughes et al., 2008). Phytase supplementation at 300 FTU/kg in an aP- and Ca-reduced layer diet (0.15% aP and 3.0% Ca) from 21 to 41 wk of age increased egg specific gravity and decreased broken eggs and soft eggshells (Lim et al., 2003). Supplementation of phytase at 300 FTU/kg in diets containing 0.10 to 0.14% aP and 2.5 to 3.3% Ca increased bone Ca and P, BBS, BMD, and BMC in aged hens (Gordon and Roland, 1998; Lei et al., 2011).

1.12.2 Impact of Phytase on Mineral Digestibility, Retention and Excretion

In aP-reduced layer diets, phytase supplementation between 300 and 600 FTU/kg increased apparent ileal digestibility (AID) of P (Gao et al., 2013b), and retention of P, Ca, Mg, Fe, and Cu, and decreased excreta N and P (Um and Paik, 1999; Keshavarz and Austic, 2004; Panda et al., 2005; Abudabos, 2012; Wang et al., 2013). Supplementation of 300 FTU phytase/kg to diets containing 3.0 to 3.6% Ca and 0.23 to 0.25% aP increased AID and retention of P (Lim et al., 2003; Bello and Korver, 2019). However, 600 and 1,200 FTU phytase/kg increased AID of P and Ca in hens fed 3.02% Ca and 0.19% aP in the diets (Pongmanee et al., 2020).

1.12.3 Impact of Phytase on Feed Costs

Phosphorus is a non-renewable and expensive resource (Summers, 1997; Biehl et al., 1998; Naves et al., 2016; Kazempour and Jahanian, 2017). Reducing the use of inorganic P in layer diets decreases feed costs. The cost of phytase supplementation in poultry diets is recovered by the reduction of inorganic P such as mono- or di-calcium phosphate; the savings are between USD $3 to 4.26/tonne of feed (Wealleans et al., 2016). Bello (2018) reported that using phytase in laying hen diet saves CDN $5/tonne of feed and allows producers to use canola meal or low quality feed ingredients such as oat hulls in corn-soy meal-based diets. Phytase supplementation in laying hen diets also increases net profit per egg and decreases overall costs

of production from reducing di-calcium phosphate, protein, and energy feed ingredients (Ponnuvel et al., 2014; Wang et al., 2014).

1.13 RESEARCH APPLICATION

The effects of phytase supplementation in aP-reduced laying hen diets have been well studied in short-term experiments. However, long-term phytase use in reduced aP and Ca diets from hatch throughout the end of the production cycle of modern laying hens is very limited. Unlike broiler studies, available information on high doses of phytase supplementation (beyond commercial recommendations) in layer diets is scant. Additionally, understanding bone mineralization over time with or without phytase supplementation of laying hens requires more information than can be provided traditional bone analytical methods. Although traditional bone analytical measures such as bone ash and BBS provide important information on bone mineralization and facture resistance, these methods do not provide information on structural or nonstructural bone tissues or even bone tissues in different locations of the long bone (Bello et al., 2020). Therefore, it is important to interpret bone mineralization beyond the limitation of the traditional bone analytical measurement. Bone assessment using QCT in cortical, trabecular, and medullary bone tissues in different regions of the long bones is essential to understand the effects of phytase on bone biology. This Ph.D. thesis was focused on the long-term phytase supplementation in aP- and Ca-reduced diets on productivity, eggshell and bone quality, and mineral retention in laying hens.

1.13.1 Thesis Objectives

The objectives of this Ph.D. thesis were:

1) To determine the short-term effects of phytase supplementation in diets with moderate reductions of aP and Ca on eggshell quality, bone traits, and digestibility of P and Ca of laying hens during peak production.
2) To investigate the long-term effects of phytase supplementation in diets with marginal reductions of aP and Ca on pullet performance and hen productivity, eggshell and bone quality, and P and Ca retention from one-d-old to 74 wk of age.

1.13.2 Thesis Hypotheses

The hypotheses of this Ph.D. thesis were:

1) Moderate reductions of aP and Ca in layer diets during peak production (from 25 to 37 wk of age) would decrease eggshell and bone quality, and the inclusion of phytase would alleviate the negative effects of moderate reduced dietary aP and Ca.
2) Marginal reductions of dietary aP and Ca during pullet rearing would decrease pullet performance, and phytase supplementation would restore performance to the same level of pullets fed aP- and Ca-adequate diets.
3) Marginal reductions of dietary aP and Ca in the long-term would decrease the quality of femur cortical, trabecular, and medullary bone tissues, and phytase supplementation would increase P and Ca availability to maintain bone quality at the same level of hens fed aP- and Ca-adequate diets.
4) Marginal reductions of dietary aP and Ca in the long-term would also consequently decrease eggshell quality, and that phytase supplementation would restore the adverse effects of eggshell quality to the similar level of hens fed aP- and Ca-adequate diets.

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1.15 FIGURES

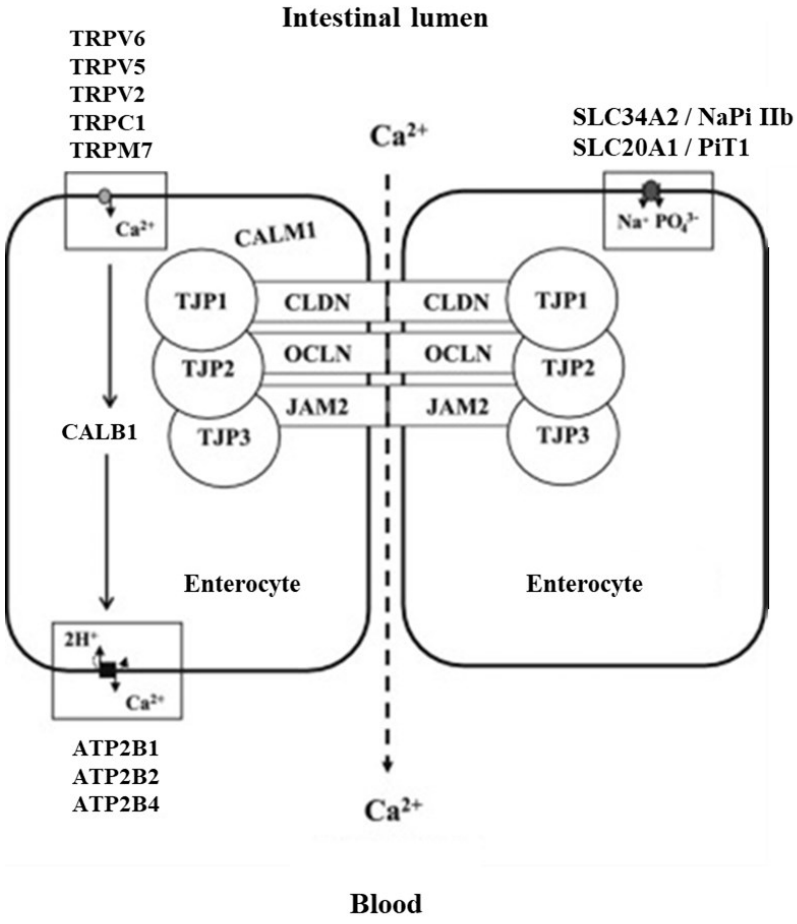

Figure 1. 1: The absorption of Ca via transcellular (black arrow) and paracellular (dotted arrow) pathways across the intestinal epithelial barrier of the laying hen. Rectangles represent the transmembrane proteins, junctional adhesion molecule (JAM), JAM2 = junctional adhesion molecule 2, claudins (CLDN), or occludin (OCLN), while the circles represent the tight junction proteins (TJP3). TJP1 = tight junction protein 1; TJP2 = tight junction protein 2; TJP3 = tight junction protein 3. TRPV6 = transient receptor potential cation channel subfamily V member 6; TRPV5 = transient receptor potential cation channel subfamily V member 5; TRPV2 = transient receptor potential cation channel subfamily V member 2; TRPC1 = transient receptor potential cation channel subfamily C member 1; TRPM7 = transient receptor potential cation channel subfamily M member 7; CALM1 = calmodulin; CALB1 = calbindin D_{28K}; ATP2B1 = ATPase plasma membrane Ca^{2+} transporting 1; ATP2B2 = ATPase plasma membrane Ca^{2+} transporting 2; ATP2B4 = ATPase plasma membrane Ca^{2+} transporting 4. SLC34A2 = solute carrier family 34 member 2 or sodium-dependent phosphate co-transporter type IIb (NaPi IIb); SLC20A1 = solute carrier family 20 member 1 or inorganic phosphate transporter 1 (PiT1; Modified from Gloux et al., 2019).

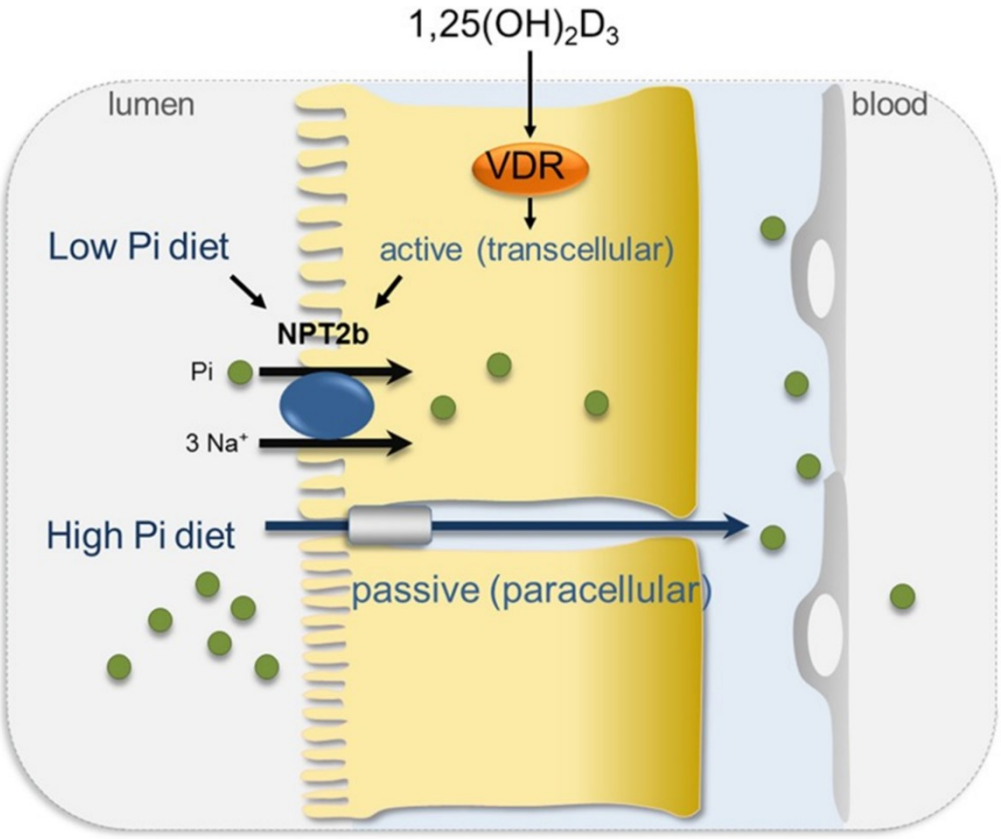

Figure 1. 2: Intestinal phosphate absorption. A large fraction of dietary phosphate intake is considered to be transported by a passive (paracellular) pathway. The active (transcellular) pathway consists of NPT2b localized at the intestinal apical brush border membrane. The expression of this transporter is increased by $1,25(OH)_2D_3$ and when dietary phosphate intake is low. NPT2b = sodium-dependent phosphate co-transporter type IIb (known as NaPi IIb or SLC34A2); VDR = vitamin D receptor (Christakos et al., 2014).

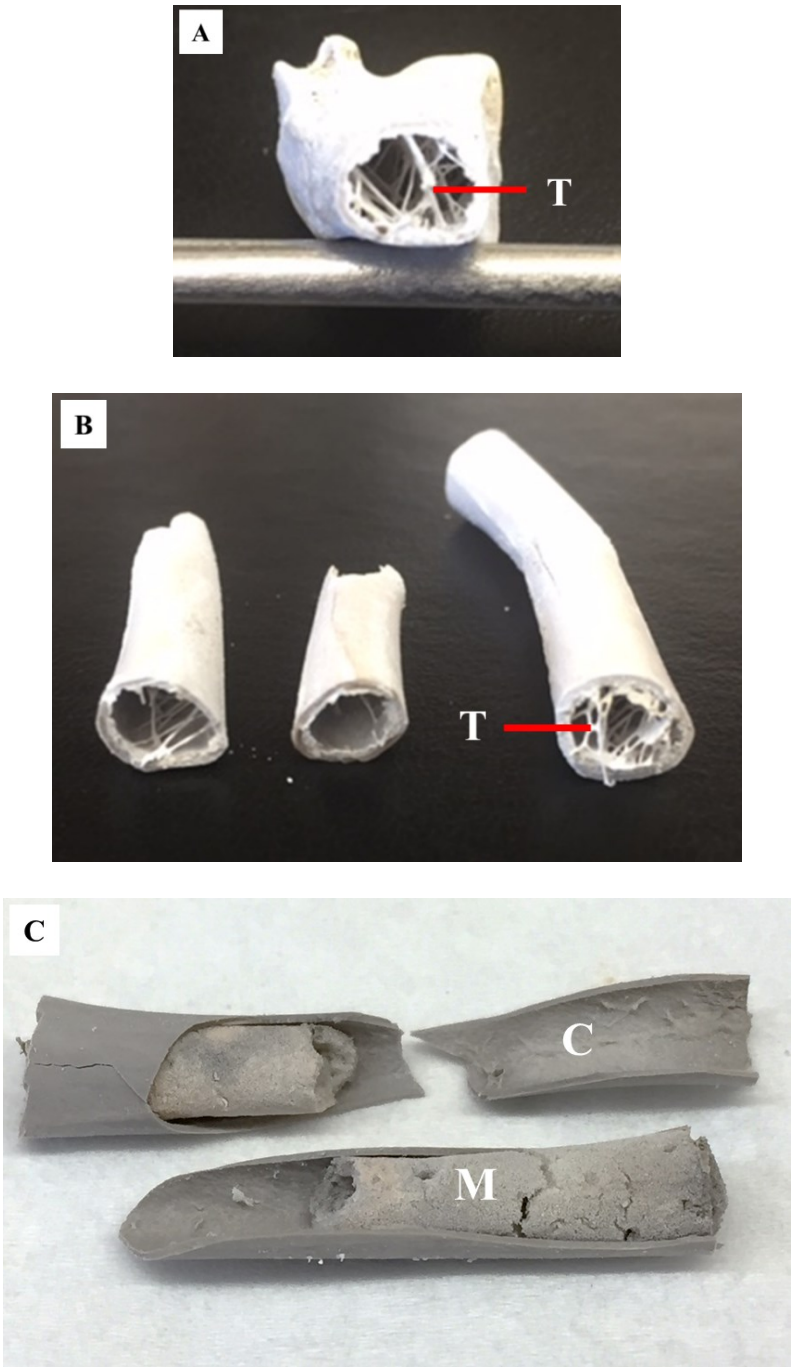

Figure 1. 3: Femur bone ash showing (A and B) trabecular network with pneumatized internal cavity at 25% proximal end of the femur bone of pullet at 6 wk of age and (C) the internal cavity filled with medullary bone at 50% mid-shaft region of the femur bone of hen at 42 wk of age. T = trabecular bone tissue; C = cortical bone; M = medullary bone.

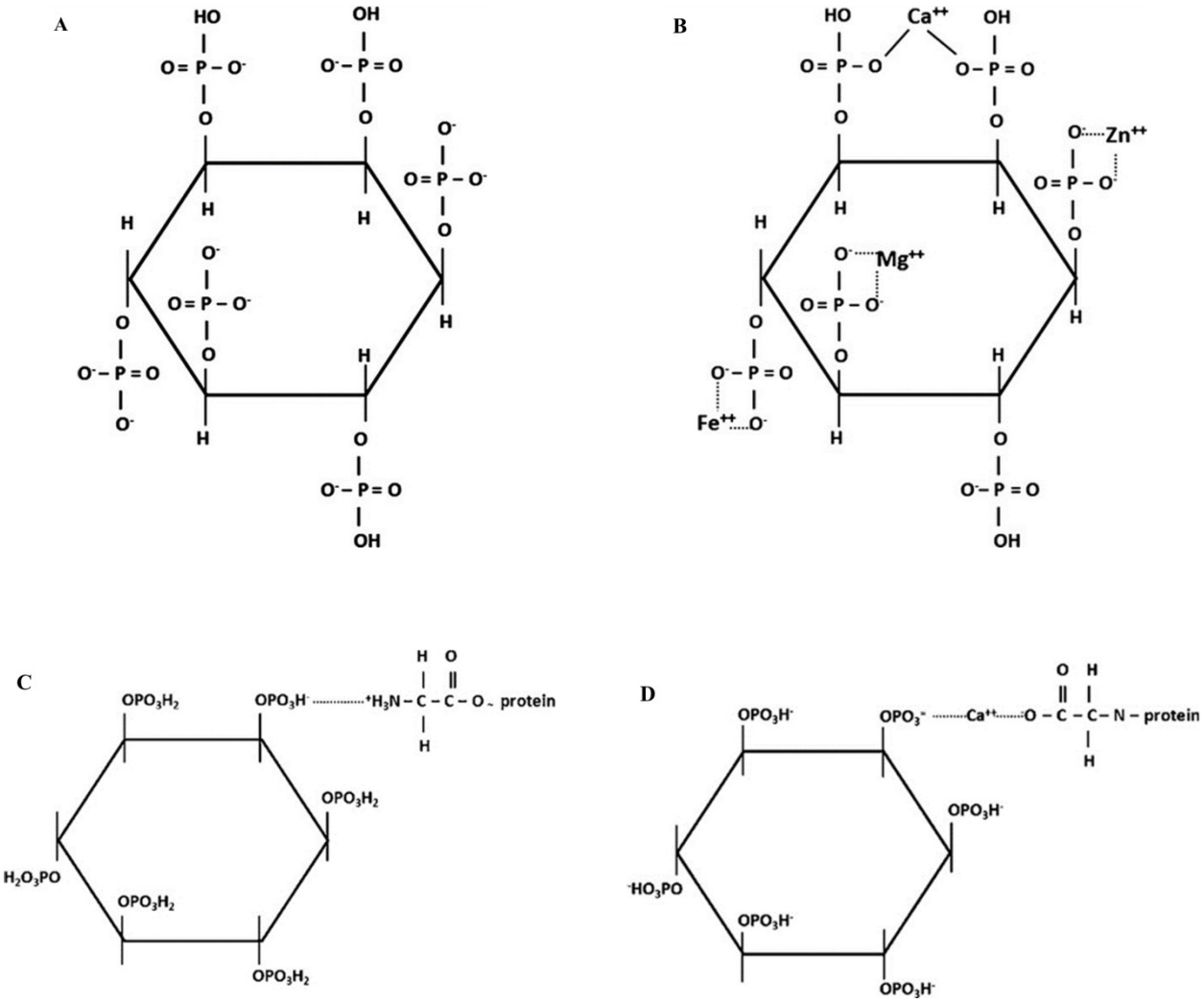

Figure 1. 4: Structures of (A) free form of phytic acid, (B) phytate-mineral complex, (C) binary phytate-protein complex, and (D) ternary phytate complex; bonds via a cationic bridge (Erdmen, 1979; Humer et al., 2015).

2. EFFECTS OF PHYTASE SUPPLEMENTATION ON EGGSHELL AND BONE QUALITY, AND PHOSPHORUS AND CALCIUM DIGESTIBILITY IN LAYING HENS FROM 25 TO 37 WEEKS OF AGE[1]

ABSTRACT

Effects of dietary available phosphorus (aP) and Ca levels, and an *Escherichia coli* 6-phytase supplementation were studied in Lohmann LSL-Lite hens from 25 to 37 wk of age. Eighty-four hens were used in a completely randomized design with 7 treatments. Treatments were: a positive control (PC) diet with 0.45% aP, 3.70% Ca and 0.16% Na from 25 to 28 wk, and 0.38% aP, 3.73% Ca and 0.15% Na from 29 to 37 wk; a negative control (NC) diet, similar to the PC diet, with 0.22% aP, 3.00% Ca and 0.13% Na from 25 to 28 wk, and 0.19% aP, 3.02% Ca and 0.13% Na from 29 to 37 wk; the NC diets supplemented with phytase at 150 (NC + 150), 300 (NC + 300), 600 (NC + 600), or 1,200 (NC + 1,200) phytase unit (FTU)/kg, respectively; and the PC diet supplemented with phytase at 1,200 (PC + 1,200) FTU/kg. Hen performance, eggshell and bone quality were measured on a 4-wk basis. Bone breaking strength (BBS) and ash, and apparent ileal digestibility (AID) of P and Ca were determined at 37 wk. One- and 2-way ANOVA were conducted, and Tukey's range test was used to compare multiple means where $P \leq 0.05$. No differences in hen performance, eggshell quality, BBS, bone ash and P digestibility were observed between the PC and the NC treatments. The NC hens had lower cortical ($P < 0.001$) and trabecular + medullary bone mineral density (BMD; $P = 0.004$), and total bone mineral content ($P < 0.001$) than the PC hens. The PC + 1,200 increased cortical BMD ($P < 0.001$). The reductions of aP and Ca in the NC diet were not deficient for performance, but

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had a minor impact on bone mineralization. The NC + 600 and NC + 1,200 increased AID of P ($P = 0.024$), and all phytase treatments except the NC + 150 increased AID of Ca ($P = 0.010$) compared to the NC diet.

Keywords: phytase, eggshell, bone, P and Ca digestibility, laying hen

2.1 INTRODUCTION

Phytate (a salt complex of myo-inositol-1,2,3,4,5,6-hexakisdihydrogenphosphate) is a P storage compound in plants (Pallauf and Rimbach, 1997) which accounts for approximately two-thirds of the total P in plant ingredients (Ravindran et al., 1995). Phytate is not well utilized by chickens as a source of P because of limited endogenous phytase in the digestive tract (Maenz and Classen, 1998) and due to P and Ca inhibition of phytate degradation at common mineral application rates (Sommerfeld et al., 2018b). Low availability of P in plant-based diets leads to inorganic P supplementation of poultry diets. Due to P being one of the most expensive nutrients in poultry diets (Naves et al., 2016), increasing inorganic P in the diet increases feed cost. Exogenous phytase has been used in poultry diets to liberate P and other minerals such as Ca, reduce P pollution in poultry excreta (Selle and Ravindran, 2007; Zyla et al., 2012), and reduce feed cost (Ponnuvel et al., 2014). Commercial phytase products differ in terms of type (3- or 6-phytase), sources (microbial source from which they are derived), characteristics (optimal pH, thermo-stability, ability to liberate P from the phytate-P complex), and catalytic and biochemical properties (Menezes-Blackburn et al., 2015). The difference in these characteristics and properties can impact the activity of phytase in the digestive tract of birds (Onyango et al., 2005). Research studies on phytase use are well documented in broilers (Hamdi et al., 2015; Gautier et al., 2018; Sommerfeld et al., 2018b; Leyva-Jimenez et al., 2019), but there has been very little work done in laying hens.

Phytase has the potential to liberate both P and Ca (Selle and Ravindran, 2007), and it is unlikely to result in a response on digestibility of these minerals if dietary requirements for available P (**aP**) and Ca are being met. Phytase supplementation in the diet containing 0.25% aP and 3.80% Ca did not cause negative effects on egg production and specific gravity (Hughes et al., 2008), ileal digestibility of protein and bone mineralization (Hughes et al., 2009). Similarly, adding phytase to diets having 0.4 or 0.3% aP with 4.0% Ca did not increase egg production, eggshell quality, and bone mineral density (Punna and Roland, 1999). Further reductions of both aP and Ca levels below the actual requirements in laying hen diets may allow us to observe the effect of phytase and better understand changes in bone mineralization in laying hens. The objective of the current study was to determine the effects of phytase supplementation in diets with substantially reduced aP and Ca on eggshell and bone quality, and ileal digestibility of P and Ca in laying hens from 25 to 37 wk of age. We hypothesized that eggshell and bone quality would decrease in hens fed a reduced aP and Ca in the negative control (**NC**) diet, and phytase supplementation would increase eggshell and bone quality to the level of the positive control (**PC**) diet.

2.2 MATERIALS AND METHODS

All animal care procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock and followed principles established by the Canadian Council on Animal Care (Canadian Council on Animal Care, 2009).

2.2.1 Animals and Housing

Eighty-four Lohmann LSL-Lite laying hens at 20 wk of age were randomly selected from the Poultry Unit flock of the University of Alberta. Each hen was weighed, wing-banded before placement and housed in individual cages in a two-tier battery (L x W; 50 x 50 cm; 43 cm high at

the front and 33 cm high at the back) located in an environmentally controlled facility. Each replicate cage was equipped with one external feed trough (L x W x D; 50 x 15 x 10 cm), two automatic water nipples, and external egg tray receiver (L x W x H; 50 x 12 x 2.5 cm). During the pre-experimental period from 20 to 25 wk of age, all the hens were fed the same diet. At 25 wk of age, hens were weighed and randomly assigned to 1 of the 7 dietary treatments with 12 replicate cages of 1 hen per treatment in a completely randomized design. Standard management practices were followed according to the primary breeder management guide (Lohmann Tierzucht, 2012). The photoperiod was 16:8D, and the room temperature was maintained at approximately 21°C throughout the experiment. Birds were checked, and eggs collected and recorded twice daily. Feed and water were provided ad libitum throughout the experiment. The experimental period lasted 12 wk from 25 to 37 wk of age.

2.2.2 Experimental Diets

The corn-soy-canola meal-based experimental diets provided approximately 2,800 kcal/kg ME (Table 2.1). Seven experimental diets were fed as mash in two dietary phases: start-lay diets from 25 to 28 wk of age and layer phase I diets from 29 to 37 wk of age. Diet 1 was a PC diet with 0.45% aP, 3.70% Ca and 0.16% Na from 25 to 28 wk of age, and 0.38% aP, 3.73% Ca and 0.15% Na from 29 to 37 wk of age. The PC diet was formulated to meet or exceed nutrient recommendations (Lohmann Tierzucht, 2012). Diet 2 was a NC, diet similar to the PC diet but reduced to 0.22% aP, 3.00% Ca and Na 0.13% from 25 to 28 wk of age and to 0.19% aP, 3.02% Ca and 0.13% Na from 29 to 37 wk of age. The reduction level of aP and Ca in the NC diet in this study were intended to provide aP and Ca levels below the respective requirements. Diets 3, 4, 5, and 6 were the NC diets supplemented with phytase at 150 (**NC + 150**), 300 (**NC + 300**), 600 (**NC + 600**), and 1,200 (**NC + 1,200**) phytase unit (**FTU**)/kg, respectively. Diet 7 was

the PC diet supplemented with phytase at 1,200 FTU/kg (**PC + 1,200**). Since 300 FTU phytase/kg is the common commercial level used in diets for laying hens, the NC + 600, the NC + 1,200 and the PC + 1,200 were included to investigate high dose effects of phytase. To observe an extra-phosphoric effect, the highest dose of 1,200 FTU/kg was also supplemented to the PC diet. The phytase used in this study was a thermotolerant, enhanced *E. coli* 6-phytase produced in *Trichoderma reesei* (Quantum Blue, AB Vista; Marlborough, UK). Phytase activity is reported in FTU; one FTU is the amount of enzyme that liberates 1 μmol of inorganic orthophosphate per minute from sodium phytate at 37°C and pH 5.5 (Engelen et al., 1994). The phytase product at 30, 60, 120, 240, and 240 g/tonne were added on top of the respective experimental diets. Feed phytase activities were analyzed by Enzyme Services Consultancy (ESC; Ystrad Mynach, UK) after feed sample extraction. Quantiplate ELISA Kits specific for Quantum Blue were used for quantification of the enzyme activity (Envirologix method AP181 with some modifications; designated ESC Standard Analytical Method SAM099). The analyzed Ca, total P and phytase activities are presented in Table 2.2. Celite (an indigestible marker; Celite Corp., Lompoc, CA) was used at 2% of the experimental diets, added on top of each diet. The Celite-containing diets were fed for 2 wk at the end of the trial from 35 to 37 wk of age. Feed samples were collected at the time of mixing, ground and stored at -20°C for further analysis.

2.2.3 Laying Performance

Individual body weights were measured on a 4-wk basis from 25 to 37 wk of age. Feed intake (**FI**) was recorded during each period to calculate feed conversion ratio (**FCR**; kg of FI per dozen eggs) on a cage basis. Eggs were collected twice daily at 0930 and 1500. Daily egg production was recorded to calculate total egg number at the end of the trial. Hen-day egg

production was calculated for each 4-wk period. At 4-wk intervals, individual fresh egg weight was recorded.

2.2.4 Eggshell Quality

At 4-wk intervals, eggs were collected from each hen for two consecutive days; one egg was used for determination of egg specific gravity (**SG**; g/cm^3), the other for determination of eggshell thickness (mm). Eggs were kept at room temperature overnight (18 h) in the same room as the saline solutions before the determination of eggshell quality. Specific gravity (n = 12 per treatment) was measured by flotation using 11 sequential saline solutions ranging from 1.060 to 1.110 in increments of 0.005 (Holder and Bradford, 1979; Wu et al., 2007). The saline solutions were calibrated before each test. For the eggshell thickness measurement (n = 6 per treatment), a 1 cm x 1 cm square was marked and cut at six different locations (broad-end, narrow-end, and 4 points around the equator) from each egg. The thickness (without membrane) of each eggshell square was measured using a digital micrometer gauge (Mitutoyo, Japan), and the mean value was calculated for each egg. At 37 wk of age, an additional egg from each hen was collected to determine eggshell breaking strength (n = 12 per treatment). The breaking force (the force required to break the egg) was measured using an Instron Materials tester (Model 4411, Instron Corp., Canton, MA) with Bluehill 2 software version 2.29 and a 200 N static load cell as described by Bello and Korver (2019).

2.2.5 Bone Characteristics

2.2.5.1 Bone Densitometry

At the beginning of the trial, 6 hens per treatment were randomly selected for in vivo bone scanning of the shank (tarsometatarsus) at 4-wk intervals throughout the trial. The birds were anaesthetized with Rompun (2 mg/kg BW of a 20 mg/mL solution) with Ketamine (20

mg/kg of BW of 100 mg/mL) to ensure the birds remained motionless during the approximately 20 minutes needed to conduct the scan (Korver et al., 2004). The right shank was scanned at 25% of the length of the shank from the proximal end to determine the total, cortical, and trabecular + medullary bone mineral density (**BMD**) and cross-sectional areas by quantitative computed tomography (**QCT**) using a Stratec Norland XCT (XCT Research SA, Norland Corp., Fort Atkinson, WI) scanner with a 50 kV x-ray tube (Saunders-Blades and Korver, 2015). The threshold used in this study was 400 mg/cm^3 to separate cortical and subcortical bone from trabecular bone (Korver et al., 2004). The total term was the weighted average of both the cortical and trabecular + medullary bone measures, and reflected the density or area of each bone compartment. Cortical BMD was the outer shell of the bone that was determined to have a density of > 500 mg/cm^3 (Saunders-Blades et al., 2009). Due to the limitation of the current QCT to distinguish between trabecular and the medullary bone (Korver et al., 2004), bone in the trabecular space was assumed to include the medullary bone. Bone mineral content (**BMC**; mg/mm) represents the amount of bone mineral in mg contained in a 1-mm thick longitudinal section of the bone, and was calculated as BMD multiplied by the cross-sectional area (Saunders-Blades et al., 2009).

2.2.5.2 Bone Breaking Strength

At the end of the experiment (37 wk of age), all 84 hens were euthanized by cervical dislocation and the right femurs removed. Femurs were cleaned out of soft tissue except for the cartilage caps and kept at -20°C for subsequent determinations of bone breaking strength (**BBS**) and ash. The frozen right femurs were thawed at 4°C for 24 h. Each femur was marked at the proximal 25%, the midpoint, and distal 25% (25%, 50%, and 75% from the proximal epiphysis of the length of the femur, respectively) determined using a digital caliper (Model CD-8”C,

Mitutoyo Corp., Japan) before BBS and ash determination. Bone breaking strength was measured as described by Riczu et al. (2004) using an Instron Materials Tester (Model 4411, Instron Corp., Canton, MA) with Bluehill 2 software version 2.29 and a 500 N static load cell.

2.2.5.3 Bone Ash

After BBS measurement, each femur was cut at 25% and 75% from the proximal epiphysis of the length of the bone using a Dremel tool (Dremel MultiPro Model 395, Racine, WI) to separate proximal end (25%), mid-diaphysis (50%), and the distal end (25%). Each bone segment was oven-dried (Despatch Oven Co., Minneapolis, MN) at 100°C for 48 h and subsequently ashed in a muffle furnace (30400 Thermolyne Furnace, Dubuque, IA) at 500°C for 48 h as described by Bello et al. (2014). The ash was weighed to determine the ash content (in g) and percent ash of each segment. Total dry bone weight and total ash content were calculated by summation of the three dry bone weights and ash content weights, respectively.

2.2.6 Phosphorus and Calcium Digestibility Assays

At 37 wk of age, the ileum was removed from 84 birds after euthanasia. Digesta from the distal part of the ileum (defined as the posterior one-third of the section between Meckel's diverticulum and 2 cm anterior to the ileocaecal junction) of each bird was collected separately by gentle squeezing and immediately frozen at -20°C until further analysis. Feed and digesta samples were analyzed for acid insoluble ash (AIA; Scott and Boldaji, 1997), P (method 935.13; AOAC, 1990) using a spectrophotometer (SpectraMax Plus 384 Microplate Reader, Molecular Devices LLC, San Jose, CA) and Ca (method 964.06; AOAC, 1990) concentrations using a nitrous oxide-acetylene fueled flame atomic absorption spectrometer (Varian AA240FS, Agilent Technologies, Santa Clara, CA) for determination of apparent ileal digestibility (**AID**) of P and Ca, respectively, as described by Bello and Korver (2019).

2.2.6.1 Calculation of Phosphorus and Calcium Digestibility

During the digestibility determination period from 35 to 37 wk of age, the analyzed AIA, total P, and Ca concentrations from the Celite-containing diets and digesta for each respective diet were used to calculate AID of P or Ca on a dry matter basis using the following equation:

$$AID\ (\%) = 100 - [((AIA_{diet}/AIA_{digesta}) \times (Mineral_{digesta}/Mineral_{diet})) \times 100]$$

Where AIA_{diet} was the initial AIA concentration in the diet; $Mineral_{diet}$ was the initial dietary concentration of the mineral (i.e. P or Ca); $AIA_{digesta}$ was the concentration of AIA in digesta; and $Mineral_{digesta}$ was the respective concentration of the mineral in digesta.

2.2.7 Statistical Analysis

The cage (individual hen) was considered the experimental unit for all measures, with 12 replicate cages per treatment. One-way ANOVA was conducted using the MIXED procedure of SAS (SAS Institute Inc., 2012) for total egg number, eggshell breaking strength, BBS, dry bone weight, bone ash content, percent bone ash, and AID of P and Ca. Two-way ANOVA was used to determine the effect of diet, age, and their interaction using the MIXED procedure of SAS (SAS Institute Inc., 2012) for FI, FCR, egg production, egg weight, SG, eggshell thickness, and BMD, bone cross-sectional area, and BMC of the total, cortical, and trabecular + medullary bone tissues. Two-way ANOVA was also conducted using the MIXED and the HPMIXED procedures of SAS (SAS Institute Inc., 2012) for BW. All data were tested for normality and normality of residuals using UNIVARIATE procedure. Since egg production is percentage data and did not fit a normal distribution, arcsine transformation was used before statistical analysis. Body weight was used as a covariate for determinations of bone traits. Means were separated using the LSMEANS statement. Tukey's range test was applied to compare multiple mean comparisons. The linear, quadratic and cubic effects of phytase supplementation among the NC treatments

were analyzed for AID of P and Ca using a polynomial regression to describe the shape of the response to increasing doses of phytase supplementation in the NC diet. The IML procedure of SAS (SAS Institute Inc., 2012) was used to generate the polynomial coefficients for unequal interval of phytase doses. Statistical significance was considered when $P \leq 0.05$. Trends were reported where $0.05 < P \leq 0.10$. Values are presented as least squares means (**LSM**) with the respective standard errors of the mean.

2.3 RESULTS

2.3.1 Analyzed Ca, P and Phytase Activity

The analyzed Ca levels were higher than the formulated levels, but overall they were consistently higher across the dietary treatments from 25 to 34 wk, except the NC + 150 and NC + 300 from 25 to 28 wk (Table 2.2). From 35 to 37 wk, the NC diet had lower analyzed Ca than the expected (2.38% vs. 3.02%) whereas the PC and PC + 1,200 had higher analyzed Ca (4.35% and 4.09%, respectively) than the planned level. The analyzed total P of the NC and NC supplemented with phytase treatments were in the expected range except low levels in the NC and NC + 300 diets (0.31% and 0.38%, respectively) from 35 to 37 wk. The analyzed total P of the PC from 29 to 34 and from 35 to 37 wk were slightly lower (0.58% and 0.59%, respectively) than the targeted level. The analyzed total P of the PC + 1,200 was lower (0.50%) than the calculated from 35 to 37 wk. Overall, feed phytase activities were higher than the planned levels but were consistent with the assumed stepwise increase of phytase activity among treatments.

2.3.2 Hen Performance

Body weight, FI, FCR, and egg production were not affected by the diet x age interaction or diet main effect (Table 2.3). Feed intake at 37 wk of age tended to be higher than at 33 wk of age ($P = 0.080$). Feed conversion ratio increased with hen age ($P < 0.001$). Hen-day egg

production, total egg number or egg weight were not affected by dietary treatments. Hens at 29 wk of age had lower egg weight (59.3 ± 0.48 g) than at 33 wk (61.5 ± 0.48 g) and 37 wk of age (61.5 ± 0.52 g; $P = 0.002$).

2.3.3 Eggshell Quality

There were no diet x age interactions for egg SG or eggshell thickness. The NC + 1,200 had greater egg SG than the NC (1.090 ± 0.001 vs. 1.088 ± 0.001 g/cm^3; $P = 0.004$). At 33 wk of age, egg SG was higher (1.090 ± 0.001 g/cm^3) than at 29 wk (1.088 ± 0.001 g/cm^3) and 37 wk (1.089 ± 0.001 g/cm^3; $P = 0.001$). Eggshell thickness was not affected by diet (0.36 ± 0.002 mm; $P = 0.105$). However, eggshell thickness at 37 wk of age tended to be higher than at the other ages ($P = 0.072$). No differences were observed among dietary treatments for eggshell breaking strength at 37 wk of age (5.06 ± 0.20 kg-force; $P = 0.402$).

2.3.4 Bone Characteristics

No diet x age interactions were observed for in vivo BMD (Table 2.4), bone cross-sectional area (Table 2.5), or BMC (Table 2.6) of the total, cortical, and trabecular + medullary bone tissues. However, the PC hens had greater total BMD than those of the NC, the NC + 300, the NC + 600, and the NC + 1,200 hens ($P < 0.001$; Table 2.4). The PC hens also had higher cortical BMD than the NC, the NC + 300, and the NC + 600 hens ($P < 0.001$). A decrease in trabecular + medullary BMD was observed in the NC, the NC + 150, and the PC + 1,200 relative to the PC treatment ($P = 0.004$). At 25 wk of age, total BMD was higher than at 33 and 37 wk of age ($P < 0.001$).

The NC had higher total bone cross-sectional area than the PC, the NC + 1,200, and the PC + 1,200 ($P = 0.003$; Table 2.5). Total bone cross-sectional area in the NC + 300 was greater than in the PC. The PC had higher cortical bone cross-sectional area than the NC + 300, the NC

+ 600, and the NC + 1,200 ($P < 0.001$). Trabecular + medullary bone cross-sectional area in trabecular space in the NC + 300 and the NC + 600 was greater than the PC, the NC + 1,200, and the PC + 1,200 ($P < 0.001$). At 25 wk of age, cortical bone cross-sectional area was higher than the other ages ($P < 0.001$). However, trabecular + medullary bone cross-sectional area at 25 wk of age was lower than at 33 and 37 wk of age ($P = 0.010$).

The PC had greater total BMC than the other treatments except NC + 1,200 and the PC + 1,200 ($P < 0.001$; Table 2.6). Cortical BMC was higher in the PC than the NC + 300, the NC + 600, and the NC + 1,200 treatments ($P < 0.001$). The NC + 600 had greater trabecular + medullary BMC than the NC + 150 and the PC + 1,200 ($P < 0.001$). Total and cortical BMC decreased with hen age ($P = 0.002$ and $P < 0.001$, respectively). At 37 wk of age, there were no differences among treatments in BBS (20.06 $\pm$ 0.47 kg-force; $P = 0.446$). Dry bone weight, bone ash content and the percent ash of the proximal, mid-bone, and distal segments, or total were not affected by dietary treatments at 37 wk of age.

2.3.5 Apparent Ileal Digestibility of Phosphorus and Calcium

A 37 wk of age, the AID of P in the NC hens was lower than the NC + 600 and the NC + 1,200 hens ($P = 0.024$; Figure 2.1A). Hens fed the NC diet had lower AID of Ca than the other treatments except the NC + 150 ($P = 0.010$; Figure 2.1B). There was a quadratic ($P < 0.001$) response in AID of P [$Y = 39.11 + 0.07X - 0.0004X^2$; $R^2 = 0.323$] with the supplementation of exogenous phytase (150, 300, 600, and 1,200 FTU/kg) to the NC diet. The quadratic ($P < 0.008$) response was also observed for the AID of Ca [$Y = 25.78 + 0.06X - 0.0004X^2$; $R^2 = 0.169$] with increasing doses of phytase supplementation.

2.4 DISCUSSION

From 25 to 34 wk of age, the analyzed Ca levels were 5 to 24% higher than the formulated while the analyzed total P levels were slightly lower than the formulated but were in the expected range. High Ca in the diet may have contributed inconsistent results to hen performance. However, the Ca and total P in the PC treatment were still higher than the NC and NC plus phytase treatments, the analyzed values still allow us to draw valid conclusions from our results. During the mineral digestibility period from 35 to 37 wk of age, the analyzed Ca and total P were in the expected range except the NC treatment. Although the analyzed Ca and total P in the NC diet were 21% and 33% lower, respectively, than formulated, this did not interfere with our interpretation. Having low analyzed Ca and total P in the NC diet during the mineral digestibility period allowed us to observe not only negative effects in the NC hens but also the efficacy of phytase.

At 37 wk of age, the BW of the NC hens (1.65 $\pm$ 0.04 kg) was within the range between 1.58 and 1.71 kg, and egg production in the NC treatment (98.12 $\pm$ 0.93 %) was higher relative to the management guide (Lohmann Tierzucht, 2012). The average daily aP intake of the NC hens in each period was approximately halved relative to the PC hens (240 vs. 490, and 210 vs. 410 mg/d per hen from 25 to 28, and 29 to 37 wk of age, respectively). Although aP intake of the NC hens was lower than the NRC recommendation of 250 mg/d per hen (NRC, 1994), hens were still performing well and no mortality was observed, indicating that aP and Ca levels in the NC diet apparently met the actual requirements for maintenance of performance. Hens were able to maintain BW throughout the 12-wk experiment without any symptoms of P or Ca deficiency, whereas bone demineralization in NC hens suggests the initial stages of a mineral shortage. This could be because the NC diet contained slightly low Ca during the last 2-wk of the experiment.

Egg production remained relatively constant from 25 to 37 wk of age but daily feed intake and FCR increased with hen age. This could be because hens gradually increased egg size from 29 to 37 wk of age.

Actively laying hens require Ca to form amorphous calcium carbonate (Rodriguez-Navarro et al., 2015) and calcium phosphate during eggshell calcification (Murakami et al., 2007). During mid-production, reducing dietary Ca to 3.60% (Bello and Korver, 2019), 3.35% (Kaur et al., 2013), or 3.25% (Rama Rao et al., 2003) did not decrease eggshell quality in White Leghorn layers. Dietary Ca at 3.22%, 4.10%, and 2.38% at each period was sufficient to maintain the eggshell quality of hens throughout this study. Others reported decreased eggshell thickness and breaking strength when fed 3% Ca in aged Lohmann LSL-Lite layers from 74 to 81 wk of age (Akbari Moghaddam Kakhki et al., 2019), possibly due to Ca absorption being inefficiency in aged laying hens (Bronner, 1987; Pelicia et al., 2009). Although 1,200 FTU phytase/kg in the NC diet increased SG, it did not increase the eggshell thickness or breaking strength relative to the NC diet. The lack of a consistent effect of phytase on eggshell quality might be due to the lack of adverse effects of the NC diet. In order to observe a response to phytase, it is necessary for the Ca or P subsequently released from phytate to contribute to meeting the requirement of the hen. If the dietary levels of Ca and aP are already adequate, there can be no response to the liberated minerals, because the hen has no further need for them. We would able to see the effect of phytase to restore eggshell quality if the levels of aP and Ca in the NC diet were reduced below the actual requirements over a longer period without any further bone demineralization when adverse effects in the NC hens are found. Typically, eggshell quality decreases as hens age (Al-Batshan et al., 1994; Machal and Simeonovova, 2002), but this was not observed in the current study. This may be because the increase in medullary bone in

bone trabecular space (trabecular + medullary) from 25 to 37 wk of age was sufficient to support eggshell quality. Also, hens in this flock were young and the experiment lasted only 12 wk.

Although the NC diet provided sufficient Ca for performance and eggshell quality, it did not appear to be sufficient to maintain bone mineralization as shown in the tarsometatarsus QCT results. From 35 to37 wk of age, 2.38% Ca in the NC diet may have contributed to the negative effect on bone quality. However, femur BBS and ash content results were not consistent with the tarsometatarsus QCT data. First, the tarsometatarsus has proportionally lower structural and medullary bone volumes, and ash than the femur in laying hens (Taylor and Moore, 1954). Therefore the tarsometatarsus may be more sensitive to changes than the femur. Secondly, BBS and ash include the total (whole) bone whereas QCT was used to measure a single point at 25% of the length of the tarsometatarsus from the proximal end. Also, the NC hens had begun to mobilize bone mineral, but not to the point where it decreased BBS or overall ash content. Reducing aP from 0.25 to 0.12% with adequate Ca at 3.50% did not decrease BBS and ash content in Hy-line W36 hens (Martinez Rojas et al., 2018). Providing sufficient level of Ca (3.51 g Ca/d per hen or 32.5 g Ca/kg diet) did not decrease serum Ca, and BBS and ash in laying hens, serum alkaline phosphatase and bone resorption were increased (Keshavarz and Nakajima, 1993; Leeson et al., 1993; Rama Rao et al., 2003), indicating that bone depletion occurred even though hens were able to maintain BBS. Total BMC and cortical, trabecular + medullary, and total BMD were lower in the NC hens than the PC hens, indicating that the NC hens started mobilizing bone mineral to support eggshell formation, maintain egg production and performance. Laying hens are able to physiologically adapt to P and Ca-reduced diets and maintain performance, depending on the degree of nutrient deficiency (Boling et al., 2000; Nie et al., 2013; Geraldo et al., 2014) and varying with strains (Hughes et al., 2009). Phytase supplementation to the NC diets did not

fully restore total BMD and BMC to the level of the PC treatment. Total Ca levels in layer diets are beyond those of broilers in which negative impact with increased Ca level on phytate degradation were shown (Sommerfeld et al., 2018b). Also, when P and Ca move to the higher pH environment of the lower gastrointestinal tract, P and Ca can form insoluble salts of calcium phosphate (Nelson and Kirby, 1987; Tamim et al., 2004; Hamdi et al., 2015; Sommerfeld et al., 2018b) and subsequently impair P and Ca absorption. High dietary Ca caused high pH in the gastrointestinal tract (Nelson and Kirby, 1987), and therefore decreased phytase efficiency (Van der Klis et al., 1997; Sommerfeld et al., 2018b). These may explain why phytase supplementation in the NC diets did not consistently restore total BMD and BMC to the level of the PC treatment. Bone quality in hens fed high dietary Ca (4.2%) was not affected when supplemented with phytase at 300, 600, or 1,200 FTU phytase/kg (Fernandez et al., 2019). The results for each measure of the NC + 600, NC + 1,200, and PC + 1,200 treatments did not differ from the commercial recommendation level at 300 FTU phytase/kg in layer diet, possibly because both the NC and the PC diets contained sufficient or even excess P and Ca relative to the actual needs of hens over this short supplementation period. It is also assumed that individual response on the selected bone measurement varies, and more replicates and a longer feeding period might be needed to determine the phytase effect on bones in older birds like laying hens.

As expected, structural bone decreased as the hens aged. The bone cross-sectional area in the trabecular space (trabecular + medullary bone) increased from 25 to 37 wk of age, reflecting an age-related increase in the accumulation of medullary bone. In actively laying hens, cortical and trabecular bone tissues can be mobilized but not formed whereas medullary bone can be deposited and mobilized (Fleming et al., 1998a; Whitehead, 2004; Fleming, 2008; Kerschnitzki et al., 2014). In a longer-lasting trial, structural bone volume in the proximal tarsometatarsus of

layers decreased from 15 to 25 wk, remained constant from 25 to 50 wk, and then decreased at 70 wk of age in hens fed Ca- and P-adequate diet (Fleming et al., 1998b). Whereas hens maintained structural bone from 30 to 70 wk of age in moderate reductions in dietary Ca and aP (Bello and Korver, 2019). However, structural bone decreased from 25 to 33 wk of age in Ca- and aP-reduced diets in our study, suggesting that greater reductions of Ca and aP in diet impaired bone quality in the short-term. Monitoring bone changes using QCT allowed us to keep the hens alive and follow individual hens throughout the 12-wk experiment period, which are some advantages of the QCT technique (Korver, 2004; Korver et al., 2004). Because of limited bird numbers, we measured BBS and ash at the end of trial only, thus limiting the opportunity to determine age effects on bone changes using traditional methods. Each segment or total of bone ash provided the same results, suggesting that femur ash determination for one segment would be sufficient for the determination of bone ash in the short-term in laying hens.

The bone architecture and structure, and metabolic behaviors in physiological processes are associated with different bone regions because trabecular bone is concentrated at the proximal (Bello and Korver, 2019) and distal ends (Sullivan et al., 2017) while cortical and medullary bone are concentrated in the mid-diaphysis (Kerschnitzki et al., 2014; Bello and Korver, 2019). Although different bone regions have different bone architecture and structure, for bone development of broilers and ducks, the epiphyseal bone mineralization was greater than at the diaphyseal region because the epiphyseal ends are responsible for linear growth and chondrocyte replication in the growth plate (Applegate and Lilburn, 2002; Van Wyhe et al., 2012). In the mouse, mRNA expression of alkaline phosphatase and osteocalcin, two key bone formation markers, was greater in the metaphyseal than the diaphyseal regions (Li et al., 2017). These authors also reported that the bone remodeling process in the tibia metaphysis was higher

than in the diaphysis, indicating that different locations of the long bones have different properties and metabolic activity. In white egg-laying hens (30 to 70 wk), the pattern of bone mineralization at the proximal metaphysis was as same as at the mid-diaphysis (Bello and Korver, 2019). This is the main reason why bone QCT measurement in the current study was conducted at only 25% of the length of the shank from the proximal end.

Laying hens are able to increase dietary P absorption via the up-regulation of the Na-P IIb transporter in the duodenum when fed a P-deficient diet (Nie et al., 2013). The AID of P in the NC hens did not differ from the PC hens, implying that the 0.31% analyzed total P in the NC diet apparently met the actual requirement of P in laying hens at this age. Phytase supplementation to the NC diet at 600 and 1,200 FTU/kg increased P digestibility relative to the NC diet. It is possible that high levels of dietary phytase increased inositol hexaphosphate (IP_6) degradation to inositol heptaphosphate (IP_5) and lower esters (Sommerfeld et al., 2018a), thereby more P was available to absorb in the small intestine (Angel et al., 2002) compared to the diet without or low level of phytase. Reducing dietary aP increased the pH at the end of the small intestine phase in an in vitro broiler digestion assay (Farhadi et al., 2019). Increased small intestine pH accelerated precipitation of mineral-phytate complexes (Tamim et al., 2004; Walk et al., 2012), and high dosages of exogenous phytase alleviated such adverse effects and increased P solubility in the small intestine phase in the in vitro digestion assay (Farhadi et al., 2019). We assumed that P digestibility in laying hens would show a similar response to high doses of phytase as in broilers as demonstrated by others when supplementation of 5,000 FTU phytase/kg in a P-reduced laying hen diet decreased phytate P content in digesta and increased P digestibility relative to 500 FTU phytase/kg (Gao et al., 2013).

Laying hens responded to a Ca-deficient diet by increasing the intestinal expression of mRNA for the Ca transporter CaBP-D_{28K} (Ieda et al., 1999) to increase Ca absorption from the diet (Pelicia et al., 2009). However, since both the dietary Ca level and the AID of Ca were lower in the NC group than the PC group, less Ca would have been absorbed from the diet. It seemed that NC hens did not up-regulate dietary Ca absorption but instead mobilized bone Ca. The analyzed Ca and total P in the NC diet during the digestibility determination period were 2.38% and 0.31%, respectively, and maintained at a Ca:total P ratio of around 8:1, which was wider than the recommended Ca:total P ratio of 6:1 (Lohmann Tierzucht, 2012). Low Ca and total P, and the widening of the Ca:total P in the diet elevated Ca excretion in the kidney (Rao and Roland, 1990). The widening of the Ca:total P ratio in the NC diet in the current study may have increased the formation of insoluble Ca phosphate and decreased the solubility of Ca in the digestive tract. Subsequently, Ca cannot be absorbed in the small intestine and is then excreted (Rao and Roland, 1990; Keshavarz and Nakajima, 1993), which may explain the decrease in AID of Ca in the NC hens. Because of this, the NC hens then started to mobilize bone to support eggshell formation and production. Phytase supplementation to the NC diet at 300, 600 and 1,200 FTU/kg increased AID of Ca. These phytase levels were able to liberate Ca from Ca-phytate complexes (Selle et al., 2009; Humer et al., 2015). However, phytase supplementation at 150 FTU/kg did not increase AID of Ca. It is assumed that 150 FTU/kg was too low a supplementation rate to reduce IP_6 to an extent that could increase Ca digestibility.

Overall, the reduction of aP and Ca in the NC diet did not cause any adverse effects on performance, production, and eggshell quality in laying hens over the 12 wk of the study. The current recommendations for Ca and aP provided by the primary breeders are likely substantially higher than actually required by hens. From a bone biology standpoint, the NC hens started

mobilizing bone at the end of the trial, resulting in decreased BMD and BMC, however this did not appear to cause osteoporosis or other bone problems. The increase of P and Ca digestibility by phytase supplementation resulted in some, but inconsistent increases in bone measures. The implications of longer term feeding of the NC diet to determine the efficacy of phytase in laying hens should be studied further. Short-term phytase studies should be designed with further reductions in Ca and aP to adequately assess the efficacy of phytases, while minimizing the risk of severe bone issues.

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2.6 TABLES

Table 2. 1: The ingredient and nutrient composition of positive control and negative control diets fed to laying hens from 25 to 37 wk of age.

	25 to 28 wk		29 to 37 wk	
	PC[1]	NC[2]	PC[1]	NC[2]
Ingredients (%)				
Corn	58.52	61.03	59.63	62.04
Soybean meal	18.96	18.96	16.47	16.47
Canola meal	10.00	10.00	12.00	12.00
Canola oil	1.44	1.44	0.96	0.96
Calcium carbonate	8.41	7.23	8.67	7.35
Dicalcium phosphate	1.76	0.50	1.37	0.33
Salt	0.32	0.24	0.29	0.24
DL-Methionine	0.10	0.10	0.10	0.10
Vitamin-mineral premix[3]	0.50	0.50	0.50	0.50
Phytase[4] (g/kg)	Variable[5]	Variable[5]	Variable[5]	Variable[5]
Calculated nutrient composition (%)				
ME (kcal/kg)	2,800	2,885	2,775	2,857
Crude protein	18.00	18.20	17.60	17.80
Calcium	3.70	3.00	3.73	3.02
Available phosphorus	0.45	0.22	0.38	0.19
Phytate phosphorus	0.25	0.26	0.26	0.27
Total phosphorus	0.70	0.48	0.64	0.46
Sodium	0.16	0.13	0.15	0.13

[1]PC = positive control diet; PC diet was mixed as a single batch and subdivided into the PC and PC + phytase treatments.

[2]NC = negative control diet; NC diet was mixed as a single batch and subdivided into the NC and various NC + phytase treatments.

[3]Vitamin-mineral premix (units per kilogram of feed): vitamin A, 12,500 IU; vitamin D_3, 3,125 IU; vitamin E, 40 IU; vitamin K (menadione), 2.5 mg; riboflavin, 7.5 mg; D-pantothenic acid, 12.5 mg; vitamin B_{12}, 0.01875 mg; pyridoxine, 5 mg; thiamine, 2.55 mg; folic acid, 0.625 mg; niacin, 37.5 mg; biotin, 0.15 mg; iodine, 1.65 mg; copper, 15 mg; iron, 80 mg; selenium, 0.3 mg; manganese, 88 mg; zinc, 100 mg.

[4]Quantum Blue phytase (5,000 FTU/g of premix; AB Vista, Marlborough, UK).

[5]Quantum Blue phytase was added on top of the NC diet at 0.03 (150 FTU/kg), 0.06 (300 FTU/kg), 0.12 g/kg (600 FTU/kg) or 0.24 (1,200 FTU/kg) g/kg for the NC phytase-containing diets, or on top of the PC diet at 0.24 g/kg (1,200 FTU/kg) for the PC phytase-containing diet.

Table 2. 2: Analyzed calcium, total phosphorus and phytase activity levels of experimental layer diets[1,2].

	Diet[3]						
	PC	NC	NC + 150	NC + 300	NC + 600	NC + 1,200	PC + 1,200
Calcium (% of the diet, as-fed basis)							
25 to 28 wk	4.64 (0.06)	3.22 (0.10)	2.74 (0.15)	2.52 (0.11)	3.64 (0.07)	3.16 (0.07)	4.13 (0.17)
29 to 34 wk	4.22 (0.08)	4.10 (0.04)	3.90 (0.09)	4.32 (0.07)	3.47 (0.03)	3.30 (0.09)	4.37 (0.13)
35 to 37 wk[4]	4.35 (0.12)	2.38 (0.11)	3.18 (0.12)	2.94 (0.13)	2.98 (0.08)	2.90 (0.12)	4.09 (0.11)
Total phosphorus (% of the diet, as-fed basis)							
25 to 28 wk	0.63 (0.03)	0.48 (0.01)	0.46 (0.01)	0.45 (0.03)	0.49 (0.01)	0.47 (0.03)	0.60 (0.01)
29 to 34 wk	0.58 (0.01)	0.45 (0.03)	0.46 (0.01)	0.44 (0.01)	0.41 (0.03)	0.47 (0.01)	0.61 (0.03)
35 to 37 wk[4]	0.59 (0.04)	0.31 (0.01)	0.45 (0.03)	0.38 (0.01)	0.41 (0.04)	0.43 (0.02)	0.50 (0.03)
Phytase activity[5] (FTU/kg)							
25 to 28 wk	<50	<50	267 (13)	421 (50)	875 (0)	2,000 (0)	1,820 (91)
29 to 37 wk	<50	<50	254 (33)	442 (35)	743 (0)	1,540 (231)	1,570 (173)

[1]Feed provided in mash form.

[2]Means of 2 replicate feed samples, standard deviations are given in parentheses. Phytase activity in the PC and NC diets were below the detection level, therefore means and standard deviations were not calculated.

[3]PC, the positive control diet, nutritionally complete diet containing 0.45% aP, 3.70% Ca and 0.16% Na (25 to 28 wk), 0.38% aP, 3.73% Ca and 0.15% Na (29 to 37 wk); NC, the negative control diet, similar to the PC diet but having 0.22% aP, 3.00% Ca and 0.13% Na (25 to 28 wk), 0.19% aP, 3.02% Ca and 0.13% Na (29 to 37 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, or 1,200 FTU/kg, respectively; and PC + 1,200, the PC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 1,200 FTU/kg.

[4]Value for each diet was used to calculate AID of P and Ca for each respective treatment.

[5]Analyzed by Enzyme Services Consultancy (ESC; Ystrad Mynach, UK) using Quantiplate ELISA Kits for Quantum Blue (Envirologix method AP181 with some modifications; designated ESC Standard Analytical Method SAM099).

Table 2. 3: Main effects of diet and age on performance in hens fed different dietary aP and Ca levels, and phytase supplementation from 25 to 37 wk of age.

	Body weight[1,2] (kg)		Feed intake[1] (g/d per hen)		Feed conversion ratio[1] (kg feed/dozen eggs)		Hen-day egg production[1,3] (%)		Total egg number[1,4]	
	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]
Diet[7]										
PC	1.64	0.01	108	1.29	1.27	0.02	98.2	0.51	82.6	0.63
NC	1.62	0.02	110	1.46	1.31	0.02	97.6	0.75	81.9	0.58
NC + 150	1.65	0.02	111	1.13	1.29	0.01	98.9	0.31	83.3	0.39
NC + 300	1.65	0.02	109	1.03	1.27	0.01	99.0	0.30	83.4	0.36
NC + 600	1.59	0.02	107	1.26	1.26	0.02	98.5	0.39	82.8	0.39
NC + 1,200	1.65	0.02	110	1.02	1.29	0.01	97.4	0.67	82.0	0.79
PC + 1,200	1.61	0.02	108	1.36	1.27	0.02	98.4	0.50	82.8	0.41
Age (wk)										
25 to 29	1.62	0.01	109	0.92	1.23[c]	0.01	98.1	0.39	-	-
29 to 33	1.62	0.01	108	0.59	1.27[b]	0.01	98.4	0.33	-	-
33 to 37	1.62	0.01	111	0.87	1.34[a]	0.01	98.4	0.29	-	-
37	1.66	0.02	-	-	-	-	-	-	-	-
Source of variation	Prob > F									
Diet	0.104		0.309		0.328		0.439		0.334	
Age	0.198		0.080		<0.001		0.924		-	
Diet x age	0.999		0.998		0.999		0.947		-	

[1]Means of 12 replicate hens for each treatment.
[2]Age means for body weight were measured at the beginning of each specific age range (25, 29, 33, and 37 wk of age).
[3]Arcsine transformation was used before statistical analysis; egg production data are presented as original values in percent.
[4]Total egg produced throughout 84 days of experiment.
[5]LSM = least squares mean.
[6]SEM = standard error of the mean.
[7]PC, the positive control diet, nutritionally complete diet containing 0.45% aP, 3.70% Ca and 0.16% Na (25 to 28 wk), 0.38% aP, 3.73% Ca and 0.15% Na (29 to 37 wk); NC, the negative control diet, similar to the PC diet but having 0.22% aP, 3.00% Ca and 0.13% Na (25 to 28 wk), 0.19% aP, 3.02% Ca and 0.13% Na (29 to 37 wk); NC + 150, NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 150, 300, 600, or 1,200 FTU/kg, respectively; and PC + 1,200, the PC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 1,200 FTU/kg.
[a-c]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

Table 2. 4: Main effects of diet and age on in vivo shank mineral density in the proximal metaphysis in hens fed different dietary aP and Ca levels, and phytase supplementation from 25 to 37 wk of age[1].

	Bone mineral density (mg/cm^3)					
	Total[2]		Cortical[3]		Trabecular + medullary[4]	
	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]
Diet[7]						
PC	513^{a}	7.34	981^{a}	3.38	65.4^{a}	4.88
NC	462^{b,c,d}	8.54	966^{b,c}	3.34	49.1^{b}	1.92
NC + 150	480^{a,b,c}	7.69	976^{a,b}	4.79	48.1^{b}	1.46
NC + 300	456^{c,d}	3.17	963^{b,c}	2.48	51.0^{a,b}	1.64
NC + 600	448^{d}	3.44	955^{c}	3.55	54.0^{a,b}	1.34
NC + 1,200	479^{b}	6.68	972^{a,b,c}	6.99	55.3^{a,b}	2.54
PC + 1,200	495^{a,b}	8.46	984^{a}	3.06	48.8^{b}	1.47
Age (wk)						
25	495^{a}	4.65	975	3.10	54.8	1.76
29	477^{a,b}	5.11	974	3.06	53.4	1.93
33	469^{b}	5.40	969	3.32	52.3	1.91
37	464^{b}	5.34	965	3.14	51.8	1.85
Source of variation	Prob > F					
Diet	<0.001		<0.001		0.004	
Age	<0.001		0.105		0.676	
Diet x age	0.999		0.887		0.986	
Body weight[8]	0.145		0.002		0.606	

[1]Means of 6 replicate hens for each treatment.
[2]Total term was the weighted average of both the cortical and trabecular + medullary bone measures.
[3]Cortical bone was the outer shell of the bone, and was defined as having a density > 500 mg/cm^3.
[4]Trabecular + medullary bone were assumed to present in the trabecular space.
[5]LSM = least squares mean.
[6]SEM = standard error of the mean.
[7]PC, the positive control diet, nutritionally complete diet containing 0.45% aP, 3.70% Ca and 0.16% Na (25 to 28 wk), 0.38% aP, 3.73% Ca and 0.15% Na (29 to 37 wk); NC, the negative control diet, similar to the PC diet but having 0.22% aP, 3.00% Ca and 0.13% Na (25 to 28 wk), 0.19% aP, 3.02% Ca and 0.13% Na (29 to 37 wk); NC + 150, NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 150, 300, 600, or 1,200 FTU/kg, respectively; and PC + 1,200, the PC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 1,200 FTU/kg.
[8]Body weight was used as a covariate.
[a-d]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

Table 2. 5: Main effects of diet and age on in vivo shank cross-sectional area in the proximal metaphysis in hens fed different dietary aP and Ca levels, and phytase supplementation from 25 to 37 wk of age[1].

	Bone cross-sectional area (mm^2)					
	Total[2]		Cortical[3]		Trabecular + medullary[4]	
	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]
Diet[7]						
PC	26.5[c]	0.21	12.5[a]	0.15	12.9[c]	0.23
NC	28.1[a]	0.41	12.1[a,b]	0.08	14.8[a,b]	0.46
NC + 150	26.7[a,b,c]	0.48	12.0[a,b,c]	0.12	13.6[a,b,c]	0.40
NC + 300	27.5[a,b]	0.24	11.7[b,c]	0.12	14.8[a]	0.21
NC + 600	27.3[a,b,c]	0.26	11.5[c]	0.14	14.7[a]	0.17
NC + 1,200	26.4[b,c]	0.30	11.8[b,c]	0.13	13.6[b,c]	0.21
PC + 1,200	26.2[b,c]	0.44	12.1[a,b,c]	0.14	13.1[b,c]	0.38
Age (wk)						
25	26.7	0.27	12.3[a]	0.11	13.3[b]	0.21
29	26.9	0.25	11.9[b]	0.10	13.9[a,b]	0.22
33	27.2	0.26	11.9[b]	0.09	14.2[a]	0.25
37	27.1	0.27	11.7[b]	0.09	14.3[a]	0.26
Source of variation	Prob > F					
Diet	0.003		<0.001		<0.001	
Age	0.621		<0.001		0.010	
Diet x age	0.999		0.999		0.999	
Body weight[8]	<0.001		<0.001		<0.001	

[1]Means of 6 replicate hens for each treatment.
[2]Total term was the weighted average of both the cortical and trabecular + medullary bone measures.
[3]Cortical bone was the outer shell of the bone, and was defined as having a density > 500 mg/cm^3.
[4]Trabecular + medullary bone were assumed to present in the trabecular space.
[5]LSM = least squares mean.
[6]SEM = standard error of the mean.
[7]PC, the positive control diet, nutritionally complete diet containing 0.45% aP, 3.70% Ca and 0.16% Na (25 to 28 wk), 0.38% aP, 3.73% Ca and 0.15% Na (29 to 37 wk); NC, the negative control diet, similar to the PC diet but having 0.22% aP, 3.00% Ca and 0.13% Na (25 to 28 wk), 0.19% aP, 3.02% Ca and 0.13% Na (29 to 37 wk); NC + 150, NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 150, 300, 600, or 1,200 FTU/kg, respectively; and PC + 1,200, the PC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 1,200 FTU/kg.
[8]Body weight was used as a covariate.
[a-c]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

Table 2. 6: Main effects of diet and age on in vivo shank mineral content in the proximal metaphysis in hens fed different dietary aP and Ca levels, and phytase supplementation from 25 to 37 wk of age[1].

	Bone mineral content[2] (mg/mm)					
	Total[3]		Cortical[4]		Trabecular + medullary[5]	
	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]
Diet[8]						
PC	13.6[a]	0.21	12.3[a]	0.17	0.83[a,b]	0.05
NC	12.9[b]	0.09	11.7[a,b,c]	0.10	0.72[a,b,c]	0.03
NC + 150	12.8[b,c]	0.13	11.7[a,b,c]	0.12	0.65[b,c]	0.02
NC + 300	12.4[b,c]	0.14	11.3[c,d]	0.12	0.75[a,b]	0.02
NC + 600	12.2[c]	0.16	11.0[d]	0.15	0.79[a]	0.02
NC + 1,200	12.7[a,b,c]	0.22	11.5[b,c,d]	0.18	0.75[a,b,c]	0.04
PC + 1,200	13.0[a,b]	0.16	11.9[a,b]	0.14	0.64[c]	0.03
Age (wk)						
25	13.2[a]	0.13	12.1[a]	0.11	0.72	0.02
29	12.8[a,b]	0.12	11.7[a,b]	0.10	0.74	0.02
33	12.7[b]	0.12	11.6[b]	0.10	0.74	0.02
37	12.5[b]	0.12	11.3[b]	0.11	0.73	0.02
Source of variation	Prob > F					
Diet	<0.001		<0.001		<0.001	
Age	0.002		<0.001		0.978	
Diet x age	0.999		0.999		0.997	
Body weight[9]	<0.001		<0.001		0.007	

[1]Means of 6 replicate hens for each treatment.
[2]Bone mineral content was calculated as bone mineral density multiplied by the bone cross-sectional area, and is the amount of bone mineral contained in a 1 mm linear section of the scanned region of the bone.
[3]Total term was the weighted average of both the cortical and trabecular + medullary bone measures.
[4]Cortical bone was the outer shell of the bone, and was defined as having a density > 500 mg/cm^3.
[5]Trabecular + medullary bone were assumed to present in the trabecular space.
[6]LSM = least squares mean.
[7]SEM = standard error of the mean.
[8]PC, the positive control diet, nutritionally complete diet containing 0.45% aP, 3.70% Ca and 0.16% Na (25 to 28 wk), 0.38% aP, 3.73% Ca and 0.15% Na (29 to 37 wk); NC, the negative control diet, similar to the PC diet but having 0.22% aP, 3.00% Ca and 0.13% Na (25 to 28 wk), 0.19% aP, 3.02% Ca and 0.13% Na (29 to 37 wk); NC + 150, NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 150, 300, 600, or 1,200 FTU/kg, respectively; and PC + 1,200, the PC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 1,200 FTU/kg.
[9]Body weight was used as a covariate.
[a-d]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

2.7 FIGURE

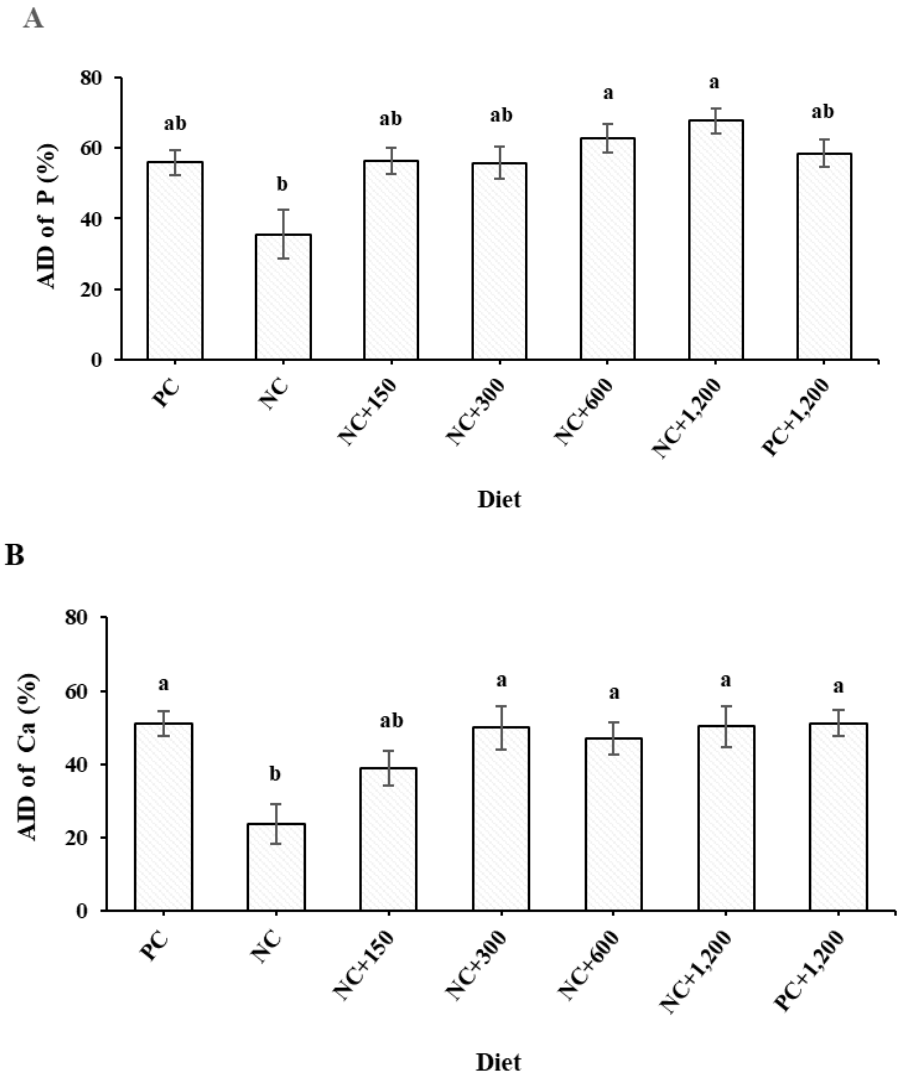

Figure 2. 1: Effect of dietary aP and Ca, and phytase supplementation on (A) apparent ileal digestibility (AID) of P ($P = 0.024$; n = 84) and (B) AID of Ca ($P = 0.010$; n = 84) at 37 wk of age. Apparent ileal digestibility of P and Ca was determined from 35 to 37 wk of age. The positive control (PC) diet, nutritionally complete diet containing 0.45% aP, 3.70% Ca and 0.16% Na (25 to 28 wk), 0.38% aP, 3.73% Ca and 0.15% Na (29 to 37 wk); NC, the negative control diet, similar to the PC diet but having 0.22% aP, 3.00% Ca and 0.13% Na (25 to 28 wk), 0.19% aP, 3.02% Ca and 0.13% Na (29 to 37 wk); NC + 150, NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with 150, 300, 600, or 1,200 FTU/kg, respectively; and PC + 1,200, the PC diet supplemented with 1,200 FTU/kg (Quantum Blue phytase, AB Vista, Marlborough, UK). Data are presented as least squares means with the respective standard errors of the mean. [a,b]Means within dietary treatment with no common superscript differ significantly ($P \leq 0.05$).

3. EFFECTS OF REDUCED DIETARY PHOSPHORUS AND CALCIUM, AND PHYTASE SUPPLEMENTATION ON PERFORMANCE, BONE CHARACTERISTICS, AND MINERAL RETENTION IN LAYER PULLETS

ABSTRACT

Although phytase is an important feed enzyme in commercial laying hen diets, there is limited information on its use during pullet rearing. Efficacy of an *Escherichia coli* 6-phytase in pullets was determined from 0 to 18 wk of age. One-d-old, White Leghorn pullets (n = 480) were randomly allocated to 6 treatments and housed in 60 pullet-rearing cages (n = 8/cage) in a completely randomized design. Treatments were: a positive control (PC) diet with a sequence of 0.48-0.45-0.37-0.45% available P (aP), 1.05-1.00-0.90-2.00% Ca, and 0.18-0.17-0.16-0.16% Na for Starter-Grower-Developer-Pre-lay phases, respectively; a negative control (NC) diet, similar to the PC but reduced in aP, Ca, and Na by 0.15%, 0.16%, and 0.035% of the diet in each phase, respectively; the NC diet supplemented with phytase at 300 (NC + 300), 600 (NC + 600), 1,200 (NC + 1,200) or 2,400 (NC + 2,400) phytase unit (FTU)/kg. Pullet performance was measured at 3, 6, 12, 15, and 18 wk of age. Bone breaking strength (BBS) and ash, total, cortical, and trabecular bone mineral density and cross-sectional areas were determined at 6, 15, and 18 wk of age. Retention of P and Ca were determined at 6 and 15 wk of age. Data were analyzed by one- and two-way ANOVA. Tukey's range test was used for multiple mean comparisons where $P \leq 0.05$. At 6 wk of age, NC birds had lower BBS ($P = 0.043$), but higher P retention ($P < 0.001$) than PC birds. The NC + 2,400 birds had greater P retention than the NC + 600 and PC birds at 6 wk. Each phytase supplementation group except NC + 300 had higher Ca retention than the NC at 15 wk ($P < 0.001$). Reduced dietary aP, Ca, and Na did not decrease pullet performance, but

decreased BBS at 6 wk. Pullets were able to adapt to the NC diet as the negative effect on BBS was no longer observed at later ages.

Keywords: layer pullet, phytase, phosphorus, bone, mineral retention

3.1 INTRODUCTION

Bone development during the pullet phase can affect laying hen bone quality in the production period (Regmi et al., 2015). Two processes of bone growth occur in young pullets; longitudinal growth of long bones starts after hatch, along with increased width (Whitehead, 2004). Both processes require P and Ca to form crystals of hydroxyapatite. Metabolism of P and Ca is closely interrelated and plays an important role in bone formation, growth, and development in laying hens.

Approximately 50 to 85% of P storage in plant-based ingredients, especially cereal grains, is bound in the form of phytate (a salt complex; Ravindran et al., 1995). Phytate refers to phytic acid which is generally chelated to various cations such as Ca^{++}, Fe^{++}, Zn^{++}, Mg^{++} and Mn^{++} (Selle et al., 2000). Chelation of phytate with amino acids, proteins, carbohydrates and lipids is also found in plant ingredients (Selle et al., 2000; Singh, 2008). Due to the chelation of phytate with other molecules, nutrient availability and utilization are reduced in chickens (Selle et al., 2000; Selle et al., 2009). Chickens can produce a very low level of phytase activity in the small intestinal brush border membrane, especially in the duodenum (Maenz and Classen, 1998). The activity of endogenous intestinal phytase increase with hen age (Marounek et al., 2010) due to the greater mucosal surface area of the small intestine (Morgan et al., 2015). In general, chickens poorly utilize phytate as a source of P (Maenz and Classen, 1998). Therefore, exogenous phytase has been used to enhance P utilization from poultry diets. Phytase supplementation reduced excretion of undigested phytate (Selle and Ravindran, 2007; Abudabos,

2012) and excessive inorganic P in manure (Zyla et al., 2012; Wang et al., 2013). Also, P has become one of the most expensive nutrients in poultry diets (Naves et al., 2016; Kazempour and Jahanian, 2017). The majority of phytase research in poultry has focused on broilers (Gautier et al., 2018; Sommerfeld et al., 2018a; Sommerfeld et al., 2018b; Bello et al., 2019; Leyva-Jimenez et al., 2019) and laying hens (Boling et al., 2000; Kim et al., 2017; Bello and Korver, 2019), with very little consideration given to the pullet phase from 0 to 18 wk. In fact, growth of structural (cortical and trabecular) bone continues up to the onset of sexual maturity during pullet rearing (Whitehead, 2004). Proper bone development during the pullet phase can be important to maintain bone quality during production cycle of laying hens (Regmi et al., 2015). There is scant published data regarding the potential of exogenous phytase in diets reduced in available P (**aP**) in growing pullets. Birds fed dietary 0.13% aP from 0 to 18 wk of age had lower body weight, and bone mineral density and mineral content relative to birds fed 0.23, 0.33, or 0.43% aP, and phytase supplementation at 300 phytase unit (**FTU**)/kg restored pullet performance and bone quality (Punna and Roland, 1997). Pullets fed 0.1% aP throughout pullet rearing or up to 36 wk of age had lower egg production and higher mortality from 21 to 36 wk of age relative to hens fed 0.2, 0.3, and 0.4% aP; 300 FTU phytase/kg negated these adverse effects (Punna and Roland, 1999). However, pullets fed 0.20, 0.15, and 0.18% aP from 0 to 6, 6 to 12, and 12 to 18 wk, respectively, did not respond to phytase supplementation because the reduced aP diet did not adversely affect pullet performance (Keshavarz, 2000). Therefore, phytase supplementation is unlikely to elicit a response if the dietary aP requirement of the bird is being met. Because of the scarcity of recent information on the use of phytase during the pullet phase, the objective of the present study was to determine the effect of an *Escherichia coli* 6-phytase in reduced aP and Ca diets on growth performance, uniformity, bone characteristics, and mineral retention in pullets

from 0 to 18 wk of age. We hypothesized that growth performance, uniformity, bone traits, and mineral retention would decrease in birds fed reduced dietary aP and Ca, and that phytase supplementation would return those parameters to the level of the nutritionally complete control diet.

3.2 MATERIALS AND METHODS

The animal protocol was approved by the University of Alberta Animal Care and Use Committee for Livestock and followed principles established by the Canadian Council on Animal Care (Canadian Council on Animal Care, 2009).

3.2.1 Animals and Housing

Four hundred and eighty H & N Nick Chick white egg pullets were obtained at one d of age and randomly assigned to six treatment groups. There were ten replicate cages of 8 chicks each (80 chicks per treatment group), housed in Specht pullet cages (L x W x H; 120 x 42 x 55 cm; Specht Canada Inc., Stony Plain, AB, Canada). The chicks were weighed individually and neck tagged before placement. Each replicate cage had one external feed trough (L x W x D; 115 x 22 x 7.5 cm) and 4 automatic nipple drinkers. The lighting program was 24L during the first four d, followed by 20L:4D for 11 d, 16L:8D for seven d, and then decreasing by 1 h of light each wk to a constant 11L:13D until 18 wk. The room temperature was thermostatically controlled and continuously monitored; temperature was 34°C at the first d, and was decreased by 0.5°C per d to a constant temperature of 21°C, which was held until the end of the experiment. Birds were checked twice daily and mortality recorded. Feed and water were provided ad libitum throughout the 18-wk experiment.

3.2.2 Experimental Diets

Six experimental diets (Table 3.1) were formulated. The positive control (**PC**) diet contained 0.48% aP, 1.05% Ca and 0.18% Na from 0 to 3 wk of age (starter), 0.45% aP, 1.00% Ca and 0.17% Na from 4 to 12 wk of age (grower), 0.37% aP, 0.90% Ca and 0.16% Na from 13 to 15 wk of age (developer), and 0.45% aP, 2.00% Ca and 0.16% Na from 16 to 18 wk of age (pre-lay). The PC diet was formulated to meet or exceed nutrient recommendations of the primary breeder (H & N International, 2012). The negative control (**NC**) diet was similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na during the starter period, 0.30% aP, 0.84% Ca and 0.13% Na during the grower period, 0.22% aP, 0.74% Ca and 0.12% Na during the developer period, and 0.30% aP, 1.84% Ca and 0.12% Na during the pre-lay phase. The remaining diets were the NC diet supplemented with 300 (**NC + 300**), 600 (**NC + 600**), 1,200 (**NC + 1,200**), or 2,400 (**NC + 2,400**) FTU phytase/kg, respectively.

All experimental diets were corn-soy-canola meal-based, fed as a mash (Table 3.1). Each experimental dietary treatment provided approximately 2,925 kcal/kg ME from 0 to 3 wk of age and 2,955 kcal/kg ME from 4 to 18 wk of age following the Nick Chick white egg layer management guide (H & N International, 2012). Dietary P and Ca were determined using methods 935.13 and 964.06, respectively (AOAC, 1990) and are presented in Table 3.2. The phytase used was a thermo-tolerant, enhanced *Escherichia coli* 6-phytase produced in *Trichoderma reesei* (Quantum Blue, AB Vista; Marlborough, UK). The phytase was added on top of the NC diets. Feed phytase activities (Table 3.2) were analyzed by Enzyme Services Consultancy (ESC; Ystrad Mynach, UK). Quantiplate ELISA Kits for Quantum Blue were used (Envirologix method AP181 with some modifications; designated ESC Standard Analytical Method SAM099). Celite (Celite Corporation, Lompoc, California USA), as an indigestible

marker, was mixed in the experimental diets at a concentration of 1%. The Celite-containing diets were fed to all birds for two weeks from 4 to 6 and from 13 to 15 wk of age. Feed samples were collected at the time of mixing, ground and stored at -20°C until further analysis.

3.2.3 Data and Sample Collection

Each bird was individually weighed for BW at 0, 3, 6, 12, 15 and 18 wk of age. Body weight gain (**BWG**) and feed intake (**FI**) were recorded within each period to calculate mortality-corrected feed conversion ratio (**FCR**; g feed/g gain) on a cage basis. Uniformity within each replicate cage was calculated as a percentage of pullets within 10 percent of the average body weight of the cage (H & N International, 2012) after each weighing. Ten birds per treatment (one bird from each replicate cage) at each of 6 and 15 wk of age, and 8 birds per treatment at 18 wk of age (randomly selected at the beginning of the trial) were euthanized by cervical dislocation, and the left and right femurs removed. Femurs were cleaned of soft tissue except for the cartilage caps and frozen at -20°C until further analysis. Total excreta output from each cage was collected for two consecutive d at each of 6 and 15 wk of age. The excreta samples were dried at 60°C overnight, ground, and stored at -20°C for future determination of P and Ca retention.

3.2.4 Bone Characteristics

3.2.4.1 Bone Breaking Strength

The frozen right femurs were thawed at 4°C for 24 h prior to determination of bone breaking strength (**BBS**) and ash. Prior to breaking, each femur was marked at the proximal 25%, the midpoint and distal 25% (25%, 50%, and 75% from the proximal epiphysis of the length of the femur, respectively) determined using a digital caliper (Model CD-8”C, Mitutoyo Corp.,

Japan). Bone breaking strength was measured as described by Pongmanee et al. (2020) using an Instron Materials Tester (Model 4411, Instron Corp., Canton, MA) with Bluehill 2 software 2.29.

3.2.4.2 Bone Ash

The right femur, after BBS measurement, was cut using a Dremel tool (Dremel MultiPro Model 395, Racine, WI) to separate proximal end (25%), mid-bone (50%), and distal end (25%). The 25% proximal end represented all bone tissue from the proximal tip of the femur to 25% of the length of the bone from the proximal end. The 25% distal end represented all bone tissue from the distal tip of the femur to 25% of the length of the femur from the distal end. The 50% mid-bone represented the remaining segment of bone between the 25% proximal and 25% distal sections. Each bone segment was oven-dried, weighed and subsequently ashed in a muffle furnace as described by Pongmanee et al. (2020). The ash was placed in a desiccator at room temperature for 2 h to cool, then weighed to obtain ash content (in g) and the percent ash of each of the proximal, mid-bone, and distal section was calculated as a percentage of dry weight. Total ash was the summation of the three individual sections.

3.2.4.3 Bone Densitometry

The frozen left femur from each of the sampled bird was thawed at 4°C for 24 h prior to quantitative computed tomography (**QCT**) analysis using a Stratec Norland XCT (XCT Research SA, Norland Corp., Fort Atkinson, WI) scanner with a 50 kV x-ray tube (Korver et al., 2004; Saunders-Blades and Korver, 2015). A 1-mm thick cross-sectional x-ray slice with a voxel size of 0.1 mm was taken at 20%, 50% (mid-point), and 80% of the length of the left femur from the proximal epiphysis of the bone. The QCT scan locations were chosen to ensure that measurements were taken within the segment cut out for the bone ash measures. Norland XMENU software version 5.40C was used to determine total, cortical, and trabecular bone

mineral density (**BMD**) and cross-sectional areas (Saunders-Blades et al., 2009). The threshold used in this study was 400 mg/cm^3 to separate cortical and subcortical bone from trabecular bone (Korver et al., 2004). The total measure was the weighted average of the cortical and trabecular bone fractions, and reflected the density or area of each bone compartment. Cortical BMD was the outer shell of the bone that was determined to have a density of > 500 mg/cm^3 (Saunders-Blades et al., 2009). The QCT technology used in the current study is not able to distinguish between trabecular and medullary bone (Korver et al., 2004). For this study, bone tissues in the trabecular space were assumed to contain only trabecular bone. At the onset of sexual maturity, osteoblasts cease structural bone formation, but begin to form medullary bone within the trabecular space (Whitehead, 2004). In the current study, the birds were not photostimulated before bone sampling, and no bird had started laying eggs. Also, upon dissection to collect bone samples at the end of the trial, no ovarian development was observed. Therefore, it was assumed that there was no medullary bone present in the trabecular space. Bone mineral content (**BMC**; mg/mm) represents the amount of bone mineral contained in a 1-mm thick slice of the bone, and was calculated by BMD multiplied by the cross-sectional area (Saunders-Blades et al., 2009).

3.2.5 P and Ca Retention Assays

Frozen diets and excreta samples were thawed at room temperature for 24 h. Two g of feed, or 1 g of excreta samples were weighed and used for each of dry matter determination (method 930.15; AOAC, 1990), acid insoluble ash (**AIA**; Scott and Boldaji, 1997), P (method 935.13; AOAC, 1990) using a spectrophotometer (SpectraMax Plus 384 Microplate Reader, Molecular Devices LLC, San Jose, CA) at 400 nm, and Ca (method 964.06; AOAC, 1990) using a nitrous oxide-acetylene fueled flame atomic absorption spectrometer (Varian AA240FS, Agilent

Technologies, Santa Clara, CA) at 422.7 nm. The procedure of each analysis was described by Bello and Korver (2019).

3.2.5.1 Calculation of P and Ca Retention

The analyzed AIA, total P, and Ca concentrations from the Celite-containing diets and excreta were used to calculate P and Ca retention on a dry matter basis using the following equation:

$$\text{Retention (\%)} = 100 - [((AIA_{diet}/AIA_{excreta}) \times (Mineral_{excreta}/Mineral_{diet})) \times 100]$$

Where AIA_{diet} was the initial AIA concentration in the diet; $Mineral_{diet}$ was the initial dietary concentration of P or Ca; $AIA_{excreta}$ was the concentration of AIA in excreta; and $Mineral_{excreta}$ was the respective concentration of the mineral (P or Ca) in the excreta.

3.2.6 Statistical Analysis

The cage was the experimental unit for all measures, with ten replicate cages assigned to each of the 6 dietary treatments. A completely randomized design was used for placing chicks in cages and in the assignment of cages to dietary treatments. One-way ANOVA was conducted using the Mixed procedure of SAS (SAS Institute Inc., 2012) for BBS within each age. Two-way ANOVA was used to determine the effect of diet, age, and their interaction using the Mixed procedure for BWG, FI, FCR, uniformity, bone ash, BMD, bone cross-sectional area, and BMC of the total, cortical, and trabecular bone, except BW using the Mixed and the HPMixed procedures of SAS (SAS Institute Inc., 2012). Body weight was used as a covariate for determination of bone characteristics. Treatment means were separated using the LSMEANS statement. Tukey's range test was used to compare multiple mean comparisons. Statistical significance was considered when $P \leq 0.05$. Trends were reported where $0.05 < P \leq 0.10$. Values are presented as least squares means (**LSM**) with the respective standard errors of the mean.

3.3 RESULTS

3.3.1 Analyzed Dietary Ca, total P and Phytase Activity

For most dietary treatments throughout the trial, the analyzed Ca levels were approximately 23% higher than the formulated levels (Table 3.2). However, the NC and NC + 600 from 0 to 3 wk, and the PC, NC and NC + 300 from 13 to 15 wk had 8 to 18% lower analyzed Ca than formulated. From 16 to 18 wk, the analyzed Ca levels in the NC phytase-containing treatments were 29 to 52% higher than the formulated. The analyzed total P levels were on average 13% (ranging from 2 to 23%) lower for all dietary treatments. On average, feed phytase activities were higher than the planned levels (505, 891, 1,755 and 3,283 vs. 300, 600, 1,200 and 2,400 FTU/kg, respectively) but were consistent with the assumed stepwise increase of phytase activity among treatments.

3.3.2 Pullet Performance

Pullet BW increased with age ($P = 0.002$; Table 3.3) in each dietary treatment. However, the 18-wk-old NC + 300 pullet weights were only greater than the 15 wk weights of the NC + 300 and NC + 600 groups ($P = 0.018$; Table 3.3), due to low rate of gain in this treatment from 7 to 18 wk of age ($P = 0.002$; Table 3.3). The NC + 1,200 pullets maintained BWG for a longer period of time than the other treatments; the NC + 1,200 from 7 to 12 wk was not different from the gain of the PC from 4 to 6 wk. The highest BWG was observed from 3 to 6 wk of age ($P < 0.001$; Table 3.3). Neither diet by age interaction nor dietary treatment affected FI (data not shown). Feed intake increased with bird age to 15 wk of age and then decreased slightly during the pre-lay period (16 to 18 wk; $P < 0.001$). Feed conversion ratio increased with pullet age ($P < 0.001$; Table 3.3). The increase in FCR from 13 to 15 wk, to 16 to 18 wk was significant for the PC and the NC treatments ($P = 0.008$; Table 3.3), but was not significant for phytase-

supplemented treatments. Neither dietary treatment nor the interaction of diet and age affected uniformity ($P > 0.05$). However, uniformity was higher at 12 wk (88.7 $\pm$ 1.90 %) and 15 wk of age (89.2 $\pm$ 1.61 %) than the other ages ($P < 0.001$). There was no mortality in the NC, NC + 300, NC + 600, and NC + 2,400 groups, but total mortality to 18 wk was 1.25% in both the PC and NC + 1,200 treatments. One bird from each of the NC and NC + 1,200 groups was removed at 6 wk of age due to sexing errors, and one bird from the NC + 2,400 was culled at 17 wk of age because of injury unrelated to dietary treatment.

3.3.3 Bone Characteristics

At 6 wk of age, the NC birds had lower femur BBS (10.52 $\pm$ 0.34 kg-force) than the PC birds (12.02 $\pm$ 0.43 kg-force; $P = 0.043$; Figure 3.1). Phytase supplementation to the NC diet resulted in similar BBS to the PC diet; each of the phytase treatments were intermediate to, and not different from the PC and NC diets. There were no dietary effects on the BBS at 15 wk (14.0 $\pm$ 0.22 kg-force) and 18 wk of age (14.4 $\pm$ 0.35 kg-force), but BBS increased as the birds aged (data not shown).

There were no interactions or diet effects on dry bone weight, nor bone ash content nor percent bone ash. Dry bone weights of the proximal ($P < 0.001$), mid-bone ($P < 0.001$), distal ($P < 0.001$), and total ($P < 0.001$) regions were highest at 15 wk of age (Table 4). Total and proximal bone ash content increased from 6 to 15 wk and plateaued from 15 to 18 wk of age. However, distal bone ash content increased with pullet age ($P < 0.001$). Mid-bone ash content increased from 6 to 15 wk of age and slightly decreased from 15 to 18 wk of age ($P < 0.001$). At 18 wk of age, the percent ash of the proximal, distal, and total was higher than at 6 and 15 wk of age (Table 3.4). The percent ash of the mid-bone was lower at 15 wk relative to those of 6 and 18 wk of age ($P < 0.001$).

Dietary treatment did not affect BMD, bone cross-sectional area, or BMC as measured by QCT, nor were there any interactions with age. However, the NC + 600 birds tended to have lower distal cortical BMD than the PC birds (876 $\pm$ 3.75 vs. 893 $\pm$ 3.08 mg/cm^3; $P = 0.051$; Table 3.5). The NC + 2,400 birds also tended to have greater distal trabecular BMD than the NC + 600 birds (83.8 $\pm$ 2.62 vs. 75.7 $\pm$ 3.80 mg/cm^3; $P = 0.052$; Table 3.5). Birds had higher proximal total BMD at 18 wk than at 15 wk of age ($P = 0.023$; Table 3.5). At 18 wk of age, birds tended to have higher distal total BMD than at 6 and 15 wk of age ($P = 0.097$). Mid-bone total BMD at 6 wk was greater than at 15 and 18 wk of age ($P < 0.001$). Pullets at 6 wk of age had lower proximal ($P < 0.001$), mid-bone ($P < 0.001$), and distal ($P < 0.001$) cortical BMD than those of birds at 15 and 18 wk of age. Birds at 15 wk of age had lower proximal ($P < 0.001$), mid-bone ($P < 0.001$), and distal ($P < 0.001$) trabecular BMD relative to at 6 and 18 wk of age. At 6 wk of age, birds had the lowest mid-bone total ($P = 0.022$; Figure 3.2A) and trabecular ($P = 0.002$; Figure 3.2C) bone cross-sectional areas, however, birds at this age had the highest mid-bone cortical bone cross-sectional area ($P = 0.035$; Figure 3.2B). Birds had a higher distal trabecular bone cross-sectional area at 15 wk of age compared to those of birds at 6 and 18 wk of age ($P < 0.001$; Figure 3.2D). Other cross-sectional area measures were not affected by age. At 18 wk of age, birds had greater proximal ($P = 0.020$) and distal ($P = 0.018$) total BMC than at 6 wk of age (Table 3.6). Birds maintained similar mid-bone total and cortical BMC from 6 to 18 wk of age. Proximal ($P < 0.001$), mid-bone ($P < 0.001$), and distal ($P < 0.001$) trabecular BMC were greater at 18 wk than at 15 wk of age, but were not different from that at 6 wk of age.

3.3.4 P and Ca Retention

There was a diet by age interaction on P retention ($P < 0.001$; Figure 3.3A). At 6 wk, birds fed the NC diet had higher P retention than that of the PC group, and birds fed the NC +

300 diet had higher P retention than those of the NC + 600 and the NC + 1,200 groups. However, at 15 wk of age, there were no differences between the PC and the NC groups. No level of phytase supplementation increased P retention as compared to the NC group, but the PC and NC + 600 groups had higher P retention than the NC + 300 and the NC + 2,400 groups. There was interaction of diet and age for Ca retention ($P < 0.001$; Figure 3.3B). There were no differences in Ca retention between the PC and the NC birds at 6 wk of age. Phytase supplementation at any level maintained Ca retention relative to the NC group except the NC + 300. At 15 wk of age, there was no difference between the PC and the NC groups. However, birds fed the NC + 600, NC + 1,200, and NC + 2,400 diets had higher Ca retention than those of the NC and the NC + 300 groups. Ca and P retention were lower at 15 wk of age than at 6 wk of age.

3.4 DISCUSSION

Proper pullet management is essential to maintain a healthy and productive layer flock, and allows a high peak, a long laying cycle and good persistency of egg production. A majority of phytase studies in laying hens have focused on the laying period, because there is a measurable return from egg production. The economic impact is less clear for pullets. However, if phytase supplementation during the pullet phase results in increased structural bone deposition prior to the onset of lay, the hens may be better able to withstand the increased demand for bone calcium mobilization associated with extended production cycles. If phytase were used effectively to reduce the need for inorganic Ca and P supplementation, the cost of pullet rearing could be reduced without compromising the long-term skeletal health of the hen. Phytase supplementation in a reduced-P diet during the pullet phase increased pullet body weight and bone quality (Punna and Roland, 1997), and increased egg production and eggshell quality from 21 to 36 wk of age (Punna and Roland, 1999). There is very limited information on the use of

phytase in the pullet rearing phase. We hypothesized that the NC diets (reduced to 0.33% aP, 0.89% Ca and 0.14% Na during the starter period, 0.30% aP, 0.84% Ca and 0.13% Na during the grower period, 0.22% aP, 0.74% Ca and 0.12% Na during the developer period, and 0.30% aP, 1.84% Ca and 0.12% Na during the pre-lay phase) would decrease growth performance and bone traits, and that phytase supplementation in the NC diets would return performance and bone quality to the level of the PC diet. However, the NC diet did not affect BW, BWG, FI, FCR or pullet uniformity. White Leghorn pullets require 0.40, 0.35, and 0.30% aP, and 0.90, 0.80, and 0.80% Ca from 0 to 6, 6 to 12, and 12 to 18 wk of age, respectively, and 0.15% Na from 0 to 18 wk of age (NRC, 1994). However, previous research has shown that the NRC (1994) requirements for aP for rearing pullets is overestimated. Pullets can adapt to moderately low dietary aP, since reductions from 0.50, 0.475, and 0.45% to 0.20, 0.175, and 0.15% dietary aP from 0 to 4, 4 to 8, and 8 to 16 wk of age, respectively, was adequate to support growth without reducing pullet performance and health (Jing et al., 2018). Similarly, reducing dietary aP from 0.40, 0.35, and 0.30% to 0.20, 0.15, and 0.10% from 0 to 6, 6 to 12, and 12 to 18 wk of age, respectively, was adequate for pullet performance (Keshavarz, 2000). The experimental diets in this study were formulated based on the primary breeder management guide (H & N International, 2012), which contained higher dietary aP, Ca, and Na levels than the recommendation by NRC (1994). Commercial recommendations result in over-feeding of aP in laying hen production (Applegate and Angel, 2014; Li et al., 201b), and a similar situation exists for pullets (Jing et al., 2018). Therefore, the levels of dietary aP, Ca, and Na in the NC diets were not sufficiently reduced to impair pullet growth.

Although the NC diet did not decrease pullet growth, BBS of pullets in this treatment was decreased at 6 wk of age. This may have been due to the analyzed Ca (0.82%) being slightly

lower than formulated (0.89%) from 0 to 3 wk, which lower than the 0.9% recommended by the NRC (1994) during the same period. Femur BBS was not affected by diet at 15 or 18 wk of age, possibly because the rapid growth of bones at a younger age could make the birds more sensitive to reductions in dietary aP and Ca than at later ages (Jing et al., 2018). Also, the birds may have been able to adapt to the reduced dietary aP and Ca over time (Ieda et al., 1999; Proszkowiec-Weglarz et al., 2019). Femur BBS increased with pullet age as the pullets deposited structural bone over time prior to the onset of sexual maturity (Whitehead, 2004; Fleming et al., 2006; Kerschnitzki et al., 2014). This is supported by our QCT data; femur cortical BMD increased from 6 to 18 wk of age and trabecular BMC accumulated from 15 to 18 wk of age. Cortical and trabecular bone tissues, which contribute to strength (Fleming et al., 1998; Webster, 2004; Bello et al., 2019; Bello and Korver, 2019), do not completely form within the first 6 wk of life, but are fully developed by the end of the pullet rearing phase (Whitehead, 2004).

The NC diet was not adequate to maintain BBS during the first 6 wk, but dry bone weight, bone ash content, percent ash, BMD, bone cross-sectional area and BMC were not influenced by the NC diet. Sequences of 0.33-0.30-0.22-0.30% aP, and 0.89-0.84-0.74-1.84% Ca from 0 to 3, 4 to 12, 13 to 15, and 16 to 18 wk, respectively, in the NC diet were sufficient to maintain overall bone quality to 18 wk of age. Reducing dietary aP from 0.40-0.35-0.30% to 0.30-0.25-0.20 or to 0.20-0.15-0.10%, with dietary Ca of 0.90-0.80-0.80% from 0 to 6, 6 to 12, and 12 to 18 wk of age, respectively, did not decrease bone weight and ash at 6, 12, or 18 wk of age (Keshavarz, 2000). A sequence of decreasing aP from 0.500-0.475-0.450% to 0.200-0.175-0.150%, with 1.10-1.10-0.95% Ca from 0 to 4, 4 to 8, and 8 to 16 wk, respectively, did not decrease BMD, BMC, or ash content (Jing et al., 2018). The analyzed total P levels in the NC diet in our study were 0.53%, 0.47%, 0.41% and 0.53% in the starter, grower, developer and pre-

lay period, and were slightly lower than the formulated levels; however, pullets performed well and the low analyzed total P did not decrease bone measures. The analyzed Ca levels in the NC diet were slightly lower than formulated (0.62 vs 0.74% Ca) from 7 to 12 wk of age. This further suggests that the NC diets in this study still met the requirements for aP and Ca of pullets for most measures, and therefore minimizing the potential for phytase to show an impact. Dry weight of each bone section was highest at 15 wk of age, suggesting that bone growth increases rapidly in pullets as osteoblasts deposit organic matrix and matrix vesicles are formed, which subsequently mineralizes with the formation of hydroxyapatite crystals (Watkins, 1992; Kerschnitzki et al., 2014) along with high BWG from 6 to 15 wk of age. The percent bone ash and total BMC increased from 6 to 18 wk of age with increasing bone deposition (Fleming et al., 1998; Whitehead, 2004; Eusebio-Balcazar et al., 2018). From 6 to 18 wk of age, mid-bone total cross-sectional area increased. Long bones rapidly increase in diameter prior to lay, leaving pores that are not filled with mineral (Riddell, 1992; Anderson and Adams, 1994; Yaissle and Lilburn, 1998; Fleming et al., 2006; Khanal et al., 2019) before the switch from structural to medullary bone deposition (Whitehead, 2004; Fleming et al., 2006; Kerschnitzki et al., 2014). Rapid bone elongation causes cortical thinning at the mid-shaft of the bone (Fleming et al., 2006). As pullets grow, the diameter of the long bones increases (Regmi et al., 2015; van der Pol et al., 2015; Akbari Moghaddam Kakhki et al., 2019b). Bone cross-sectional area increases by approximately 12 and 25% in Lohmann Brown-Classic and Bovans White, respectively, from 13 to 18 wk of age (Eusebio-Balcazar et al., 2018). Proximal, mid-shaft, and distal trabecular BMD and BMC decreased from 6 to 15 wk, likely because bone within the trabecular space is resorbed to and re-deposited on the periosteal surface, thus increasing the size of the trabecular cavity (Whitehead, 2004; Fleming et al., 2006). However, trabecular BMD and BMC of the proximal,

mid-shaft, and distal regions increased from 15 to 18 wk of age. The pullets were close to the beginning of sexual maturity, and the level of estrogen may have been increasing at this stage (Fleming et al., 1998), in which case the pullets were preparing to deposit medullary bone to support eggshell formation. Medullary bone formation begins at 10 to 14 d before the onset of lay (Hurwitz, 1964; Taylor and Dacke, 1984; Singh et al., 1986) or at about 16 wk of age; abundant medullary bone was observed around 17 wk of age (Deng et al., 2010).

The assessment of specific bone regions has been reported to provide a better characterization of responses to dietary treatments in layers (Akbari Moghaddam Kakhki et al., 2019a; Khanal et al., 2019) than whole bone measurements. However, in the current study, treatments effects on dry bone weight, ash content and the percent ash within each of the individual bone regions were similar to the responses for the entire bone at each age. Therefore, the assessment of a single bone segment would be simpler, yet adequate for determining effects on bone mineralization in pullets (Khanal et al., 2019). Using QCT, the cortical and trabecular BMD at the distal end of the femur was more responsive than at the proximal and mid-shaft regions to the dietary treatments. The mid-bone and distal locations were more responsive than the proximal region to the effects of pullet age. Taken together, the distal metaphysis and mid-diaphysis scan locations may be more sensitive to dietary treatment and age than the proximal location. Tibia cortical density and thickness of the mid-bone and distal regions are more response to treatments than the proximal end in 16 wk White Leghorn pullets (Regmi et al., 2015). However, in white egg-laying hens (30 to 70 wk), the pattern of bone mineralization at the proximal metaphysis was the same as in the diaphyseal region (Bello and Korver, 2019).

The P retention at 6 wk of age was higher in pullets fed the NC diet relative to the PC diet, indicating that the NC birds likely responded by increasing P absorption in the small

intestine (Murer et al., 2001; Huber et al., 2015) and decreasing renal P excretion (Manangi et al., 2018; Munoz et al., 2018). The NC birds received lower dietary P than the NRC (1994) recommendation during this short period of time (0.43% total P from 4 to 6 wk of age). In response, the increase in efficiency of P absorption by the NC birds was adequate to support growth performance, but not BBS at 6 wk of age. Feeding 0.30 or 0.20% aP to 5 wk of age in Babcock B300 pullets increased total P retention and decreased total P excretion relative to the NRC (1994) recommended level of 0.40% aP (Keshavarz, 2000). In laying hens and broilers, reduced dietary aP results in up-regulation of intestinal NaPi IIb co-transporter (Li et al., 2012; Nie et al., 2013) and renal NaPi IIa co-transporter mRNA levels (Huber et al., 2006). These mechanisms would also be expected to occur in pullets. Phytase supplementation at any level to the NC diet did not increase P retention compared to the NC group at 6 and 15 wk of age. This could be because dietary P was adequate at both ages, and therefore the phytate P liberated by phytase was not needed and excreted. Pullets had high P retention (approximately 40%) at 6 wk, but it was decreased to 20% at 15 wk of age in this study. Younger pullets require higher dietary P levels (NRC, 1994), and have higher P retention than at older ages (Keshavarz, 2000; Jing et al., 2018) for bone formation, nerve function, phospholipid synthesis and energy metabolism through ATP (Li et al., 2017). In diets without phytase supplementation, pullets at 5 wk retained approximately 50% of dietary P, which decreased to about 30% at 18 wk of age (Keshavarz, 2000). The effect of phytase supplementation on Ca retention was inconsistent in the current study, likely because of the lack of response in the NC birds. At 6 wk of age, the NC + 300 birds had lower Ca retention than the NC birds. The reason for this is unclear and there is scant published information on phytase use during the pullet rearing period. However, an increase in unbound P in the gastrointestinal tract interferes with Ca utilization during the laying period

(Pelicia et al., 2009). In broilers, excesses or deficiencies of either P or Ca directly interfere with the homeostasis of the other (Li et al., 2015; Majeed et al., 2020). Analyzed total P in the NC + 300 diet (0.54%) was slightly higher than in the NC diet (0.46%), perhaps contributing to the lower Ca retention at 6 wk in the NC + 300 birds. High doses of phytase (600, 1,200, or 2,400 FTU/kg) supplementation resulted in greater Ca retention than at 300 FTU/kg at 15 wk, suggesting that high doses of phytase may have retained a high residual phytase activity in the gastrointestinal tract (Bello et al., 2019), increased phytate P solubility and hydrolysis (Tran et al., 2011), and increased Ca availability for absorption and subsequently increased Ca digestibility and retention (Adeola and Walk, 2013; Babatunde et al., 2019a; Babatunde et al., 2019b).

In conclusion, reducing aP by 0.15% and Ca by 0.16% of the diet up to 18 wk of age after placement did not cause adverse effects on pullet performance, indicating that the birds were able to meet their P and Ca requirements for growth. Although a minor negative effect of reduced aP and Ca in the NC diet on BBS was observed at 6 wk of age, the NC birds were able to overcome that effect by increasing the efficiency of intestinal Ca and P absorption at later ages. Therefore, the lack of a phytase response in this scenario was not surprising. However, exogenous phytase could allow the poultry industry to increase the use of high-phytate ingredients (e.g. canola meal) in pullet diets. Further reductions in dietary Ca and total P can also be achieved in order to take advantage of the ability of phytase to degrade phytate to increase P retention. This would reduce inorganic P usage which would subsequently decrease P excretion to the environment. Current primary breeder recommendations for pullet dietary Ca and aP are above the actual requirements of the birds. To make use of more of Ca and P that are already in

the diet by exogenous phytase, Ca and P levels could be substantially below that recommended by the primary breeders, without compromising pullet performance or bone quality.

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3.6 TABLES

Table 3. 1: The ingredient and nutrient composition of positive control and negative control diets from 0 to 18 wk of age.

	Starter (0 to 3 wk)		Grower (4 to 12 wk)		Developer (13 to 15 wk)		Pre-lay (16 to 18 wk)	
	PC[1]	NC[2]	PC[1]	NC[2]	PC[1]	NC[2]	PC[1]	NC[2]
Ingredients (%)								
Corn	62.67	59.86	66.07	63.86	66.27	64.63	61.52	63.12
Soybean meal	27.92	31.84	15.87	19.12	10.43	9.04	12.12	11.96
Canola meal	5.00	5.00	7.00	7.00	10.00	12.00	8.00	8.00
Canola oil	0.00	0.00	0.00	0.00	0.00	0.00	1.64	1.09
Triticale DDGS	0.00	0.00	7.00	7.00	10.00	12.00	10.00	10.00
Calcium carbonate	1.55	1.42	1.44	1.44	1.40	1.41	4.08	4.09
Dicalcium phosphate	1.87	1.03	1.58	0.74	1.07	0.18	1.54	0.72
Salt	0.38	0.29	0.36	0.29	0.33	0.24	0.33	0.24
L-lysine HCl	0.00	0.00	0.13	0.02	0.00	0.00	0.08	0.08
DL-Methionine	0.10	0.06	0.05	0.02	0.00	0.00	0.19	0.19
Vitamin-mineral premix[3,4]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Phytase[5] (g/kg)	0.00	variable[6]	0.00	variable[6]	0.00	variable[6]	0.00	variable[6]
Calculated nutrient composition (%)								
ME (kcal/kg)	2,925	2,925	2,955	2,955	2,955	2,955	2,955	2,955
Crude protein	20.91	22.62	18.17	19.49	17.38	17.93	17.28	17.33
Calcium	1.05	0.89	1.00	0.84	0.90	0.74	2.00	1.84
Available phosphorus	0.48	0.33	0.45	0.30	0.37	0.22	0.45	0.30
Phytate phosphorus	0.26	0.27	0.25	0.26	0.26	0.28	0.25	0.25
Total phosphorus	0.74	0.60	0.70	0.56	0.63	0.50	0.70	0.55
Sodium	0.18	0.14	0.17	0.13	0.16	0.12	0.16	0.12

[1]PC = positive control diet.

[2]NC = negative control diet; NC diet was mixed as a single batch and subdivided into the NC and various NC phytase-containing diets for each phase.

[3]Vitamin-mineral premix provided (units per kilogram of feed) from 0 to 15 wk of age: vitamin A, 10,000 IU; vitamin D_3, 4,000 IU; vitamin E, 50 IU; vitamin K (menadione), 4 mg; riboflavin, 10 mg; D-pantothenic acid, 15 mg; vitamin B_{12}, 0.02 mg; pyridoxine, 5 mg; thiamine, 4 mg; folic acid, 2 mg; niacin, 65 mg; biotin, 0.2 mg; iodine, 1.65 mg; copper, 20 mg; iron, 80 mg; selenium, 0.3 mg; manganese, 120 mg; zinc, 100 mg.

[4]Vitamin-mineral premix provided (units per kilogram of feed) from 16 to 18 wk of age: vitamin A, 12,500 IU; vitamin D_3, 3,125 IU; vitamin E, 40 IU; vitamin K (menadione), 2.5 mg; riboflavin, 7.5 mg; D-pantothenic acid, 12.5 mg; vitamin B_{12}, 0.01875 mg; pyridoxine, 5 mg; thiamine, 2.55 mg; folic acid, 0.625 mg; niacin, 37.5 mg; biotin, 0.15 mg; iodine, 1.65 mg; copper, 15 mg; iron, 80 mg; selenium, 0.3 mg; manganese, 88 mg; zinc, 100 mg.

[5]Quantum Blue phytase (5,000 FTU/g of premix; AB Vista, Marlborough, UK).

[6]Quantum Blue phytase was added to the NC diet at 0.06 (300 FTU/kg), 0.12 (600 FTU/kg), 0.24 (1,200 FTU/kg) or 0.48 g/kg diet (2,400 FTU/kg) for the NC phytase-containing diets.

Table 3. 2: Analyzed Ca, total P and phytase activity levels of experimental pullet diets[1].

	Diet[2]					
	PC	NC	NC + 300	NC + 600	NC + 1,200	NC + 2,400
Calcium (% of the diet, as-fed basis)						
0 to 3 wk	1.19	0.82	1.07	0.74	0.93	0.97
4 to 6 wk[3]	1.32	1.19	1.06	1.08	1.04	1.05
7 to 12 wk	1.12	0.83	0.94	1.08	0.97	0.96
13 to 15 wk[3]	0.74	0.62	0.64	1.01	0.93	0.96
16 to 18 wk	2.11	1.90	2.50	2.38	2.79	2.64
Total phosphorus (% of the diet, as-fed basis)						
0 to 3 wk	0.57	0.53	0.52	0.49	0.52	0.52
4 to 6 wk[3]	0.63	0.46	0.54	0.46	0.47	0.47
7 to 12 wk	0.54	0.47	0.48	0.49	0.48	0.50
13 to 15 wk[3]	0.57	0.41	0.44	0.42	0.42	0.42
16 to 18 wk	0.64	0.53	0.54	0.49	0.49	0.53
Phytase activity[4] (FTU/kg)						
0 to 3 wk	<50	<50	489	848	1,700	3,300
4 to 12 wk	<50	<50	602	973	1,960	3,550
13 to 15 wk	<50	<50	456	951	1,810	3,360
16 to 18 wk	<50	<50	471	793	1,550	2,920

[1]Feed provided in mash form.

[2]PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.

[3]Analyzed values for each diet were used to calculate retention of P and Ca for each respective treatment.

[4]Analyzed by Enzyme Services Consultancy (ESC; Ystrad Mynach, UK) using Quantiplate ELISA Kits for Quantum Blue (Envirologix method AP181 with some modifications; designated ESC Standard Analytical Method SAM099).

Table 3. 3: Interaction of diet and age on performance of pullets fed different dietary aP and Ca, and phytase supplementation.

	Diet[1]											
	PC		NC		NC + 300		NC + 600		NC + 1,200		NC + 2,400	
	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]
Age (wk)						Body weight (g)						
1[4]	36[g]	0.33	36[g]	0.31	36[g]	0.38	36[g]	0.31	37[g]	0.32	37[g]	0.31
3[4]	196[f]	2.68	185[f]	2.50	189[f]	1.91	186[f]	2.43	190[f]	1.91	192[f]	2.04
6[4]	441[e]	6.29	454[e]	3.96	440[e]	4.30	449[e]	3.29	444[e]	5.79	446[e]	5.64
12[5]	979[d]	10.54	967[d]	10.42	967[d]	10.33	961[d]	7.25	989[d]	9.65	975[d]	8.94
15[5]	1,143[b,c]	11.81	1,137[b,c]	10.25	1,121[c]	10.52	1,116[c]	9.48	1,153[b,c]	8.90	1,145[b,c]	8.90
18[6]	1,238[a]	15.69	1,231[a]	16.96	1,209[a,b]	17.10	1,225[a]	13.73	1,261[a]	14.82	1,242[a]	16.97
Source of variation						Prob > F						
Diet						0.002						
Age						<0.001						
Diet x age						0.018						
Age (wk)						Body weight gain (g/d per bird)						
0 to 3[4]	7.2[e,f,g]	0.13	6.7[f,g,h]	0.11	6.9[e,f,g]	0.09	6.8[f,g,h]	0.10	7.0[e,f,g]	0.09	7.04[e,f,g]	0.09
4 to 6[4]	11.7[a,b]	0.34	12.8[a]	0.16	12.0[a]	0.19	12.5[a]	0.08	12.1[a]	0.23	12.1[a]	0.24
7 to 12[5]	10.0[c]	0.25	9.4[c]	0.23	9.8[c]	0.25	9.3[c]	0.20	10.2[b,c]	0.20	9.8[c]	0.28
13 to 15[5]	7.8[d,e]	0.22	8.1[d]	0.18	7.7[d,e,f,g]	0.28	7.4[d,e,f,g]	0.18	7.8[d,e,f]	0.27	8.1[d]	0.16
16 to 18[6]	4.2[i]	0.36	4.3[i]	0.54	4.6[h,i]	0.61	5.2[i]	0.34	5.0[e,f,g,h,i]	0.71	4.9[g,h,i]	0.72
Source of variation						Prob > F						
Diet						0.812						
Age						<0.001						
Diet x age						0.002						
Age (wk)						Feed conversion ratio (g feed/g gain)						
0 to 3[4]	2.16[f]	0.02	2.30[f]	0.03	2.22[f]	0.04	2.29[f]	0.04	2.22[f]	0.03	2.24[f]	0.07
4 to 6[4]	3.11[e]	0.06	2.87[e]	0.04	2.97[e]	0.04	2.95[e]	0.03	2.98[e]	0.05	3.02[e]	0.07
7 to 12[5]	6.36[d]	0.14	6.68[d]	0.11	6.27[d]	0.10	6.68[d]	0.13	6.23[d]	0.15	6.44[d]	0.14
13 to 15[5]	8.36[c]	0.22	8.06[c]	0.20	8.38[b,c]	0.30	8.68[b,c]	0.16	8.34[c]	0.17	8.27[c]	0.17
16 to 18[6]	13.12[a]	0.77	14.57[a,b]	1.62	13.29[a,b,c]	1.39	10.91[a,b]	0.63	13.17[a,b,c]	1.47	13.03[a,b,c]	1.62
Source of variation						Prob > F						
Diet						0.562						
Age						<0.001						
Diet x age						0.008						

[1]PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.
[2]LSM = least squares mean.
[3]SEM = standard error of the mean.
[4]Means of 10 replicates of 8 pullets for each treatment.
[5]Means of 10 replicates of 7 pullets for each treatment.
[6]Means of 10 replicates of 6 pullets for each treatment.
[a-i]Means within column and within row for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).

Table 3. 4: Main effect of age on percent bone ash of pullets fed different dietary aP and Ca levels, and phytase supplementation[1].

	Proximal 25%[2]		Mid-bone 50%[3]		Distal 25%[4]		Total[5]	
	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]
Age (wk)				Dry bone weight (g)				
6[8]	0.94[c]	0.04	1.02[b]	0.04	0.84[b]	0.04	2.82[b]	0.09
15[8]	1.27[a]	0.02	1.31[a]	0.02	1.05[a]	0.02	3.63[a]	0.05
18[9]	1.10[b]	0.03	0.93[b]	0.03	0.86[b]	0.03	2.88[b]	0.08
Source of variation				Prob > F				
Diet	0.340		0.351		0.410		0.337	
Age	<0.001		<0.001		<0.001		<0.001	
Diet x age	0.788		0.405		0.260		0.366	
Body weight[10]	<0.001		<0.001		<0.001		<0.001	
Age (wk)				Bone ash content (g)				
6[8]	0.27[b]	0.01	0.42[b]	0.02	0.26[c]	0.01	0.94[b]	0.03
15[8]	0.38[a]	0.01	0.49[a]	0.01	0.31[b]	0.01	1.17[a]	0.02
18[9]	0.39[a]	0.01	0.46[b]	0.01	0.33[a]	0.01	1.18[a]	0.03
Source of variation				Prob > F				
Diet	0.113		0.238		0.159		0.183	
Age	<0.001		<0.001		0.005		<0.001	
Diet x age	0.446		0.113		0.437		0.237	
Body weight[10]	<0.001		<0.001		<0.001		<0.001	
Age (wk)				Percent bone ash (% of dry bone weight)				
6[8]	27.6[b]	0.92	42.9[a]	1.30	29.4[b]	0.91	33.9[b]	0.91
15[8]	29.6[b]	0.47	38.2[b]	0.64	30.2[b]	0.46	33.1[b]	0.45
18[9]	33.9[a]	0.72	48.1[a]	1.01	37.2[a]	0.77	39.9[a]	0.74
Source of variation				Prob > F				
Diet	0.653		0.424		0.334		0.683	
Age	<0.001		<0.001		<0.001		<0.001	
Diet x age	0.422		0.323		0.460		0.304	
Body weight[10]	0.995		0.093		0.811		0.188	

[1]Pullets were fed different dietary treatments: PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.
[2]Proximal 25% represented all bone tissue from the proximal tip of the femur to 25% of the length of the bone from the proximal end.
[3]Mid-bone 50% represented the remaining segment of bone between the proximal 25% and distal 25% sections.
[4]Distal 25% represented all bone tissue from the distal tip of the femur to 25% of the length of the femur from the distal end.
[5]Total term was calculated by summation of three sections of proximal 25%, mid-bone 50% and distal 25% femur.
[6]LSM = least squares mean.
[7]SEM = standard error of the mean.
[8]Means of 10 replicate birds for each treatment.
[9]Means of 8 replicate birds for each treatment.
[10]Body weight was used as a covariate.
[a-c]Means within column for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).

Table 3. 5: Main effect of age on femur bone mineral density of pullets fed different dietary aP and Ca levels, and phytase supplementation[1].

	Bone mineral density (mg/cm^3)					
	Proximal 20%[2]		Mid-bone 50%[3]		Distal 20%[4]	
	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]
Age (wk)			Total[7]			
6[8]	317[a,b]	14.31	543[a]	16.15	329	13.04
15[8]	303[b]	7.57	407[b]	8.39	331	6.90
18[9]	332[a]	14.56	417[b]	14.29	353	13.50
Source of variation			Prob > F			
Diet	0.908		0.928		0.686	
Age	0.023		<0.001		0.097	
Diet x age	0.429		0.809		0.680	
Body weight[10]	0.879		0.128		0.599	
Age (wk)			Cortical[11]			
6[8]	803[b]	10.02	987[b]	9.64	810[b]	8.95
15[8]	906[a]	5.37	1,117[a]	5.13	921[a]	4.62
18[9]	902[a]	7.91	1,120[a]	7.89	925[a]	7.06
Source of variation			Prob > F			
Diet	0.397		0.621		0.051	
Age	<0.001		<0.001		<0.001	
Diet x age	0.340		0.481		0.776	
Body weight[10]	0.843		0.424		0.600	
Age (wk)			Trabecular[12]			
6[8]	87.7[a]	7.21	71.7[a]	8.14	93.5[a]	5.87
15[8]	51.6[b]	3.93	30.9[b]	3.56	59.1[b]	3.29
18[9]	86.3[a]	5.54	49.0[a]	6.13	85.1[a]	4.46
Source of variation			Prob > F			
Diet	0.241		0.116		0.052	
Age	<0.001		<0.001		<0.001	
Diet x age	0.763		0.569		0.379	
Body weight[10]	0.206		0.066		0.073	

[1]Pullets were fed different dietary treatments: PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.
[2]A single 1 mm-thick slice taken at a point 20% along the length of the femur from the proximal end.
[3]A single 1 mm-thick slice taken at a point 50% along the length of the femur from the proximal end.
[4]A single 1 mm-thick slice taken at a point 80% along the length of the femur from the proximal end.
[5]LSM = least squares mean.
[6]SEM = standard error of the mean.
[7]Total term was the weighted average of both the cortical and trabecular bone measures.
[8]Means of 10 replicate birds for each treatment.
[9]Means of 8 replicate birds for each treatment.
[10]Body weight was used as a covariate.
[11]Cortical bone was the outer shell of the bone, and was defined as having a density of > 500 mg/cm^3
[12]Bone in the trabecular space was defined as having a density of $\leq$ 400 mg/cm^3
[a,b]Means within column for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).

Table 3. 6: Main effect of age on femur bone mineral content of pullets fed different dietary aP and Ca levels, and phytase supplementation[1].

	Bone mineral content[2] (mg/mm)					
	Proximal 20%[3]		Mid-bone 50%[4]		Distal 20%[5]	
	LSM[6]	SEM[7]	LSM[6]	LSM[7]	SEM[6]	LSM[7]
Age (wk)			Total[8]			
6[9]	14.6[b]	0.57	14.2	0.43	14.9[b]	0.58
15[9]	15.2[a,b]	0.31	13.5	0.25	15.6[a,b]	0.32
18[10]	17.0[a]	0.67	14.1	0.45	17.3[a]	0.63
Source of variation			Prob > F			
Diet	0.493		0.228		0.468	
Age	0.020		0.145		0.018	
Diet x age	0.479		0.831		0.452	
Body weight[11]	<0.001		<0.001		<0.001	
Age (wk)			Cortical[12]			
6[9]	11.2	0.47	13.6	0.46	11.5	0.44
15[9]	13.0	0.25	12.6	0.25	13.1	0.24
18[10]	13.3	0.68	12.6	0.46	13.7	0.61
Source of variation			Prob > F			
Diet	0.339		0.131		0.191	
Age	0.138		0.536		0.142	
Diet x age	0.434		0.807		0.668	
Body weight[11]	<0.001		<0.001		<0.001	
Age (wk)			Trabecular[13]			
6[9]	2.81[a]	0.24	1.05[a,b]	0.17	2.71[a,b]	0.24
15[9]	1.65[b]	0.14	0.68[b]	0.08	1.85[b]	0.13
18[10]	3.07[a]	0.21	1.16[a]	0.14	2.92[a]	0.22
Source of variation			Prob > F			
Diet	0.324		0.271		0.318	
Age	<0.001		<0.001		<0.001	
Diet x age	0.565		0.715		0.569	
Body weight[11]	<0.001		0.010		<0.001	

[1]Pullets were fed different dietary treatments: PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.
[2]Bone mineral content was calculated as bone mineral density multiplied by the bone cross-sectional area, and is the amount of bone mineral contained in a 1 mm linear section of the scanned region of the bone.
[3]A single 1 mm-thick slice taken at a point 20% along the length of the femur from the proximal end.
[4]A single 1 mm-thick slice taken at a point 50% along the length of the femur from the proximal end.
[5]A single 1 mm-thick slice taken at a point 80% along the length of the femur from the proximal end.
[6]LSM = least squares mean.
[7]SEM = standard error of the mean.
[8]Total term was the weighted average of both the cortical and trabecular bone measures.
[9]Means of 10 replicate birds for each treatment.
[10]Means of 8 replicate birds for each treatment.
[11]Body weight was used as a covariate.
[12]Cortical bone was the outer part of the bone, and was defined as having a density of > 500 mg/cm^3
[13]Bone in the trabecular space was defined as having a density of $\leq$ 400 mg/cm^3

[a,b]Means within column for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).

3.7 FIGURES

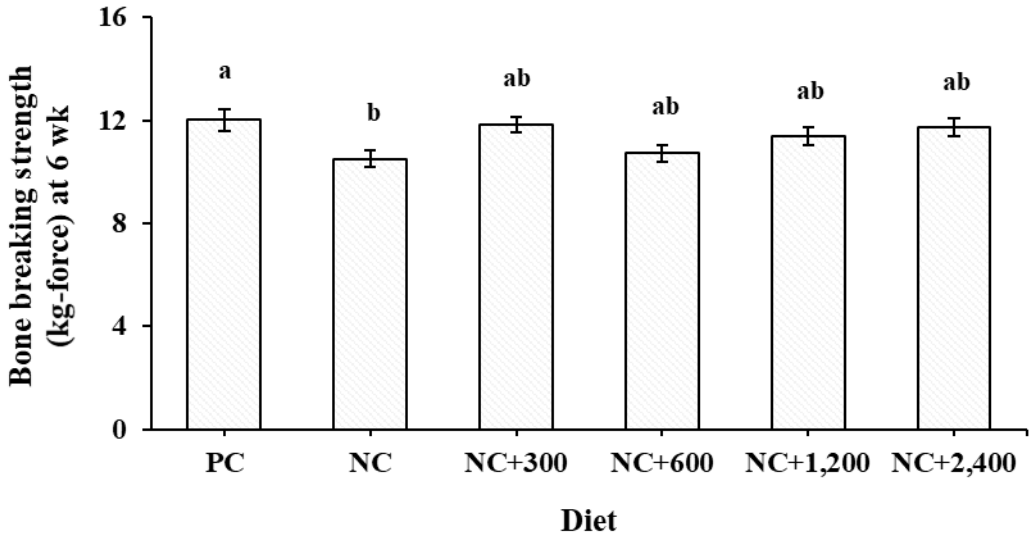

Figure 3. 1: Effect of diet on femur bone breaking strength of pullets fed different dietary aP and Ca levels, with or without phytase supplementation at 6 wk ($P = 0.043$; n = 60). The positive control (PC) diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively. Data are presented as least squares means with the respective standard errors of the mean. [a,b]Means with no common superscript differ significantly ($P \leq 0.05$). Effect of diet on femur bone breaking strength at each of 15 wk or 18 wk was not significant.

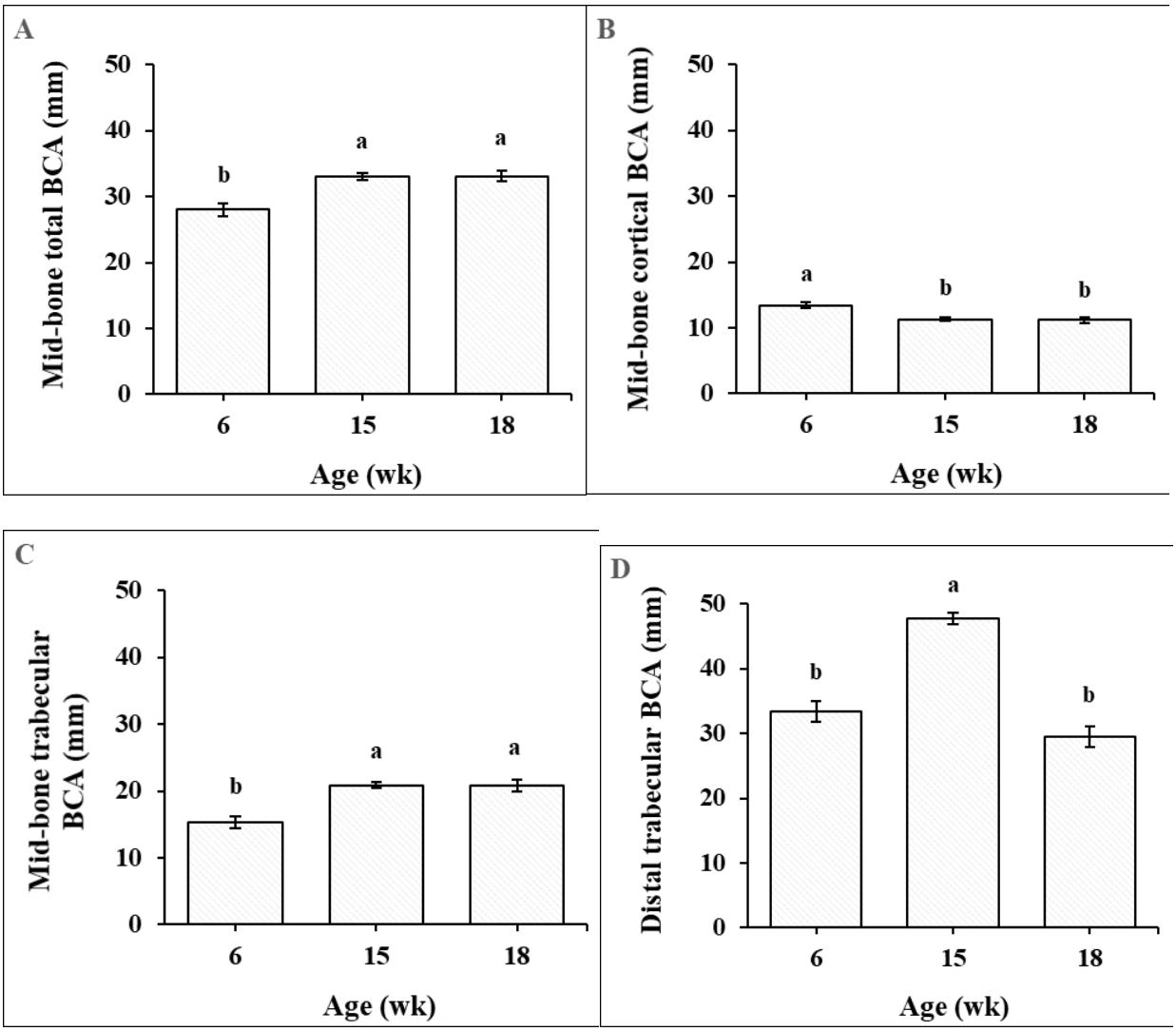

Figure 3. 2: Main effect of age on femur bone cross-sectional area (BCA) of pullets fed different dietary aP and Ca levels, with or without phytase supplementation at different ages (n = 168). A) Total BCA of the mid-bone ($P = 0.022$); B) cortical BCA of the mid-bone ($P = 0.035$); C) trabecular BCA of the mid-bone ($P = 0.002$); and D) trabecular BCA of the distal end of the femur ($P < 0.001$). Data are presented as least squares means with the respective standard errors of the mean. [a,b]Means with no common superscript differ significantly ($P \leq 0.05$). Neither diet by age interaction nor dietary treatment affected BCA.

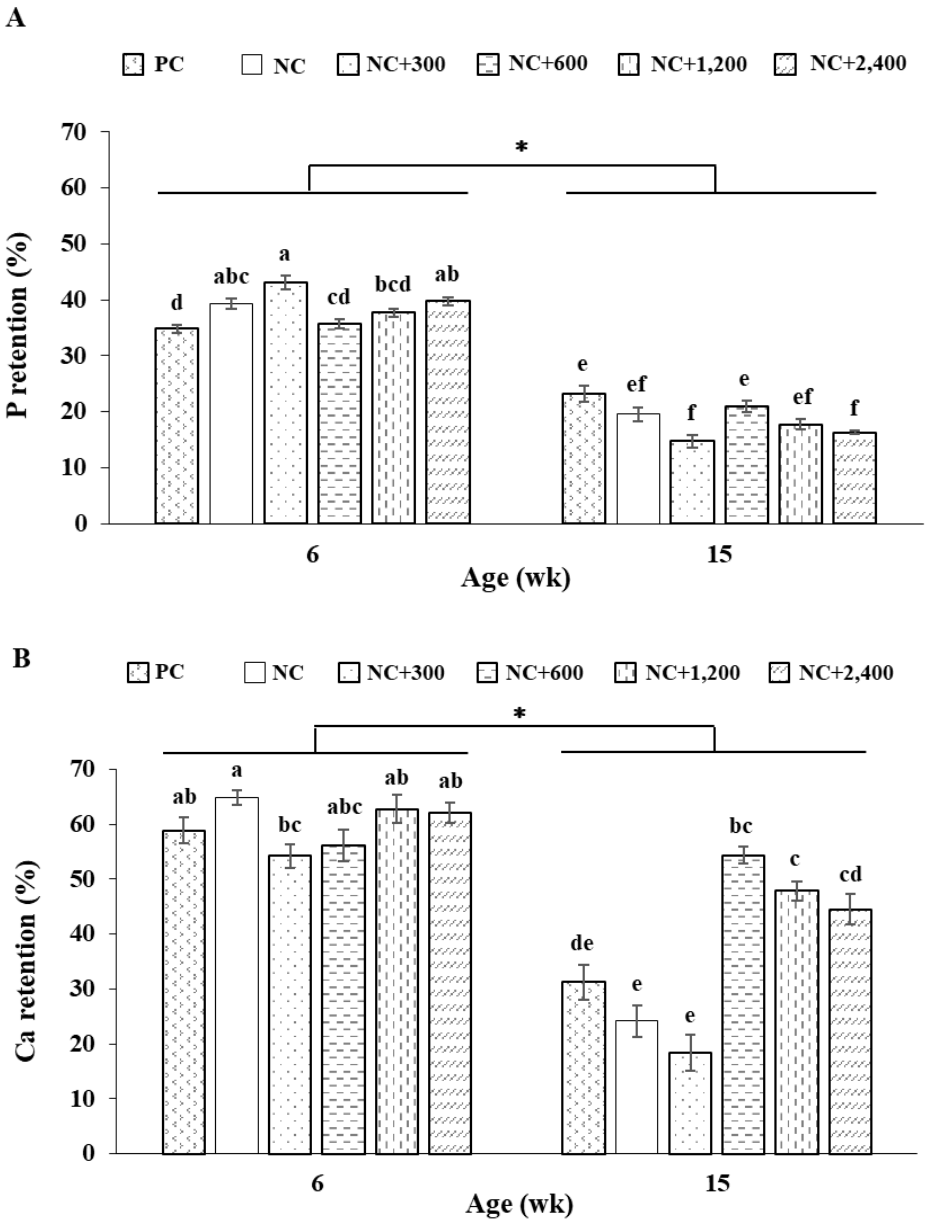

Figure 3. 3: Interaction of diet and age on (A) P retention ($P < 0.001$; n = 120) and (B) Ca retention ($P < 0.001$; n = 120) of pullets. The positive control (PC) diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively. Data are presented as least squares means with the respective standard errors of the mean. [a-f]Means with no common superscript differ significantly ($P \leq 0.05$). An asterisk (*) indicates significant difference between pullet ages ($P \leq 0.05$).

4. LONG-TERM PHYTASE SUPPLEMENTATION IN LAYER DIETS REDUCED IN AVAILABLE PHOSPHORUS AND CALCIUM: 1. HEN PERFORMANCE AND MINERAL RETENTION

ABSTRACT

Proper P and Ca nutrition is essential for long-term performance and egg production of laying hens. The effects of an *Escherichia coli* 6-phytase on performance and mineral retention in laying hens from 19 to 74 wk was investigated. Hens (n = 256) were maintained on 5 respective dietary treatments differing in aP and Ca diets fed from hatch to 19 wk in a previous part of the study. Treatments were: a positive control (PC) diet with 0.43% aP, 3.71% Ca and 0.17% Na from 19 to 54 wk, and 0.40% aP, 3.73% Ca and 0.16% Na from 55 to 74 wk; a negative control (NC) diet, the PC with aP and Ca reduced by 0.15 and 0.16% of the diet, respectively; the NC diet supplemented with phytase at 300 (NC + 300), 600 (NC + 600), or 1,200 (NC + 1,200) phytase unit (FTU)/kg, respectively. Body weight, feed intake (FI), feed conversion ratio (FCR), hen-day egg production (HDEP), and P and Ca retention were determined. Two-way ANOVA was conducted, and differences were considered significant at $P \leq 0.05$. Feed intake, FCR, HDEP, or P retention in the NC hens did not differ from the PC hens, indicating that aP and Ca in the NC diet were not deficient for those measures. However, BW in the NC was lower than the PC (1.58 $\pm$ 0.01 vs. 1.63 $\pm$ 0.01 kg); this may have been the initial signs of P or Ca deficiency, but the NC + 1,200 restored BW ($P < 0.001$). The NC hens had greater ($P < 0.001$) Ca retention than all other treatments except NC + 300. At 74 wk, the NC + 600 hens had higher ($P < 0.001$) P retention than NC + 300 hens. Overall, the NC diet did not decrease hen performance nor mineral retention, but caused a subtle reduction in BW. The NC

hens were able to compensate for moderately reduced dietary aP and Ca through physiological adaptations in the long-term.

Keywords: phytase, available phosphorus, performance, mineral retention, laying hen

4.1 INTRODUCTION

Phytate is an anti-nutritional factor in plant-based ingredients (Ravindran et al., 1995; Singh, 2008) that reduces the availability of minerals such as P and Ca. Phytate also has the potential to bind amino acids, carbohydrate, and lipids, reducing the efficiency of nutrient utilization (Selle et al., 2000). Phytate is not well utilized by birds as a source of P because of limited phytase production in the digestive tract (Maenz and Classen, 1998). As a consequence, inorganic P is added to the diets to facilitate growth and production. However, a large portion of inorganic P and undigested phytate are not utilized by birds and are excreted in the feces (Abudabos, 2012), which leads to P pollution in the environment (Selle and Ravindran, 2007; Selle et al., 2009; Lei et al., 2011). Also, P has become much more expensive to add in poultry diets (Biehl et al., 1998; Naves et al., 2016). Therefore, phytase has been used in poultry diets to enhance P utilization and subsequently reduce P excretion (Plumstead et al., 2007; Deniz et al., 2013; Wang et al., 2013). Phytase can degrade phytate and liberate proteins, amino acids and minerals, such as P and Ca (Selle and Ravindran, 2007). Many research studies have investigated the use of phytase in layer diets with reduced available P (**aP**) but containing adequate Ca. Phytase supplementation in reduced aP but in Ca-sufficient diets decreased mortality, feed to egg mass ratio, and increased BW, egg weight, egg production, phytate degradation, and P retention in laying hens (Punna and Roland, 1999; Boling et al., 2000b; Panda et al., 2005; Hughes et al., 2008; 2009). However, research on phytase use in laying hen diets reduced in both P and Ca in is less extensive. Supplementation of phytase in diets reduced in aP and Ca increased apparent ileal

digestibility of P (Bello and Korver, 2019), and reduced P excretion in laying hens (Lim et al., 2003). Previous phytase studies on hen performance have been done over short durations and using hens of varying age. There is a lack of information regarding on long-term phytase used in laying hens throughout the life of the birds from the pullet phase (Chapter 3), continuing on to the laying phase from 19 to 74 wk of age. Therefore, the present study was conducted to determine the long-term effects of phytase in diets reduced in aP and Ca on performance and mineral retention throughout the laying cycle. We hypothesized that the long-term feeding of a negative control (**NC**) diet low in aP and Ca would impair hen performance and mineral retention, and that phytase supplementation would overcome those deficiencies and return hen performance and mineral retention to the level of the positive control (**PC**) diet.

4.2 MATERIALS AND METHODS

The animal protocol was approved by the University of Alberta Animal Care and Use Committee for Livestock and followed the Canadian Council on Animal Care guidelines and policies (Canadian Council on Animal Care, 2009).

4.2.1 Animals and Housing

Two hundred and fifty-six H&N Nick Chick white egg layers were transferred from pullet cages at 19 wk of age and maintained on the respective pullet dietary treatments. Each pullet was weighed and housed in an individual cage (L x W; 50 x 50 cm; 43 cm high at the front and 33 cm at the back) in a double-tier laying cage system in an environmentally-controlled facility. There were 51, 51, 52, 52, and 50 hens for the PC, the NC, the NC supplemented with phytase at 300 (**NC + 300**), 600 (**NC + 600**), and 1,200 (**NC + 1,200**) phytase unit (**FTU**)/kg treatments, respectively. Each individual cage was equipped with one external feed trough (L x W x D; 50 x 15 x 10 cm), two automatic water nipples, and one external egg tray receiver (L x

W x H; 50 x 12 x 2.5 cm). At 19 wk of age, the lighting program was 13L:11D for the first two days, and followed by 14L:10D, increased to 15L:9D at 20 wk of age and then increased to a constant of 16L:8D from 21 to 74 wk of age. The room temperature was maintained at approximately 21 to 22°C throughout the study. Eggs were collected twice daily at 0930 and 1500. Hens were checked twice daily and mortality recorded. Feed and water were provided ad libitum throughout the 55-wk study from 19 to 74 wk of age.

4.2.2 Experimental Diets

Individual birds were maintained on the respective experimental dietary treatments used in the pullet phase (Chapter 3), except that the NC +2,400 treatment was excluded from the layer study because this treatment did not show any additional effects and given the lack of effect, the extra cost of enzyme supplementation was not likely to be commercially practical. The five treatments were as follows: a positive control (PC) diet with 0.43% aP, 3.71% Ca and 0.17% Na from 19 to 54 wk of age, and 0.40% aP, 3.73% Ca and 0.16% Na from 55 to 74 wk of age; the PC diet was formulated to meet or exceed nutrient recommendations (H & N International, 2012); a negative control (NC) diet similar to the PC diet, but reduced to 0.28% aP, 3.55% Ca and 0.14% Na from 19 to 54 wk of age, and 0.25% aP, 3.57% Ca and 0.13% Na from 55 to 74 wk of age; the NC diet supplemented with 300 (NC + 300), 600 (NC + 600), or 1,200 (NC + 1,200) phytase unit (FTU)/kg diet, respectively. Since 300 FTU phytase/kg diet is the common commercial level used in diets for laying hens, the NC + 600 and the NC + 1,200 were included to investigate high dose effects of phytase.

All experiment diets were corn-soy-canola-DDGS based, and fed as mash form. The ingredient and nutrient composition of the experimental diets are shown in Table 4.1. Each dietary treatment provided approximately 2,728 from 19 to 54 wk of age, and 2,705 kcal/kg ME

from 55 to 74 wk of age. The hens were managed following the primary breeder recommendations (H&N International, 2012). The phytase used in this study was a thermotolerant, enhanced *Escherichia coli* 6-phytase produced in *Trichoderma reesei* (Quantum Blue, AB Vista, Marlborough, UK). Phytase activity is reported in FTU; one FTU is the amount of enzyme that liberates 1 μmol of inorganic orthophosphate per minute from sodium phytate at 37°C and pH 5.5 (Engelen et al., 1994). The phytase was added to the NC diets at 0.06 (300 FTU/kg), 0.12, (600 FTU/kg), and 0.24 (1,200 FTU/kg) g/kg diet for the NC + 300, the NC + 600, and the NC + 1,200 groups, respectively. Feed phytase activities were analyzed by Enzyme Services Consultancy (ESC; Ystrad Mynach, UK) after feed sample extraction. Quantiplate ELISA Kits specific for Quantum Blue were used for quantification of the enzyme activity (Envirologix method AP181 with some modifications; designated ESC Standard Analytical Method SAM099). Celite (Celite Corporation, Lompoc, California USA) was mixed in the experimental diets at a concentration of 1%. The Celite-containing diets were fed for two wk from 40 to 42, from 52 to 54, and from 72 to 74 wk of age. Feed samples were collected at the time of mixing, ground and stored at -20°C until further analysis. The analyzed Ca, total P and phytase activities are presented in Table 4.2.

4.2.3 Performance Data

Individual hen BW was measured at 4 wk intervals from 19 to 54, and at 5 wk intervals from 55 to 74 wk of age. Average daily feed intake (**FI**) was determined for each period, and mortality-corrected feed conversion ratio (**FCR**; kg of feed intake per dozen eggs produced) was calculated. Hen-day egg production (**HDEP**) was calculated each day from each of the cages and mortality corrected hen-day egg production calculated on a 4-wk interval from 19 to 54 and a 5-wk basis from 55 to 74 wk of age.

4.2.4 Mineral Retention Assays

At each of 42, 54, and 74 wk of age, total excreta output from an individual hen in eight pre-identified cages per dietary treatment was collected for two consecutive days, and immediately frozen at -20°C for subsequent determinations of dry matter, acid insoluble ash (**AIA**), P and Ca concentrations. Before chemical analysis, excreta outputs were thawed overnight, placed in the oven-dried (Model V-31, Style II, Despatch Industries, Inc., Minneapolis, MN) at 60°C overnight and subsequently ground. The Celite-containing diets were thawed at room temperature overnight before analysis. Two gram of diets (n = 30: 5 diets x 3 periods; duplicates) and 1 g of excreta samples (n = 240: 5 diets x 8 hens x 3 periods; duplicates) were used for each of dry matter analysis (method 930.15; AOAC, 1990), AIA (Scott and Boldaji, 1997), total P (method 935.13; AOAC, 1990) using a spectrophotometer (SpectraMax Plus 384 Microplate Reader, Molecular Devices LLC, San Jose, CA) and Ca (method 964.06; AOAC, 1990) concentrations using a nitrous oxide-acetylene fueled flame atomic absorption spectrometer (Varian AA240FS, Agilent Technologies, Santa Clara, CA) as described by Bello and Korver (2019). The analyzed AIA, total P, and Ca concentrations from the Celite-containing diets and excreta for each respective diet from 40 to 42, 52 to 54, and 72 to 74 wk of age were calculated for P or Ca retention on a dry matter basis using the following equation:

$$\text{Retention (\%)} = 100 - [((AIA_{diet}/AIA_{excreta}) \times (Mineral_{excreta}/Mineral_{diet})) \times 100]$$

Where AIA_{diet} was the initial AIA concentration in the diet; $Mineral_{diet}$ was the initial dietary concentration of P or Ca; $AIA_{excreta}$ was the concentration of AIA in excreta; and $Mineral_{excreta}$ was the respective concentration of P or Ca in the excreta.

4.2.5 Statistical Analysis

The individual cage of one hen was the experimental unit for all outcomes. Two-way ANOVA was conducted using the MIXED procedure of SAS (SAS Institute, 2009) on BW, FI, FCR, HDEP, P and Ca retention to determine the effects of diet, age and their interactions. All data were tested for normality and normality of residuals using UNIVARIATE procedure. Egg production did not fit a normal distribution, therefore arcsine transformation was performed before statistical analysis. Means were separated using the LSMEANS statement. Differences between means among treatments were determined by the Tukey's range test. Statistical significance was considered when $P \leq 0.05$. Trends were reported where $0.05 < P \leq 0.10$. Values are presented as least squares means (**LSM**) with the respective standard errors of the mean.

4.3 RESULTS

4.3.1 Analyzed Dietary Ca, Total P and Phytase activity

The analyzed dietary Ca levels were 2 to 11% higher than formulated for most dietary treatments from 19 to 74 wk (Table 4.2). However, the NC diet had 4% lower analyzed Ca than formulated (3.40 vs 3.55%) from 19 to 54 wk. The analyzed Ca level in the NC + 600 diet was 12% lower than formulated (3.14 vs. 3.57%) from 55 to 74 wk. The analyzed total P levels in all dietary treatments were 1 to 16% lower than formulated from 19 to 54 wk of age, and from 18 to 22% lower from 55 to 74 wk of age. Phytase activities were higher than the targeted levels but were consistent with the assumed stepwise increase of phytase activity. On average, the phytase activities were 410, 680, and 1,420 in the NC + 300, NC + 600, and NC + 1,200 diets, respectively.

4.3.2 Hen Performance

There was no diet x age interaction on BW. Across ages, BW of the NC, NC + 300, and NC + 600 hens was lower than the PC hens, however, 1,200 FTU phytase/kg diet restored BW (Figure 4.1A). Across dietary treatments, hen BW was 1.35 ± 0.01 kg at 19 wk of age, and gradually increased with age to 1.65 ± 0.01 kg at 38 wk of age. Hens maintained BW from 38 to 74 wk of age (Figure 4.1B), however, a slight decrease in BW was observed at 64 wk of age. The NC hens had greater FI than the NC + 1,200 hens from 55 to 59 wk of age ($P = 0.004$; Table 4.3), but there was no effect of dietary treatment at any other age. Feed intake increased from 97.18 ± 0.56 g/d per hen at 22 wk of age to 112.01 ± 0.46 g/d per hen at 34 wk of age ($P < 0.001$), after which it remained relatively constant. Higher FCR were observed in the NC hens relative to the NC + 600 hens and the NC + 1,200 hens from 55 to 59 wk of age, but was not affected by diet at other ages (Table 4.4). Total mortality in this study was low, with mortality to 74 wk of age of 3.9, 0.0, 0.0, 1.9, and 2.0% in the PC, NC, NC + 300, NC + 600, and NC + 1,200 groups, respectively. In addition to mortality, two hens from the NC group and one hen from each of the PC and NC + 300 groups were culled because of injury unrelated to dietary treatment. There were no diet x age interactions or diet main effect (Figure 4.2A) on HDEP. Peak production was observed from 30 to 34 wk of age (98.7 ± 0.18%; Figure 4.2B). Hens maintained egg production between 88.2 to 98.2% from 35 to 74 wk of age. Interestingly, HDEP was higher from 69 to 74 wk of age (92.7 ± 0.67%) than from 64 to 69 wk of age (88.2 ± 0.86%).

4.3.3 P and Ca Retention

Phosphorus retention was higher in the NC + 600 hens (27.88 ± 4.99%) than the NC + 300 hens (6.42 ± 1.99%) at 74 wk of age, but was not affected by diet at other ages ($P < 0.001$;

Figure 4.3A). There was no diet x age interaction on Ca retention. Across ages, Ca retention was greater in the NC hens than the other treatments except for the NC + 300 hens (Figure 4.3B). At 42 wk of age, hens had higher Ca retention than at 54 and 74 wk of age (Figure 4.3C).

4.4 DISCUSSION

In order to assess phytase efficacy, it is necessary that phytate is present as a substrate for phytase, and that the Ca and P liberated by the phytase is needed by the bird. In the present study, the PC diet was formulated to meet or exceed nutrient recommendations (H & N International, 2012), and canola meal and triticale DDGS were used to ensure that the experimental diet contained an appreciable amount of phytate P. The NC diet was formulated to contain aP and Ca reduced by 0.15% and 0.16%, respectively, compared to the PC diet. However, the analyzed Ca levels were slightly higher whereas the levels of total P were slightly lower than the formulated (Table 4.2). The analyzed aP and Ca levels were still substantially higher in the PC diet than the NC diet and the NC diets supplemented with phytase. These differences still allow a determine of any potential negative effects of reductions of aP and Ca in the NC diet, and the effects of phytase supplementation. Although feed phytase activities were 13 to 37% higher than the targeted levels, there was a consistent trend with the assumed stepwise increase (direction and spacing) of phytase activity among dietary treatments. Therefore, the variances of analyzed Ca, total P and phytase from formulated values do not interfere with the ability to draw valid conclusions in regards to original hypotheses.

Across ages, the reduced levels of aP and Ca had a subtle effect on BW as seen a significantly lower BW in the NC hens relative to the PC hens, suggesting that aP and Ca levels in the NC diet were only marginally deficient. The decrease in hen BW may be an initial stage of signs of P or Ca deficiency. On average, the NC hens consumed less Ca (3.74 g/d per hen) and

total P (0.54 g/d per hen) than the PC hens (4.31 g/d Ca per hen and 0.62 g/d P per hen) from 19 to 54 wk of age. However, the NC hens had numerically higher egg production than the PC hens. The NC hens may have maintained high productivity and bone quality at the expense of BW (Bello et al., 2020). This may explain why the NC hens had slightly lower BW than the PC hens. However, diets with 3.0 to 3.5% Ca, and 0.19 to 0.23% aP were adequate to support hen BW (Chapter 2; An et al., 2016; Bello and Korver, 2019). In Ca-adequate diets, reduction of aP to 0.2% did not reduce hen BW, but further reductions to 0.15% or 0.10% decreased hen weight (Boling et al., 2000b; Hughes et al., 2008). Notably, NC hen BW was slightly lower than PC hen BW by approximately 20 g at 19 wk of age, and the difference in BW was increased to 100 g from 46 to 54 wk of age, but was not significantly different. This may have contributed to the significant dietary treatment effect on BW over the duration of the experiment.

The NC, NC + 300, and NC + 600 diets reduced hen BW relative to the PC diet, but phytase at 1,200 FTU/kg restored hen BW. Body weight of hens fed reduced aP, but adequate Ca was lower than the PC hens; phytase supplementation at 400 and 600 FTU/kg, but not 200 FTU/kg restored hen BW (Hughes et al., 2008). We did not see the positive effects of 300 and 600 FTU phytase/kg on BW in the current study. The variable results between experiments may be associated with the degree of aP and Ca reductions. The reductions were moderate in the current study whereas Hughes et al. (2008) provided a severe reduction of aP to 0.15%, with adequate Ca. Other factors such as sources of phytase, duration of feeding, strains and age of hens are also contribute the differences among studies (Dersjant-Li et al., 2015). Phytase can dephosphorylate phytate complexes and release energy, amino acids, P, Ca and *myo*-inositol (Cowieson et al., 2011; Gao et al., 2013; Gehring et al., 2013). A higher phytase dose exhibited greater degradation of *myo*-inositol hexakisphosphate (IP_6) to lower esters relative to lower

phytase doses (Li et al., 2016; Taylor et al., 2018; Walk et al., 2018; Bello et al., 2019; Walk and Olukosi, 2019). A high dose of phytase may have a faster rate and greater extent of hydrolysis of IP_6 than a lower dose, as 0.3 FTU/ml phytase liberated 0.35 mM of phosphate in 6 minutes whereas 0.1 FTU/ml released same amount of phosphate in 10 minutes in an in vitro assay (Tran et al., 2011). A higher dose of phytase also provided greater residual activity in each segment of the gastrointestinal tract than a low phytase dose (Nyannor et al., 2009). Phytase supplementation at 1,500 FTU/kg dramatically decreased IP_6 and IP_5 and increased *myo*-inositol in the gizzard and ileum of laying hens relative to 300 FTU/kg (Taylor et al., 2018). *Myo*-inositol has an insulin-mimetic effect on glucose transport, gluconeogenesis and protein deposition (Cowieson et al., 2015; Lee and Bedford, 2016; Bedford and Rousseau, 2017), and may have contributed to the higher BW in the NC + 1,200 hens relative to the NC hens.

The lack of diet effects on FI, FCR and egg production in the NC hens indicated that the reductions in aP and Ca in the NC diet were not severe enough to adversely affect those measures. Reductions of aP to 0.224% and Ca to 3.506% did not decrease egg production from 30 to 70 wk of age (Bello and Korver, 2019). In other long-term laying hen studies, reduction of aP to 0.2% (Boling et al., 2000a) or 0.25% (Hughes et al., 2008) in Ca-adequate diets did not reduce production performance, but reductions to 0.15% or less decreased egg production (Boling et al., 2000a; Boling et al., 2000b; Hughes et al., 2008). However, 0.15% aP from 22 to 34 wk (Jing et al., 2018) or 0.12% aP from 28 to 33 wk of age (Nie et al., 2018) in a Ca-adequate diet did not reduce hen performance and egg production. In Ca-adequate diets, approximately 14% mortality was observed in hens fed 0.15% aP (Hughes et al., 2008), and mortality increased to 19% (Boling et al., 2000b), 22% (Jalal and Scheideler, 2001) and 30% (Punna and Roland, 1999) when hens consumed aP-deficient (0.1%) diets. No mortality was observed in the NC hens

in the current study, suggesting the moderate reductions of aP and Ca in the NC diet were not severe. Feed intake was not different between the NC and the PC hens, and was not different among phytase treatments. However, the NC + 1,200 hens had lower FI and FCR than the NC hens from 55 to 59 wk, indicating that a high dose of phytase liberates more P, Ca, amino acids, energy and *myo*-inositol than a low dose of phytase or without phytase supplementation (Selle and Ravindran, 2007; Cowieson et al., 2013; Cowieson et al., 2015). However, this only happened during one specific short-term period (55 to 59 wk of age).

Laying hens are able to physiologically adapt to P-reduced or P-deficient diets, depending on the degree of P deficiency (Boling et al., 2000b; Nie et al., 2013; Geraldo et al., 2014) and strains of hens (Hughes et al., 2009). Laying hens fed 0.2% aP had greater NaP-IIb mRNA expression than hens fed 0.4% aP, implying that hens maintain P homeostasis by increasing P absorption through NaP-IIb co-transporter in the small intestine when aP is reduced (Nie et al., 2013). The NaP-IIb co-transporter is responsible for sodium-dependent phosphate absorption by the small intestine whereas NaP-IIa regulates renal phosphate excretion to maintain P balance (Marks et al., 2010). In the present study, the NC diet did not increase P retention in the NC hens relative to the PC hens at 42, 54, and 74 wk of age, indicating that aP in the NC diet was adequate. Phytase supplementation in P-deficient diets increased P digestibility and retention (Panda et al., 2005), and decreased P excretion (Deniz et al., 2013). Generally, higher doses of phytase increase phytate degradation and increase P digestibility and retention relative to a lower dose in laying hens (Van der Klis et al., 1997; Panda et al., 2005; Gao et al., 2013). However, the NC + 600 hens had greater P retention than the NC + 300 hens, but there was no difference in P retention relative to the NC + 1,200 hens. The mechanism of the effect of phytase on P retention is unclear in this study. The Ca level in the NC + 300 diet (3.63%) was

higher than in the NC + 600 (3.49%) during the retention determination period from 72 to 74 wk of age. A higher dietary Ca can decrease P utilization (Pelicia et al., 2009; Li et al., 2015) and retention when supplemented with phytase (Gautier et al., 2018) because the excess of Ca concentration can form insoluble Ca-phytate complexes in the small intestine (Nelson and Kirby, 1987) which limits phytase activity (Van der Klis et al., 1997; Beutler, 2009; Selle et al., 2009; Sommerfeld et al., 2018). The NC + 300 also had a wide Ca:total P ratio (10.1:1) compared to NC + 600 (8.7:1). Al-Masri (1995) reported that a wide Ca:P ratio (high Ca and low P) decreased P retention because high Ca depressed P absorption in the small intestine, and therefore P is excreted through the kidney. The wide Ca:P ratio also increased pH in the digestive tract (not suitable for phytase), leading to P precipitation, likely with phytate, in the small intestine. The insoluble precipitation phytate complexes are then excreted (Walk, 2016). This may explain why phytase at 600 FTU/kg had higher P retention than 300 FTU/kg. However, P retention in the NC + 1,200 was not greater than the NC + 600. This could be due to Ca:total P ratio in the NC + 1,200 (9.2:1) was slightly higher than in the NC + 600 (8.7:1), and therefore this wide ratio of Ca:total P may have decreased phytase activity (Al-Masri, 1995; Selle and Ravindran, 2007).

The NC diet as mixed had 2% and 10% lower analyzed Ca than formulated at 42 wk and 54 wk, respectively. As a result, the NC hens had lower Ca intake than the PC hens but were able to maintain similar egg production as the PC hens (approximately 92%). Therefore, the NC hens increased Ca retention through the increase in the rate of Ca absorption via increase the synthesis of calbindin and reduced renal Ca excretion (Wideman, 1987; Bar et al., 1992; Ieda et al., 1999; Pelicia et al., 2009). Dietary P levels may have influenced Ca retention. The analyzed total P levels in the NC diets were 0.44%, 0.45% and 0.33% at 42, 54 and 74 wk, respectively, which were 16% to 35% lower than the planned. The calculated aP from total P based on the feed

formulation values (Table 4.1), were approximately 50 to 60% of total P, therefore the aP levels during the retention determination period would be approximately 0.23% or less. Nie et al. (2013) demonstrated that reducing dietary aP from 0.40 to 0.25% increased duodenal calbindin mRNA, duodenal vitamin D receptor mRNA, and duodenal vitamin D receptor protein; this was speculated to increase Ca absorption and retention. This may also explain why the NC hens had higher Ca retention than the PC hens. Among phytase treatments, the NC + 600 and NC + 1,200 hens had lower Ca retention than the NC + 300, but the mechanism for this is not clear. Gao et al. (2013) reported that phytate degradation increases with phytase dose. Phytase liberates not only P, but also Ca. However, Ca liberated by high doses of phytase can form insoluble salts of Ca in the small intestine (Tamim et al., 2004; Selle and Ravindran, 2007; Hughes et al., 2009; Hamdi et al., 2015; Li et al., 2016; Sommerfeld et al., 2018), and therefore reduce Ca absorption and increase Ca excretion. A high Ca (4.0%) layer diet impaired phytate degradation by phytase compared to 3.0% Ca (Van der Klis et al., 1997). Since the analyzed Ca levels in the NC + 600 and NC + 1,200 diets during the mineral retention assay were slightly higher than the NC + 300 diet, higher dietary Ca in the NC + 600 and NC + 1,200 may have reduced phytate degradation, and therefore decreased Ca retention relative to NC + 300 treatment. Calcium retention decreased with hen age in the current study. This could be due to the decrease in renal calbindin-D_{28k} and less synthesis of 1,25-dihydroxy-cholecalciferol in older hens (Elaroussi et al., 1994).

Although the NC hens maintained egg production relative to the other dietary treatments, BW in the NC hens was approximately 55 g lower than the PC hens. Our findings indicate that the reduced levels of aP and Ca in this study were still adequate to maintain egg production, but may have been marginally deficient in the long-term. Since the laying hens in this study had

been fed the experimental treatments from hatch, there were likely some subtle cumulative effects on BW of the NC hens. However, hens were able to adapt to the reduced Ca and aP diets. Because of the physiological adaptation of laying hens to reduced aP and Ca in diet, and the reductions were not severe, the effects of phytase were not pronounced in the present study. Besides, the PC diet was formulated following the primary breeder recommendations, which contains a considerable safety margin. This also limited the opportunity for phytase supplementation to alleviate adverse effects of aP and Ca reductions, because the NC diet was not deficient. In order to reduce total P in layer diets and minimize the environmental impact of excessive P excretion, without compromising hen performance, the actual aP and Ca requirements of laying hens should be evaluated. Dietary aP and Ca for commercial layers can likely be substantially reduced, and even more so if an effective phytase is supplemented.

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4.6 TABLES

Table 4. 1: The ingredient and nutrient composition of positive control (PC) and negative control (NC) diets fed to laying hens from 19 to 74 wk of age.

	19 to 54 wk		55 to 74 wk	
	PC	NC[1]	PC	NC[1]
Ingredients (%)				
Corn	57.20	58.95	59.64	58.65
Soybean meal	12.69	12.40	8.88	6.92
Canola meal	8.00	8.00	8.05	12.00
Triticale DDGS	10.00	10.00	12.00	12.00
Canola oil	0.68	0.11	0.00	0.00
Calcium carbonate	8.53	8.54	8.69	8.66
Dicalcium phosphate	1.44	0.62	1.24	0.40
Salt	0.35	0.26	0.33	0.24
Choline chloride	0.50	0.50	0.50	0.50
L-Lysine HCl	0.01	0.01	0.08	0.06
DL-Methionine	0.10	0.10	0.09	0.07
Vitamin-mineral premix[2]	0.50	0.50	0.50	0.50
Phytase[3] (g/kg)	0.00	Variable[4]	0.00	Variable[4]
Calculated nutrients (%)				
ME (kcal/kg)	2,728	2,728	2,705	2,705
Crude protein	17.14	17.14	16.12	16.54
Calcium	3.71	3.55	3.73	3.57
Available phosphorus	0.43	0.28	0.40	0.25
Phytate phosphorus	0.25	0.25	0.24	0.26
Total phosphorus	0.68	0.53	0.64	0.51
Sodium	0.17	0.14	0.16	0.13

[1]NC diet was mixed in a single batch and subdivided into the NC and various NC phytase-containing diets.
[2]Vitamin-mineral premix (units per kilogram of feed): vitamin A, 12,500 IU; vitamin D_3, 3,125 IU; vitamin E, 40 IU; vitamin K (menadione), 2.5 mg; riboflavin, 7.5 mg; D-pantothenic acid, 12.5 mg; vitamin B_{12}, 0.01875 mg; pyridoxine, 5 mg; thiamine, 2.55 mg; folic acid, 0.625 mg; niacin, 37.5 mg; biotin, 0.15 mg; iodine, 1.65 mg; copper, 15 mg; iron, 80 mg; selenium, 0.3 mg; manganese, 88 mg; zinc, 100 mg.
[3]Quantum Blue phytase 5G product (5,000 FTU/g of premix); an enhanced *E. coli* 6-phytase, AB Vista, Marlborough, UK.
[4]Quantum Blue phytase was added on top to the NC diet at 0.06 (300 FTU/kg), 0.12 (600 FTU/kg) or 0.24 g/kg (1,200 FTU/kg) for the NC phytase-containing diets.

Table 4. 2: Analyzed Ca, total P and phytase activity levels of experimental layer diets[1].

	Diet[2]				
	PC	NC	NC + 300	NC + 600	NC + 1,200
Calcium (% of the diet, as-fed basis)					
19 to 54 wk	3.96	3.40	3.70	3.93	3.49
55 to 74 wk	4.13	3.97	3.53	3.14	3.53
Total phosphorus (% of the diet, as-fed basis)					
19 to 54 wk	0.57	0.49	0.52	0.52	0.53
55 to 74 wk	0.52	0.40	0.41	0.42	0.41
Phytase activity[4] (FTU/kg diet)					
19 to 54 wk	<50	<50	419	671	1,410
55 to 74 wk	<50	<50	401	689	1,430

[1]Feed provided in mash form.

[2]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.

Table 4. 3: Interaction of diet and age on feed intake of laying hens fed different levels of dietary aP, Ca and phytase supplementation.

	Diet[1]									
	PC		NC		NC + 300		NC + 600		NC + 1,200	
	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]
Age (wk)	Feed intake (g/d per hen)									
19 to 22[4]	97	1.18	96	1.39	99	0.87	97	1.26	96	1.32
23 to 26[4]	106	1.03	104	1.21	106	1.16	106	1.33	106	1.18
27 to 30[4]	107	1.21	110	1.24	112	1.14	110	1.12	109	1.13
31 to 34[5]	110	1.14	112	1.15	112	0.74	112	1.01	113	1.06
35 to 38[5]	113	1.26	116	1.23	112	1.10	113	1.28	114	1.13
39 to 42[5]	112	1.30	115	1.49	112	1.19	112	1.46	113	1.15
43 to 46[6]	114	1.59	112	1.58	115	1.39	113	1.37	115	1.37
47 to 50[6]	111	1.65	111	1.52	112	1.41	110	1.35	113	1.36
51 to 54[6]	110	1.78	113	1.27	113	1.59	112	1.34	114	1.51
55 to 59[7]	117[a,b]	2.75	120[a]	2.86	109[a,b]	3.41	104[a,b]	2.88	103[b]	2.75
60 to 64[8]	106	3.58	106	3.27	118	2.51	117	2.95	114	3.54
65 to 69[9]	112	2.71	112	1.64	112	2.03	111	2.35	113	2.28
70 to 74[9]	113	2.37	115	2.25	114	1.86	114	1.92	116	1.94
Source of variation	Prob > F									
Diet	0.174									
Age	<0.001									
Diet x age	0.004									

[1]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.
[2]LSM = least squares mean.
[3]SEM = standard error of the mean.
[4]Means of 51 replicate hens (PC and NC), 52 replicates (NC + 300 and NC + 600), and 50 replicates (NC + 1,200).
[5]Means of 50 replicate hens (PC), 51 replicates (NC), 52 replicates (NC + 300 and NC + 600), and 50 replicates (NC + 1,200).
[6]Means of 42 replicate hens (PC), 43 replicates (NC), 44 replicates (NC + 300 and NC + 600), and 42 replicates (NC + 1,200).
[7]Means of 34 replicate hens (PC), 33 replicates (NC), 36 replicates (NC + 300 and NC + 600), and 33 replicates (NC + 1,200).
[8]Means of 33 replicate hens (PC and NC), 36 replicates (NC + 300 and NC + 600), and 33 replicates (NC + 1,200).
[9]Means of 24 replicate hens (PC), 25 replicates (NC), 27 replicates (NC + 300 and NC + 600), and 25 replicates (NC + 1,200).
[a,b]Means within row with no common superscript differ significantly ($P \leq 0.05$).

Table 4. 4: Interaction of diet and age on feed conversion ratio of laying hens fed different levels of dietary aP, Ca and phytase supplementation.

	Diet[1]									
	PC		NC		NC + 300		NC + 600		NC + 1,200	
	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]
Age (wk)	Feed conversion ratio (kg feed/dozen eggs)									
19 to 22[4]	2.40	0.31	2.66	0.34	2.20	0.08	2.38	0.16	3.15	0.61
23 to 26[4]	1.29	0.01	1.27	0.02	1.30	0.02	1.29	0.02	1.30	0.01
27 to 30[4]	1.30	0.02	1.33	0.02	1.36	0.01	1.33	0.01	1.33	0.02
31 to 34[5]	1.34	0.02	1.36	0.01	1.37	0.01	1.37	0.01	1.38	0.02
35 to 38[5]	1.38	0.02	1.43	0.02	1.37	0.01	1.37	0.02	1.38	0.01
39 to 42[5]	1.38	0.02	1.42	0.02	1.39	0.02	1.38	0.02	1.39	0.01
43 to 46[6]	1.40	0.02	1.38	0.02	1.42	0.02	1.40	0.01	1.43	0.02
47 to 50[6]	1.37	0.02	1.38	0.02	1.40	0.02	1.37	0.02	1.40	0.02
51 to 54[6]	1.39	0.02	1.41	0.02	1.41	0.02	1.41	0.01	1.42	0.02
55 to 59[7]	1.50[a,b]	0.04	1.54[a]	0.05	1.38[a,b,c]	0.04	1.30[b,c]	0.03	1.29[c]	0.04
60 to 64[8]	1.41	0.06	1.38	0.04	1.51	0.04	1.52	0.04	1.47	0.05
65 to 69[9]	1.60	0.04	1.53	0.03	1.50	0.03	1.51	0.05	1.55	0.04
70 to 74[9]	1.50	0.05	1.50	0.03	1.47	0.02	1.46	0.02	1.51	0.04
Source of variation	Prob > F									
Diet	0.397									
Age	<0.001									
Diet x age	0.002									

[1]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.

[2]LSM = least squares mean.

[3]SEM = standard error of the mean.

[4]Means of 51 replicates hens (PC and NC), 52 replicates (NC + 300 and NC + 600), and 50 replicates (NC + 1,200).

[5]Means of 50 replicate hens (PC), 51 replicates (NC), 52 replicates (NC + 300 and NC + 600), and 50 replicates (NC + 1,200).

[6]Means of 42 replicate hens (PC), 43 replicates (NC), 44 replicates (NC + 300 and NC + 600), and 42 replicates (NC + 1,200).

[7]Means of 34 replicate hens (PC), 33 replicates (NC), 36 replicates (NC + 300 and NC + 600), and 33 replicates (NC + 1,200).

[8]Means of 33 replicate hens (PC and NC), 36 replicates (NC + 300 and NC + 600), and 33 replicates (NC + 1,200).

[9]Means of 24 replicate hens (PC), 25 replicates (NC), 27 replicates (NC + 300 and NC + 600), and 25 replicates (NC + 1,200).

[a-c]Means within row with no common superscript differ significantly ($P \leq 0.05$).

4.7 FIGURES

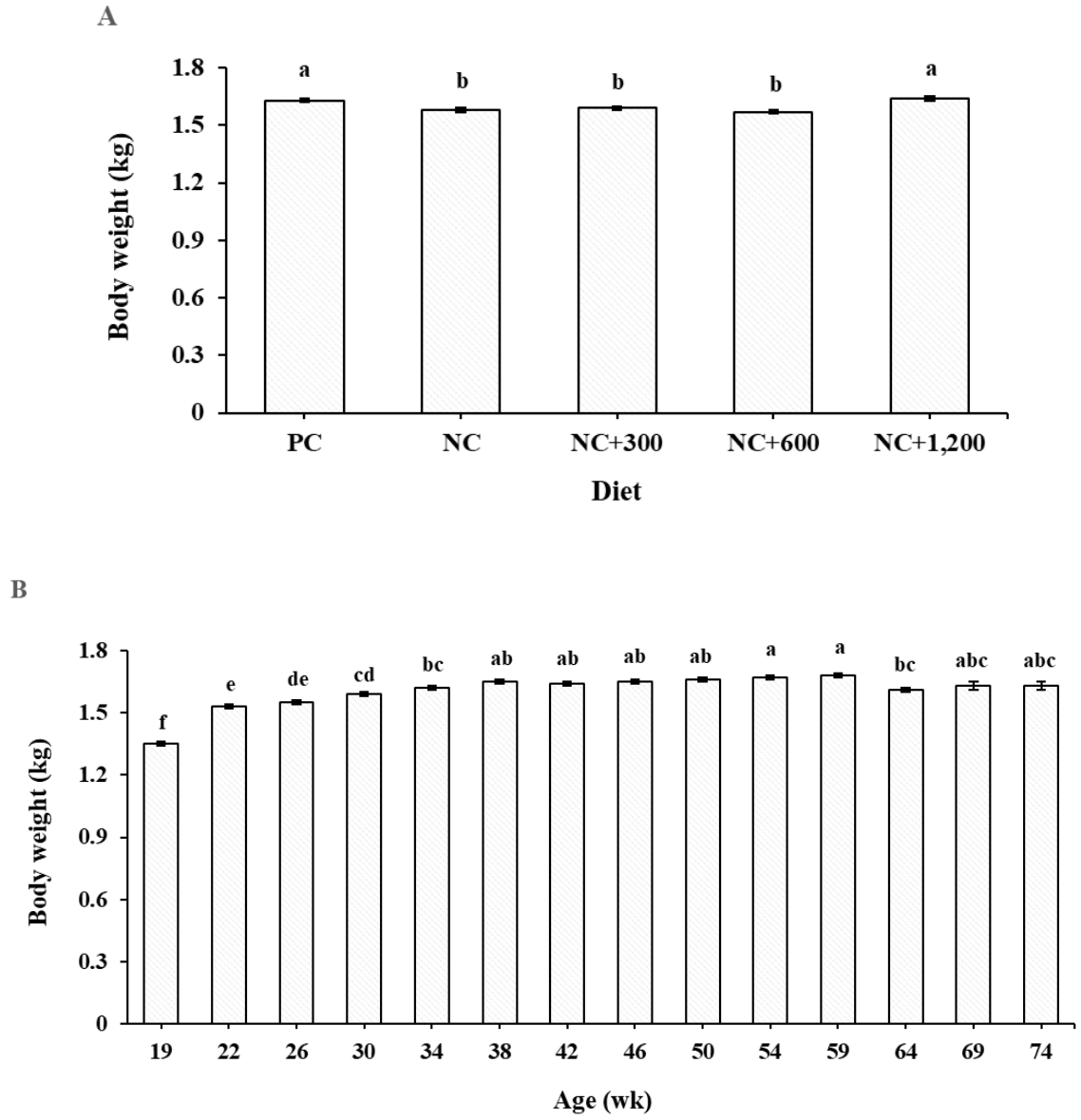

Figure 4. 1: Diet effect (A) and age effect (B) on body weight of laying hens fed different levels of dietary aP, Ca and phytase supplementation from 19 to 74 wk of age. The positive control (PC) diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively. Data are presented as least squares means with the respective standard errors of the mean. [a-f]Means with no common superscript differ significantly ($P \leq 0.05$). The interaction of diet and age was not significant.

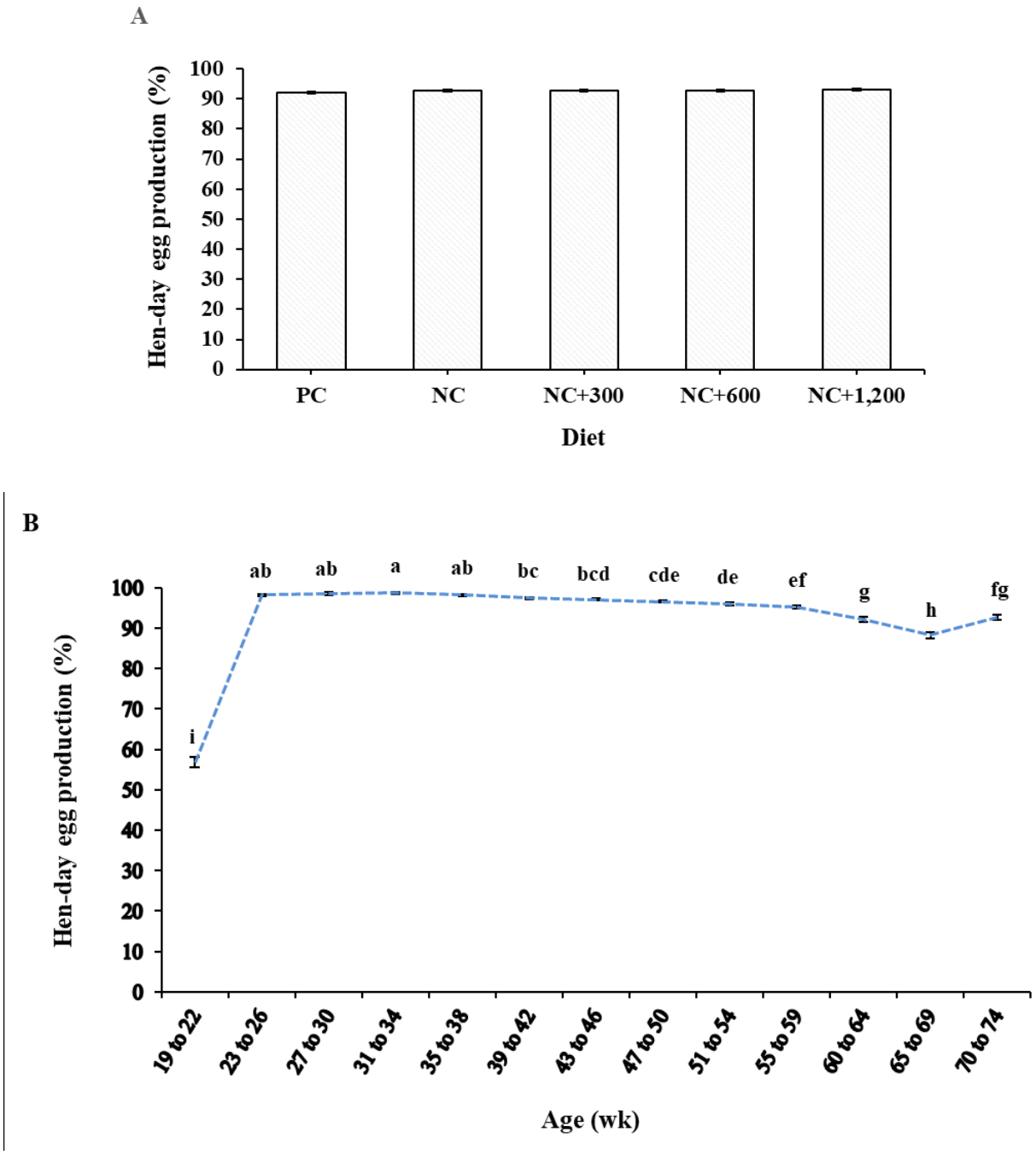

Figure 4. 2: Effects of dietary aP, Ca and phytase supplementation on egg production by diet (A) and by age (B) of laying hens. The positive control (PC) diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet containing 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively. Data are presented as least squares means with the respective standard errors of the mean. [a-i]Means with no common superscript differ significantly ($P \leq 0.05$). The interaction of diet and age was not significant.

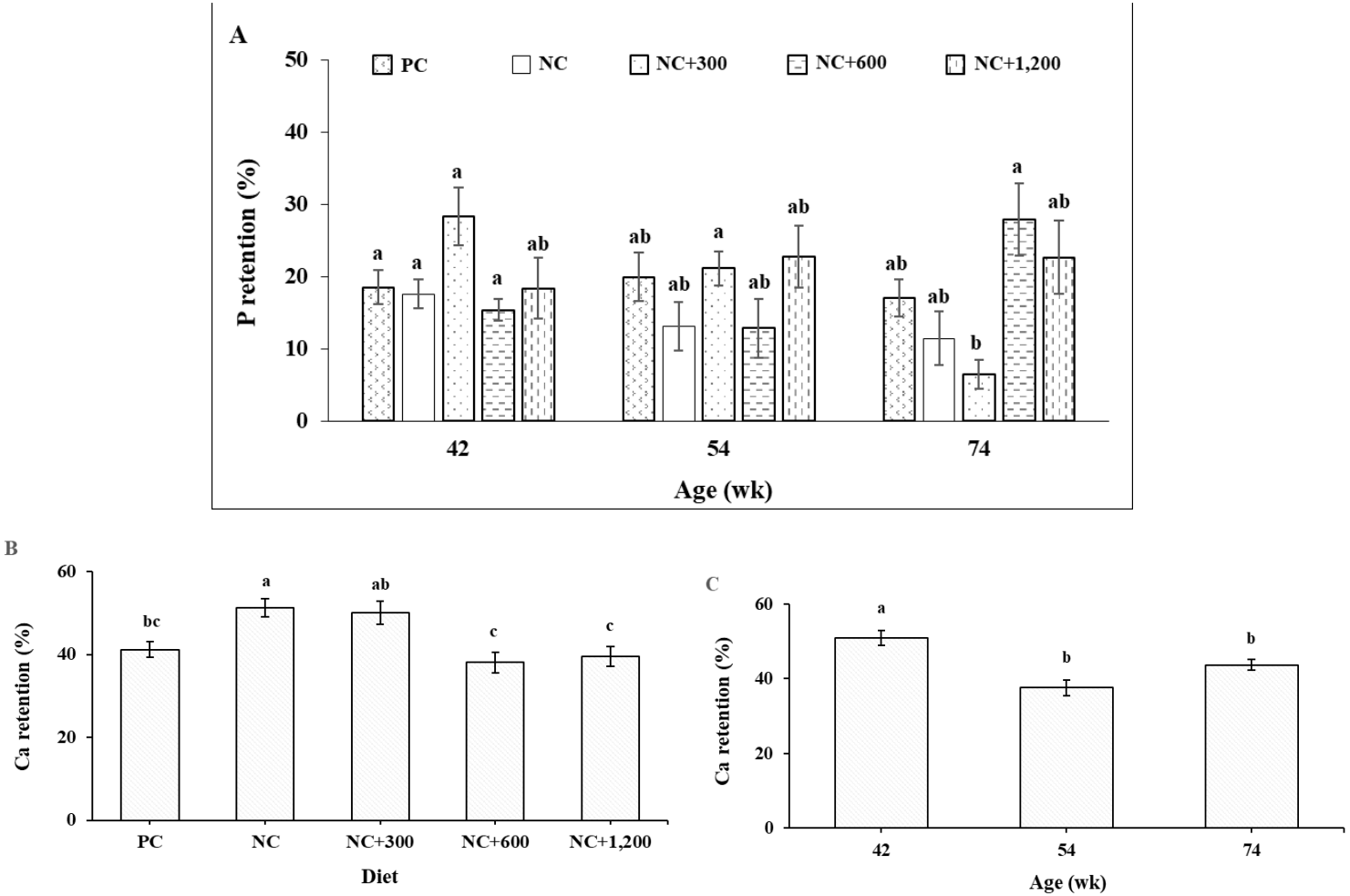

Figure 4. 3: Interaction of diet and age on P retention ($P < 0.001$; A). Main effects of diet ($P < 0.001$; B) and age ($P < 0.001$; C) on Ca retention of laying hens fed different levels of dietary aP, Ca and phytase supplementation. The positive control (PC) diet contained 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet contained 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively. Data are presented as least squares means with the respective standard errors of the mean. [a,b]Means with no common superscript differ significantly ($P \leq 0.05$). The interaction of diet and age on Ca retention was not significant.

5. LONG-TERM PHYTASE SUPPLEMENTATION IN LAYER DIETS REDUCED IN AVAILABLE PHOSPHORUS AND CALCIUM: 2. EGGSHELL AND BONE QUALITY

ABSTRACT

The long-term effect of phytase supplementation to diets reduced in available phosphorus (aP) and Ca on eggshell and bone quality of laying hens was investigated. Pullets reared on diets varying in aP and Ca from 0 to 19 wk of age were continued on their respective diets: a positive control (PC) diet with 0.43% aP, 3.71% Ca and 0.17% Na from 19 to 54 wk of age, and 0.40% aP, 3.73% Ca and 0.16% Na from 55 to 74 wk of age; a negative control (NC) diet, similar to the PC diet but with 0.28% aP, 3.55% Ca and 0.14% Na from 19 to 54 wk of age, and 0.25% aP, 3.57% Ca and 0.13% Na from 55 to 74 wk of age; and the NC diet supplemented with phytase at 300 (NC + 300), 600 (NC + 600), or 1,200 (NC + 1,200) phytase unit (FTU)/kg, respectively. Data were analyzed by two-way ANOVA and means separated using Tukey's range test where $P \leq 0.05$. Egg specific gravity, eggshell thickness and breaking strength, bone breaking strength (BBS), ash, mineral density (BMD), cross-sectional area, and mineral content (BMC) of three distinct sections of the femur were determined. There were no diet x age interactions for eggshell measures or BBS. At 74 wk, the NC + 600 hens had greater distal femur ash than the NC hens ($P = 0.013$). The NC + 600 and NC + 1,200 hens had increased total BMD of the proximal ($P = 0.001$) and distal ($P = 0.002$) relative to the NC hens. Birds fed the NC + 600 diet had increased proximal ($P = 0.002$) and mid-bone ($P = 0.008$) total femur BMC relative to the NC birds. Hens at 74 wk had greater total BMD and BMC than at 42 wk, likely due to an increase of medullary bone. Phytase supplementation at 600 and 1,200 FTU/kg restored bone quality in laying hens fed reduced dietary aP and Ca.

Keywords: phytase, available phosphorus, eggshell, bone, laying hen

5.1 INTRODUCTION

Bones play a vital role in egg production of laying hens by providing a source of Ca for eggshell formation (Orban and Roland, 1990) when dietary Ca is limiting. Bone formation and resorption (i.e. remodeling) occur continuously during a daily egg-laying cycle, and Ca metabolism is extremely intense (Kerschnitzki et al., 2014). Laying hens deposit daily approximately 2.2 g of Ca to form eggshell (Josling et al., 2019). Hens mobilize medullary bone, a non-structural type of woven bone and labile Ca storage (Dacke et al., 1993), to form calcium carbonate microcrystals and crystals through distinct regions of the oviduct during eggshell calcification (Nys et al., 2004; Nys et al., 2007). In the long bones of laying hens such as the femur or tibia, the proximal and distal epiphyses contain a high concentration of trabecular tissue whereas cortical and medullary tissues are concentrated in the mid-diaphysis (Dacke et al., 1993; Shipov et al., 2010; Kerschnitzki et al., 2014). Cortical and trabecular tissues (structural bone tissues which provide strength) can be resorbed by osteoclasts, but neither cortical and trabecular bone can be re-deposited while the hen is actively laying (Fleming et al., 1998b). However, medullary bone tissue can be mobilized and formed while the hen is in lay (Whitehead, 2004; Kerschnitzki et al., 2014). Over the laying period, accumulation of medullary bone occurs whereas cortical and trabecular bone tissues decrease (Whitehead, 2004). In the long-term, the cumulative effects of structural bone loss can lead to osteoporosis and increase the risk of bone fracture in laying hens, especially late in the production cycle (Fleming et al., 1998a; Fleming et al., 1998b; Whitehead, 2004).

Traditional analytical methods such as bone ash or bone breaking strength (**BBS**) of long bones have been commonly used to assess bone quality in poultry (Boling et al., 2000b; Korver, 2004; Molnár et al., 2018). Bone ash provides information on the amount of bone mineral

present (Sari et al., 2012; Tahmasbi et al., 2012; Deniz et al., 2013), but no distinction between non-structural and structural bone tissues. Bone ash is also confounded by the presence of medullary bone during the laying period (Bello and Korver, 2019). A hen could have severe osteoporosis with very low in structural bone, but still have high bone ash because of high medullary bone accumulation (Whitehead, 2004; Fleming et al., 2006). Three-point bending BBS, a structural mechanical test, tests the mechanical property of the mid-diaphysis (Panda et al., 2005; Lei et al., 2011), but does not provide information on the mechanical properties of the proximal and distal epiphyses, which have a higher proportion of trabecular bone and more susceptible to fracture (Fleming et al., 1998b; Reich and Gefen, 2006). Although much of the current knowledge of laying hen bone dynamics has been obtained using traditional analytical methods, the use of technologies such as quantitative computed tomography (**QCT**), along with conventional bone assessment may provide a more complete picture of bone metabolism.

Phytase studies in laying hens are normally conducted by reducing dietary available phosphorus (**aP**) and Ca levels by the matrix values for those nutrients expected to be liberated by phytase, and then phytase is added back. Previous research on phytase supplementation in laying hens fed aP- and Ca-reduced diets have only been done in the short-term, either during early or mid-lay production (Punna and Roland, 1999; Panda et al., 2005; Kozłowski and Jeroch, 2011; Sari et al., 2012), late production (Gordon and Roland, 1998; Lei et al., 2011; Tahmasbi et al., 2012; Deniz et al., 2013; Fernandez et al., 2019), or even 40 wk in long-term studies (Hughes et al., 2008; 2009; Bello and Korver, 2019). In those studies, bone and eggshell quality tended to be unaffected by moderate dietary reductions in either aP and Ca, or the subsequent addition of phytase. One possible limiting factor might be the duration of time of the study. A longer time of feeding diets with reductions in Ca and aP may be required to observe the changes in bone

characteristics and the efficacy of phytase in laying hens. There is limited information on the effect of phytase on laying hen bone mineralization over an entire production cycle from hatch. Therefore, the present study was conducted to determine the long-term effects of reduced dietary aP and Ca, and phytase supplementation on eggshell and bone quality in laying hens from 19 to 74 wk of age. It was hypothesized that reduced dietary aP and Ca in the long-term would decrease eggshell and bone quality, and phytase supplementation would restore eggshell and bone quality.

5.2 MATERIALS AND METHODS

The protocol was approved by the University of Alberta Animal Care and Use Committee for Livestock and followed principles established by the Canadian Council on Animal Care guidelines and policies (Canadian Council on Animal Care, 2009).

5.2.1 Animals and Housing

Two hundred and fifty-six H&N Nick Chick white egg layers were transferred from pullet cages at 19 wk of age and maintained on the respective dietary treatments (Chapter 4). There were 50 to 52 hens per dietary treatment. Hens were weighed and housed in individual cages in a double-tier cage system in an environmentally-controlled facility. Each replicate cage was equipped with one external feed trough, two automatic water nipples, and one external egg tray receiver (Pongmanee et al., 2020). The lighting program, room temperature, and general management were as described in Chapter 4. Hens were checked twice daily and mortality recorded. Feed and water were provided ad libitum throughout the experiment. Hens were managed as recommended by the primary breeder company (H & N International, 2012). The experimental period lasted 55 wk from 19 to 74 wk of age.

5.2.2 Experimental Diets

The five dietary treatment were: a positive control (**PC**; formulated to meet or exceed nutrient recommendations (H & N International, 2012)) diet with 0.43% aP, 3.71% Ca and 0.17% Na from 19 to 54 wk of age, and 0.40% aP, 3.73% Ca and 0.16% Na from 55 to 74 wk of age; a negative control (**NC**) diet similar to the PC diet, but reduced to 0.28% aP, 3.55% Ca and 0.14% Na from 19 to 54 wk of age, and 0.25% aP, 3.57% Ca and 0.13% Na from 55 to 74 wk of age; the NC diets supplemented with 300 (**NC + 300**), 600 (**NC + 600**), or 1,200 (**NC + 1,200**) phytase unit (**FTU**)/kg, respectively. The ingredient and nutrient composition of the experimental diets, and the 6-phytase product (Quantum Blue, AB Vista, Marlborough, UK) used were described in Chapter 4.

5.2.3 Eggshell Quality

At 4 wk and 5 wk intervals from 19 to 54 and 55 to 74 wk of age, respectively, eggs were collected from each hen for three consecutive days; one egg was used for determination of egg specific gravity (**SG**), one egg for determination of eggshell thickness, and the last one for determination of eggshell breaking strength. All eggs for eggshell quality measurement were kept in a constant environment (room temperature at 22°C) overnight (approximately 18 h) before testing. Egg SG (g/cm^3) was measured by flotation using 11 sequential saline solutions ranging from 1.060 to 1.110 in increments of 0.005 (Holder and Bradford, 1979; Wu et al., 2007). The saline solutions were calibrated before each test. Eight eggs from eight pre-specified hens were randomly chosen from each dietary treatment to determine eggshell thickness. A 1 cm x 1 cm square was marked and cut at three different locations (broad-end, equator, and narrow-end) from each egg. The eggshell thickness without membrane (mm) of each square was measured using a digital caliper (Model CD-8”C, Mitutoyo Corp., Japan) and the mean value

was calculated for each egg. The force required to break the eggshell (kg-force) was measured using an Instron Materials Tester (Model 4411, Instron Corp., Canton, MA) with a static load cell (200 N) and Bluehill software version 2.29 as described by Bello and Korver (2019).

5.2.4 Bone Characteristics

At each of 42, 54, 64, and 74 wk of age, eight pre-identified hens per dietary treatment were randomly selected and euthanized by cervical dislocation, and the left and right femurs removed. Femurs were cleaned of soft tissue except the cartilage caps and frozen at -20°C until further analysis.

5.2.4.1 Bone Breaking Strength

The frozen right femurs were thawed at 4°C for 24 h before measurement. Each femur was marked at proximal (25% from the proximal epiphysis of the length of the femur), the midpoint (50% from the proximal epiphysis of the length of the femur), and distal (75% from the proximal epiphysis of the length of the femur) locations, determined using a digital caliper (Model CD-8”C, Mitutoyo Corp., Japan). Bone breaking strength was measured using an Instron Materials tester (Model 4411, Instron Corp., Canton, MA) with a 500 N static load cell and Bluehill software version 2.29 as described by Riczu et al. (2004).

5.2.4.2 Bone Ash

The right femur, after BBS measurement, was cut at 25% and 75% from the proximal epiphysis of the bone using a Dremel tool (Dremel MultiPro Model 395, Racine, WI) to separate proximal end (25%), mid-bone (50%), and distal end (25%). Each bone segment was oven-dried (Despatch Oven Co., Minneapolis, MN) at 100°C for 48 h to determine dry bone weight, and subsequently ashed in a muffle furnace (30400 Thermolyne Furnace, Dubuque, IA) at 500°C for 48 h (Bello et al., 2014) to determine ash content (in g) and the percent ash of each of the

proximal, mid-bone, and distal sections. Total dry bone weight and total bone ash were calculated by summation of the three section dry bone weights and ash content weights, respectively (Pongmanee et al., 2020).

5.2.4.3 Bone Densitometry

The frozen left femurs were thawed at 4°C for 24 h prior to QCT analysis. Each femur was marked at proximal 20%, mid-bone 50%, and distal 20% femur (20%, 50%, and 80% from the proximal epiphysis of the length of the femur, respectively), using a digital caliper (Model CD-8"C, Mitutoyo Corp., Japan). The proximal 20%, mid-bone 50% and distal 20% of femur were scanned by QCT using a Stratec Norland XCT (XCT Research SA, Norland Corp., Fort Atkinson, WI) scanner with a 50 kV x-ray tube (Korver et al., 2004; Saunders-Blades and Korver, 2015) to determine total, cortical, and trabecular + medullary bone mineral density (**BMD**) and cross-sectional area (Saunders-Blades et al., 2009). The QCT scan locations were chosen to ensure that measurements were taken within the segment cut out for the bone ash measures. The total measure was the weighted average of the cortical and trabecular bone fractions, and reflected the density or area of each bone compartment. Cortical BMD was the outer shell of the bone that was determined to have a density of > 500 mg/cm^3 (Saunders-Blades et al., 2009). Due to the limitation of the QCT equipment to distinguish trabecular from medullary bone (Korver et al., 2004), the bone in the trabecular space reported by the software was assumed to include both trabecular and medullary bone tissues, and is reported as "trabecular + medullary." Bone mineral content (**BMC**; mg/mm) represents the amount of bone mineral contained in a 1-mm thick slice of the bone, and was calculated by BMD multiplied by the cross-sectional area (Saunders-Blades et al., 2009; Pongmanee et al., 2020).

5.2.5 Statistical Analysis

The cage (individual hen) was the experimental unit for all outcomes. All data were analyzed by a two-way ANOVA, with the model including diet, age, and their interaction using the MIXED procedure of SAS for bone traits and eggshell thickness, except egg SG and eggshell breaking strength using the MIXED and the HPMIXED procedures of SAS (SAS Institute Inc., 2012). All data were tested for normality and normality of residuals using the UNIVARIATE procedure. Body weight was used as a covariate for determinations of bone traits. Means were separated using the LSMEANS statement. Tukey's range test was applied to compare multiple means. Statistical significance was considered when $P \leq 0.05$. Trends were reported where $0.05 < P \leq 0.10$. Values are presented as least squares means (**LSM**) with the respective standard errors of the mean.

5.3 RESULTS

5.3.1 Eggshell Quality

There was no diet x age interaction for egg SG, eggshell thickness nor breaking strength (Table 5.1). The NC + 1,200 diet had lower egg SG than the NC + 300 and the PC diets. Egg SG was the highest at 26 wk of age and lowest at 74 wk of age. The NC + 600 diet had lower eggshell thickness than the others except for the NC + 1,200 treatment. Across dietary treatments, eggshell thickness was higher at 22 wk of age than the other ages except at 54 wk of age. Eggshell thickness was lowest from 26 to 30, at 50, and from 59 to 74 wk of age. Dietary treatment did not affect eggshell breaking strength. The eggshell breaking strength was highest from wk 22 to 46, but was lower from 50 wk to the end of the trial. Eggshell breaking strength was lower at 74 wk of age than all other ages except 69 wk of age ($P < 0.001$).

5.3.2 Bone Characteristics

5.3.2.1 Bone Breaking Strength

There was no diet by age interaction for BBS. Although birds fed the NC diet had similar BBS to the PC birds, phytase supplementation at 600 and 1,200 FTU/kg increased BBS (P = 0.014) relative to the NC treatment (Figure 5.1A). Bone breaking strength at 74 wk of age was greater than at 42 wk of age (P = 0.042; Figure 5.1B).

5.3.2.2 Dry Bone Weight and Ash

Across ages, the proximal and distal region dry bone weights were higher in the NC + 1,200 hens than the NC hens; total dry bone weights of the NC + 600 and NC + 1200 hens were greater than that of the NC hens (Table 5.2). At the mid-bone region, the NC + 300 hens had greater dry bone weight than the NC hens (P = 0.026). Across dietary treatment, proximal, distal, and total dry bone weights were higher at 74 wk than at 42 and 54 wk of age. The NC + 600 and NC + 1,200 diets increased bone ash content of the total and each of bone regions. Bone ash content of the proximal, mid-bone, distal, and total increased with hen age. The NC + 600 hens had greater percent distal bone ash than the NC hens at 74 wk of age, but there was no treatment effect at the other ages (P = 0.013; Figure 5.2). Across ages, the percent bone ash for total and at each bone region was higher in the NC + 600 hens than the NC hens. At 42 wk, the percent bone ash of the mid-bone and total bone ash were lower than at other ages (P < 0.001). No diet x age interactions were observed for dry bone weight nor bone ash content for any of the bone sections. At 74 wk, the NC hens tended to have lower proximal (P = 0.086) and mid-bone (P = 0.066) dry bone weight, and lower mid-bone (P = 0.092) ash content than the PC hens (Table 5.2).

5.3.2.3 Bone Densitometry

There were no diet x age interactions on BMD at any of the scan locations (Table 5.3). Birds fed the NC diet had decreased proximal ($P = 0.001$) and distal ($P = 0.002$) femur total BMD compared to those fed the PC diet, and feeding the NC + 300, NC + 600 or NC + 1,200 diets restored proximal and distal total BMD to the level of the PC diet. Although there was no difference in total BMD at the mid-bone region between the NC and PC diets, the NC + 600 and the NC + 1,200 diets increased mid-bone total BMD ($P < 0.001$) relative to the NC diet. Although there were no differences between the PC and NC hens, the NC + 600 hens had greater distal trabecular + medullary BMD than the NC and NC + 300 hens ($P = 0.023$). Across dietary treatment, proximal, mid-bone, and distal total BMD at 42 wk of age were lower than at 64 and 74 wk of age. At 42 wk of age, hens had lower proximal ($P < 0.001$), mid-bone ($P < 0.001$), and distal ($P < 0.001$) trabecular + medullary BMD than at 74 wk of age, and also lower values for the mid-bone region than at 64 wk of age. Neither dietary treatment nor age affected cortical BMD.

There were no diet x age interactions for proximal, mid-bone, and distal femur total, cortical or trabecular + medullary bone cross-sectional area (data not shown). There were no treatment effects on proximal, mid-bone, or distal cortical bone cross-sectional area between the PC and the NC hens. However, the NC + 600 diet increased cortical bone cross-sectional area at the proximal (22.2 $\pm$ 1.18 vs. 17.8 $\pm$ 0.35 mm^2; $P = 0.001$), mid-bone (16.8 $\pm$ 0.91 vs. 13.9 $\pm$ 0.28 mm^2; $P = 0.011$), and distal (22.1 $\pm$ 1.22 vs. 18.4 $\pm$ 0.24 mm^2; $P = 0.006$) relative to the NC diet. Conversely, the NC + 600 diet decreased trabecular + medullary bone cross-sectional area at the proximal (36.0 $\pm$ 1.55 vs. 41.4 $\pm$ 0.86 mm^2; $P = 0.020$), mid-bone (20.1 $\pm$ 1.15 vs. 25.0 $\pm$ 0.74 mm^2; $P = 0.008$), and distal (34.8 $\pm$ 0.96 vs. 38.6 $\pm$ 0.91 mm^2; $P = 0.021$) relative to the NC

diet. Age did not affect the proximal, mid-bone, or distal femur total, cortical, or trabecular + medullary cross-sectional area.

No diet by age interactions were observed for any of the trabecular + medullary BMC measures (Table 5.4). Birds fed the NC diet had lower proximal ($P = 0.002$) and mid-bone ($P = 0.008$) total BMC than birds fed the PC diet. Phytase supplementation at 600 FTU/kg increased proximal and mid-bone total BMC to the level of the PC; this was also true of 1,200 FTU/kg for the proximal region. There were no differences between the PC and NC hens, but the NC + 600 hens had greater distal femur total BMC than the NC hens ($P = 0.012$). Although there were no differences in proximal, mid-bone or distal cortical bone BMC between the PC and the NC diets, the NC + 600 diet increased proximal ($P = 0.002$), mid-bone ($P = 0.010$), and distal ($P = 0.003$) cortical BMC compared to that of the NC treatment. The NC + 1200 diet also increased proximal cortical BMC relative to the NC diet. There was no effect of diet on proximal, mid-bone, or distal trabecular + medullary BMC. However, the NC hens tended to have higher ($P = 0.069$) distal trabecular + medullary BMC than that of the NC + 300 hens. At 74 wk of age, hens had greater proximal ($P = 0.002$), mid-bone ($P = 0.003$), and distal ($P = 0.006$) femur total BMC than at 42 wk of age. At 64 wk of age, the mid-bone total BMC was also greater than at 42 wk of age. Hens at 74 wk of age also tended to have higher proximal ($P = 0.086$) and distal ($P = 0.100$) femur cortical BMC than at 42 wk of age. Mid-bone trabecular + medullary BMC of hens at 74 wk of age was higher than at 42 and 64 wk of age ($P = 0.003$).

5.4 DISCUSSION

The reduced dietary aP and Ca in the NC diet did not decrease egg SG, eggshell thickness, and breaking strength in this study. This indicates that the decreases were not sufficient to negatively affect eggshell quality. At the tested levels, hens were able to maintain

egg production and eggshell quality in the long term. In Ca-adequate diets, reductions of aP to 0.20 or 0.10% (Punna and Roland, 1999; Boling et al., 2000b; Keshavarz, 2003; Hughes et al., 2008; Lei et al., 2011; Nie et al., 2013; Jing et al., 2018) did not adversely affect eggshell quality. Moderate reductions of Ca (to 3.506%) and aP (to 0.224%) did not decrease eggshell quality (Bello and Korver, 2019). Also, reductions of dietary Ca to 3.0% and aP to 0.19% did not impair eggshell thickness and breaking strength (Pongmanee et al., 2020). Eggshells contain only 0.1% P, but between 38 to 42% Ca (Masuda and Hiramatsu, 2008; Kulshreshtha et al., 2018), or approximately 2.5 g of Ca (Bar et al., 1996). Severe reductions of Ca in layer diets reduce eggshell quality. When hens were fed 0.54% Ca with adequate aP in the diet for five days, eggshell weight and plasma Ca decreased compared to hens fed 3.5% Ca (Ieda et al., 1999). Also, reduction of dietary Ca to 2.62% significantly decreased eggshell breaking strength (Jiang et al., 2013). Since aP and Ca levels in the NC diet were not deficient to what the hens actually require, the negative effect on eggshell quality was not observed in the present study. It was not surprising that egg SG, eggshell thickness and breaking strength decreased with age in the current study. Eggshell thickness decreases as hens age (De Ketelaere et al., 2002) because egg size increases with hens age, but the amount of shell remains relatively constant (Saunders-Blades and Korver, 2015). Also, the activity of renal 25-hydroxylase, renal calbindin-D_{28k} and level of active vitamin D_3 metabolites decrease in older hens, resulting in the efficiency of Ca absorption and Ca deposition in eggshell decreased (Elaroussi et al., 1994; Świątkiewicz et al., 2017). This also reduces eggshell breaking strength in aged hens (Lichovnikova, 2007; Akbari Moghaddam Kakhki et al., 2019).

The NC diet seemed to have only a subtle effect on traditional methods of bone measures (i.e. dry bone weight, bone ash, and BBS). However, the negative effect of the NC diet were

more clearly observed in the QCT measurements. Therefore, the reduced levels of aP and Ca in the NC diet were only marginally deficient, which is consistent with the decreased BW in these birds (Chapter 4). With a marginal deficiency, the NC hens mobilized structural bone (cortical and trabecular bone tissues) to support the intensive of egg production and eggshell formation, resulting in increased cortical thinning, but a thicker area of bone in the trabecular space as indicated by the decrease in cortical bone cross-sectional area and increase in trabecular + medullary bone cross-sectional area in each of the QCT scan locations. As a result, the NC hens had low total and cortical BMC. Although serum pyridinoline (as a bone resorption marker) was not determined in our study, elevated serum pyridinoline has been observed in hens fed moderate and severe reductions of aP and Ca, which caused hens to mobilize bone Ca to support eggshell formation, and subsequently reduced total and trabecular BMC (Bello et al., 2020).

Laying hens are able to physiologically adapt to the reductions of aP and Ca in diet and maintain productive performance, depending on the degree of deficiency (Boling et al., 2000b; Nie et al., 2013; Geraldo et al., 2014) and varying by strain (Hughes et al., 2009). Reducing either dietary Ca or aP can decrease bone quality. In a Ca-adequate diet, reducing non-phytate P to 1.2 g/kg (Panda et al., 2005) or 1.0 g/kg (Boling et al., 2000a) decreased egg production, hen BW and bone quality. In an aP-sufficient diet, reduction of Ca to 32 g/kg (Świątkiewicz et al., 2015), 26.2 g/kg (Jiang et al., 2013), or 25 g/kg (Roland et al., 1996) decreased BBS, BMD, and stiffness. Reducing dietary Ca to 26.2 g/kg also decreased osteoprotegerin mRNA expression, indicating that hens increased osteoclast differentiation and bone resorption (Jiang et al., 2013). Moderate reductions in aP (to 0.163%) and Ca (to 3.341% Ca) maintained egg production from 68 to 78 wk but decreased BW from 76 wk of age, while severe reductions to 0.119% aP and 3.225% Ca decreased egg production and hen BW from 72 wk of age and decreased BMD and

BMC (Bello et al., 2020). Although the moderate reductions of aP and Ca in the NC diet in the current study were fed from hatch until 74 wk of age, the hens were able to maintain overall productivity, but it was associated with decreases in some bone measures.

Because the NC diet decreased total BMD and BMC in the NC hens, this scenario allowed us to observe the efficacy of exogenous phytase on bone mineralization. Phytase supplementation at 600 FTU/kg (and 1,200 FTU/kg for some bone measures) restored total BMD and BMC, and maintained the cortical bone cross-sectional area to the level of the PC treatment. Phytase addition to the NC diet alleviated those subtle effects on bone mineralization, suggesting that it was liberating Ca and P from phytate (Selle and Ravindran, 2007; Tahmasbi et al., 2012; Humer et al., 2015) and reduced bone loss (Hughes et al., 2009). Therefore, if the reductions in dietary aP and Ca were more severe, it is likely that phytase would have been more efficacious. Supplementation of 300 FTU phytase/kg in a diet containing 1.2 g/kg non-phytate P restored BBS (Panda et al., 2005). Reducing aP to 0.119% and Ca to 3.225% decreased total BMD, and 600 FTU phytase/kg feed restored bone quality (Bello et al., 2020). With the moderate reductions of aP and Ca in the NC diet, both 600 and 1,200 FTU phytase/kg resulted in positive effects, but 600 FTU phytase/kg completely alleviated detrimental effects on most of the bone traits measures. In the case of more severe reductions, 600 FTU phytase/kg did not completely restore bone quality (Bello et al., 2020), indicating that more than 600 FTU phytase /kg feed may be necessary to maintain bone health.

Total BMD, BMC and BBS increased with hen age in the present study although cortical bone measures did not change up to 74 wk of age, which is surprising. Bone in the trabecular space increased with age, almost certainly due to the accretion of medullary bone (Whitehead and Fleming, 2000; Whitehead, 2004; Shahnazari et al., 2006; Bello and Korver, 2019) and this

may account for the increase in total bone mineralization. Medullary bone contributes to only a limited to strength and fracture resistance (Whitehead, 2004; Rodriguez-Navarro et al., 2018), and with the lack of change in the cortical bone, may explain the increase in BBS. In the past, a depletion of structural bone coupled with a decrease in BBS was observed in hens from 50 to 70 wk of age (Fleming et al., 1998b). However, genetic selection for longer laying cycles has increased the resistance to bone loss over time (Harlander-Matauschek et al., 2015; Stratmann et al., 2016; Hardin et al., 2019). Also, modern genetics, nutrition, age, and housing systems contribute to bone quality and fracture resistance (Riczu et al., 2004; Hughes et al., 2009; Candelotto et al., 2017). Therefore, modern laying hens are able to maintain structural bone health and are capable of long production cycles compared to what was normal in the past two decades.

Bone ash represents the total amount of mineral present in the bone, but does not distinguish cortical, trabecular or medullary bone. Also, bone ash does not provide information on the strength of the bone (Bello and Korver, 2019), especially when medullary bone is present (Whitehead, 2004). Whereas three-point bending BBS measures the strength of the mid-diaphysis, it does not provide information on mechanical properties of the metaphysis, which is more susceptible to fracture (Fleming et al., 1998b; Reich and Gefen, 2006). Therefore, having a variety of data from traditional bone quality methods in conjugation with bone QCT assessment gives a more complete picture of bone mineralization.

In the current study, bone ash was determined for each of the 25% proximal, 50% mid-shaft, and 25% distal bone regions, whereas QCT measures were taken at a specific location within each of the proximal (20%), mid-diaphysis (50%), and distal (20%) of the length of the femur, respectively. The three different locations of the femur were selected because different

locations have different bone structure. Typically, the mid-diaphysis contains primarily cortical and medullary bone tissues (Fleming et al., 1998b; Shahnazari et al., 2006; Kerschnitzki et al., 2014; Bello and Korver, 2019), whereas trabecular bone is primarily found in the proximal and distal regions of long bones (Fleming et al., 1998b; Loveridge, 1999; Barak, 2010; Shipov et al., 2010).

The most responsive location of the long bone to dietary treatment was not reported in previous studies (Casey-Trott et al., 2017; Guo et al., 2017; Jing et al., 2018). Although different regions of the bones have different bone architecture, each the bone ash sections provided similar responses to dietary treatment. Total, cortical, and trabecular + medullary BMD, bone cross-sectional area and BMC at each of the proximal, mid-bone and distal scan locations had the same response to moderate reductions of aP and Ca in diet, but the distal scan location was the most sensitive to dietary treatment in the present study. The mechanism of the responsiveness for the site-specific effect at the distal location remains unknown in hens. Regmi et al. (2016) found that BMD at the distal and mid-bone regions are responsive to the housing system whereas Bello and Korver (2019) reported that the proximal and mid-bone regions were equally responsive to dietary treatment. However, the distal region is further from the centre of gravity of hens, and is likely to support more weight or total loading under usual activity than the proximal location, although no information is available in laying hens or in poultry. The response of trabecular bone volume/total tissue volume (BV/TV) to exercise was higher in the distal metaphysis than the proximal metaphysis in rats (Iwamoto et al., 1999), possibly because the distal region received more mechanical loading than the proximal region during exercise (Iwamoto et al., 2005). We assumed that bone mineralization at the distal location in laying hens would show a similar response as in rats. Trabecular bone is predominantly presented at the proximal and distal

metaphyses of the long bones, and consists of a network of bony struts (Loveridge, 1999). However, the distal location contains much more progenitor cells than the proximal location in rats (Iwamoto et al., 2004). Progenitor cells are multi-potent stem cells, which can differentiate into adipocytes, osteoblasts, and chondrocytes (Li et al., 2015). Taken together, this may explain the apparently greater responsiveness to dietary treatment at the distal location. Therefore, assessment of bone ash and QCT at the distal region are recommended for future research to determine bone mineralization in laying hens. This may reduce the costs and increase the sensitivity of bone quality measurement in future studies. Further investigation in the site-specific responses of the long bones to dietary phytase is recommended.

Moderate reductions of aP and Ca in the short-term did not cause the adverse effect on bone mineralization (Frost and Roland, 1991; Gordon and Roland, 1997; Punna and Roland, 1999; Hughes et al., 2008; 2009; Jing et al., 2018). However, fed the moderate reductions of aP and Ca had only subtle long-term (from hatch to 74 wk of age) effects on bone quality in the present study. The long-term reductions allowed us to observe the cumulative subtle effect and the efficacy of phytase. To more readily observe adverse effects on structural bone, hens may need to be fed the moderate reductions of aP and Ca diet longer than 74 wk because cortical BMD, cross-sectional area, and BMC at 74 wk were maintained at the same levels relative to at 42, 54, and 64 wk of age. However, we tended to see the decrease in egg SG and eggshell breaking strength at 74 wk of age. A more severe degree of Ca and aP reductions might also be necessary to better study the effects of phytase in laying hens. Severe reductions of aP and Ca showed negative effects on productivity and bone mineralization faster (within 10 wk) than the moderate reductions as demonstrated by the work of Bello (2018). Severe reduction of aP at 0.1% caused 55% mortality and decreased egg production to 19% within approximately 15 wk

(Punna and Roland, 1999). In the context of commercial layer nutrition, more substantial reductions in dietary Ca and aP relative to the primary breeder recommended levels are likely possible, particularly if an effective phytase is included.

Overall, moderate, long-term reductions of aP and Ca resulted in only marginal deficiencies for bone traits, but hens were able to maintain eggshell quality through 74 wk of age. Thus, the hens adapted to the reductions of aP and Ca by sacrificing bone Ca to support eggshell formation without resulting in clinical signs of P or Ca deficiency or excessive depletion of cortical bone. The marginal deficiencies of aP and Ca are shown by the subtle effects on bone quality and BW. However, addition of 600 and 1,200 FTU phytase/kg to the NC diet alleviated the moderate adverse effects and supported medullary bone remodeling in actively laying hens. Therefore, it is likely that dietary aP and Ca can be reduced in laying hen diets to a greater degree than in the current study with the inclusion of exogenous phytase.

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5.6 TABLES

Table 5. 1: Main effects of diet and age on eggshell quality of laying hens fed different levels of dietary aP and Ca, and phytase supplementation from 19 to 74 wk of age.

	Egg specific gravity (g/cm^3)		Eggshell thickness[1] (mm)		Eggshell breaking strength (kg-force)	
Diet[4]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]
PC	1.084[a]	<0.001	0.353[a]	0.005	3.90	0.04
NC	1.084[a,b]	<0.001	0.352[a]	0.004	3.88	0.04
NC + 300	1.084[a]	<0.001	0.352[a]	0.003	3.99	0.04
NC + 600	1.083[a,b]	<0.001	0.344[b]	0.003	3.85	0.04
NC + 1,200	1.083[b]	<0.001	0.346[a,b]	0.005	3.91	0.04
Age (wk)						
22[5]	1.083[c,d]	<0.001	0.372[a]	0.005	4.33[a]	0.06
26[5]	1.091[a]	<0.001	0.347[c,d]	0.004	4.16[a]	0.05
30[5]	1.082[d,e]	<0.001	0.345[c,d]	0.004	4.26[a]	0.06
34[6]	1.084[c]	<0.001	0.352[b,c]	0.004	4.22[a]	0.06
38[6]	1.087[b]	<0.001	0.353[b,c]	0.004	4.07[a,b]	0.05
42[6]	1.086[b]	<0.001	0.353[b,c]	0.004	4.08[a,b]	0.07
46[7]	1.084[c]	<0.001	0.353[b,c]	0.004	4.13[a,b]	0.09
50[7]	1.082[e,f]	<0.001	0.337[d]	0.005	3.76[c]	0.06
54[7]	1.081[f,g]	<0.001	0.363[a,b]	0.005	3.81[b,c]	0.06
59[8]	1.084[c]	<0.001	0.343[c,d]	0.005	3.72[c]	0.07
64[9]	1.081[f,g]	<0.001	0.340[c,d]	0.005	3.73[b,c,d]	0.10
69[10]	1.081[e,f,g]	<0.001	0.344[c,d]	0.006	3.34[d,e]	0.08
74[10]	1.079[g]	<0.001	0.345[c,d]	0.005	3.16[e]	0.07
Source of variation			Prob > F			
Diet	0.006		0.003		0.212	
Age	<0.001		<0.001		<0.001	
Diet x age	0.298		0.444		0.118	

[1]Means of 8 replicate eggs for each diet at each age.
[2]LSM = least squares mean.
[3]SEM = standard error of the mean.
[4]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.
[5]Means of 51 replicate eggs (PC and NC), 52 replicates (NC + 300 and NC + 600), and 50 replicates (NC + 1,200) for SG and eggshell breaking strength.
[6]Means of 50 replicate eggs (PC), 51 replicates (NC), 52 replicates (NC + 300 and NC + 600), and 50 replicates (NC + 1,200) for SG and eggshell breaking strength.
[7]Means of 42 replicate eggs (PC), 43 replicates (NC), 44 replicates (NC + 300 and NC + 600), and 42 replicates (NC + 1,200) for SG and egggshell breaking strength.
[8]Means of 34 replicate eggs (PC), 33 replicates (NC), 36 replicates (NC + 300 and NC + 600), and 33 replicates (NC + 1,200) for SG and eggshell breaking strength.
[9]Means of 33 replicate eggs (PC and NC), 36 replicates (NC + 300 and NC + 600), and 33 replicates (NC + 1,200) for SG and eggshell breaking strength.
[10]Means of 24 replicate eggs (PC), 25 replicates (NC), 27 replicates (NC + 300 and NC + 600), and 25 replicates (NC + 1,200) for SG and eggshell breaking strength.
[a-g]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

Table 5. 2: Main effects of diet and age on dry bone weight, ash content and percent ash of laying hens fed different levels of dietary aP and Ca, and phytase supplementation from 19 to 74 wk of age[1].

	Proximal 25%[2]		Mid-bone 50%[3]		Distal 25%[4]		Total[5]	
	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]
Diet[8]				Dry bone weight (g)				
PC	1.53[a,b]	0.03	1.31[a,b]	0.02	1.27[a,b]	0.02	4.10[a,b]	0.07
NC	1.45[b]	0.02	1.24[b]	0.02	1.19[b]	0.02	3.89[b]	0.06
NC + 300	1.50[a,b]	0.04	1.32[a]	0.02	1.20[a,b]	0.04	4.03[a,b]	0.09
NC + 600	1.57[a,b]	0.03	1.31[a,b]	0.02	1.29[a,b]	0.03	4.18[a]	0.08
NC + 1,200	1.58[a]	0.03	1.31[a,b]	0.02	1.32[a]	0.03	4.21[a]	0.08
Age (wk)								
42	1.48[b]	0.02	1.28	0.02	1.24[b]	0.02	4.01[b]	0.04
54	1.45[b]	0.02	1.29	0.02	1.16[c]	0.03	3.91[b]	0.05
64	1.54[a,b]	0.03	1.29	0.02	1.26[a,b]	0.03	4.09[a,b]	0.07
74	1.64[a]	0.04	1.34	0.03	1.35[a]	0.03	4.32[a]	0.09
Source of variation				Prob > F				
Diet	0.023		0.026		0.026		0.015	
Age	0.001		0.421		0.001		0.003	
Diet x age	0.086		0.066		0.746		0.230	
Body weight[9]	<0.001		<0.001		<0.001		<0.001	
Diet[8]				Bone ash content (g)				
PC	0.75[a,b]	0.02	0.80[a,b]	0.02	0.63[a,b]	0.02	2.18[a,b]	0.06
NC	0.69[b]	0.01	0.75[b]	0.01	0.58[b]	0.01	2.02[b]	0.04
NC + 300	0.74[a,b]	0.03	0.79[a,b]	0.02	0.59[a,b]	0.02	2.13[a,b]	0.07
NC + 600	0.80[a]	0.02	0.82[a]	0.02	0.66[a]	0.02	2.29[a]	0.06
NC + 1,200	0.78[a]	0.02	0.80[a]	0.02	0.66[a]	0.02	2.25[a]	0.05
Age (wk)								
42	0.71[b]	0.01	0.75[b]	0.01	0.59[b]	0.01	2.06[b]	0.03
54	0.72[b]	0.02	0.78[a,b]	0.01	0.58[b]	0.02	2.08[b]	0.04
64	0.77[a,b]	0.02	0.80[a]	0.02	0.64[a,b]	0.02	2.21[a,b]	0.06
74	0.81[a]	0.03	0.84[a]	0.02	0.68[a]	0.02	2.33[a]	0.07
Source of variation				Prob > F				
Diet	0.004		0.014		0.004		0.003	
Age	0.006		0.001		0.003		0.002	
Diet x age	0.215		0.092		0.601		0.235	
Body weight[9]	<0.001		<0.001		<0.001		<0.001	
Diet[8]				Percent bone ash (% of dry bone weight)				
PC	48.9[a,b]	0.61	60.9[a,b]	0.54	49.7[a,b]	0.68	53.0[a,b]	0.57
NC	47.5[b]	0.53	60.5[b]	0.44	48.4[b]	0.54	51.9[b]	0.47
NC + 300	48.9[a,b]	0.72	60.1[b]	0.60	49.0[a,b]	0.68	52.6[a,b]	0.62
NC + 600	50.5[a]	0.61	62.3[a]	0.48	51.5[a]	0.64	54.5[a]	0.54
NC + 1,200	49.2[a,b]	0.65	61.2[a,b]	0.43	49.8[a,b]	0.66	53.1[a,b]	0.53

Table 5. 2: *Continued*.

	Proximal 25%[2]		Mid-bone 50%[3]		Distal 25%[4]		Total[5]	
	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]
Age (wk)								
42	48.1	0.51	58.4[c]	0.43	47.8[b]	0.45	51.3[b]	0.43
54	49.3	0.58	60.7[b]	0.48	50.1[a]	0.60	53.3[a]	0.50
64	49.7	0.56	62.1[a,b]	0.43	50.6[a]	0.65	53.9[a]	0.51
74	48.9	0.58	62.8[a]	0.45	50.4[a]	0.58	53.7[a]	0.50
Source of variation	Prob > F							
Diet	0.021		0.044		0.007		0.016	
Age	0.176		<0.001		< 0.001		<0.001	
Diet x age	0.159		0.479		0.013		0.124	
Body weight[9]	0.752		0.467		0.502		0.772	

[1]Means of 8 replicate hens for each diet at each age.
[2]Proximal 25% represented the entire section of bone from the proximal tip of the femur to a point 25% of the length of the bone from the proximal end.
[3]Mid-bone 50% represented the remaining segment of bone between the proximal 25% and distal 25% sections.
[4]Distal 25% represented the entire section of bone from the distal tip of the femur to a point 25% of the length of the femur from the distal end.
[5]Total term was calculated by summation of three sections of proximal 25%, mid-bone 50% and distal 25% femur.
[6]LSM = least squares mean.
[7]SEM = standard error of the mean.
[8]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.
[9]Body weight was used as a covariate.
[a-c]Means within column and within main effect for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).

Table 5. 3: Main effects of diet and age on bone mineral density of laying hens fed different levels of dietary aP and Ca, and phytase supplementation from 19 to 74 wk of age[1].

	Bone mineral density (mg/cm^3)					
	Proximal 20%[2]		Mid-bone 50%[3]		Distal 20%[4]	
	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]
Diet[7]			Total[8]			
PC	463[a]	9.89	574[a,b,c]	10.87	489[a]	9.18
NC	428[b]	6.96	538[c]	7.77	457[b]	6.88
NC + 300	449[a,b]	10.53	552[b,c]	11.23	478[a,b]	11.83
NC + 600	484[a]	12.78	607[a]	13.87	516[a]	12.87
NC + 1,200	467[a]	9.17	583[a,b]	9.99	490[a]	8.38
Age (wk)						
42	433[b]	6.54	541[b]	6.88	464[b]	6.49
54	451[a,b]	8.79	562[a,b]	7.94	477[a,b]	9.24
64	468[a]	8.40	581[a]	9.23	494[a]	8.52
74	480[a]	11.49	599[a]	13.64	509[a]	11.16
Source of variation			Prob > F			
Diet	0.001		<0.001		0.002	
Age	0.002		<0.001		0.004	
Diet x age	0.715		0.673		0.339	
Body weight[9]	0.437		0.136		0.012	
Diet[7]			Cortical[10]			
PC	912	7.93	1,109	11.48	935	6.83
NC	928	6.48	1,134	8.00	932	5.86
NC + 300	918	8.20	1,117	12.04	927	7.42
NC + 600	907	9.26	1,097	13.42	921	8.63
NC + 1,200	909	8.35	1,112	11.99	931	7.25
Age (wk)						
42	906	5.61	1,112	9.39	922	5.20
54	917	7.57	1,111	10.34	925	7.76
64	921	7.92	1,120	9.26	935	5.64
74	915	7.61	1,111	12.02	934	7.01
Source of variation			Prob > F			
Diet	0.282		0.168		0.768	
Age	0.431		0.895		0.303	
Diet x age	0.247		0.451		0.108	
Body weight[9]	0.323		0.363		0.492	
Diet[7]			Trabecular + medullary[11]			
PC	235	6.66	226	9.63	248[a,b]	6.77
NC	215	6.20	216	6.91	233[b]	5.29
NC + 300	217	7.12	201	9.29	227[b]	6.44
NC + 600	233	7.93	229	9.67	256[a]	6.51
NC + 1,200	234	6.99	225	8.72	244[a,b]	7.40
Age (wk)						
42	205[c]	5.36	191[b]	7.27	222[c]	5.39
54	220[b,c]	6.64	215[a,b]	9.30	233[b,c]	6.60
64	236[a,b]	6.50	228[a]	5.70	251[a,b]	5.53
74	247[a]	6.47	244[a]	9.04	260[a]	5.72

Table 5. 3: *Continued.*

	Bone mineral density (mg/cm^3)					
	Proximal 20%[2]		Mid-bone 50%[3]		Distal 20%[4]	
	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]
Source of variation			Prob > F			
Diet	0.103		0.237		0.023	
Age	<0.001		<0.001		<0.001	
Diet x age	0.716		0.687		0.146	
Body weight[9]	0.632		0.688		0.039	

[1]Means of 8 replicate hens for each diet at each age.
[2]A single 1 mm-thick slice taken at a point 20% along the length of the femur from the proximal end.
[3]A single 1 mm-thick slice taken at a point 50% along the length of the femur from the proximal end.
[4]A single 1 mm-thick slice taken at a point 80% along the length of the femur from the proximal end.
[5]LSM = least squares mean.
[6]SEM = standard error of the mean.
[7]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.
[8]Total term was the weighted average of both the cortical and trabecular + medullary bone.
[9]Body weight was used as a covariate.
[10]Cortical bone was the outer part of the bone, and was defined as having a density of > 500 mg/cm^3
[11]Trabecular + medullary bone represents bone within the trabecular space, and is assumed to contain unknown proportions of trabecular and medullary bone.
[a-c]Means within column and within main effect for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).

Table 5. 4: Main effects of diet and age on bone mineral content of laying hens fed different levels of dietary aP and Ca, and phytase supplementation from 19 to 74 wk of age[1].

	Bone mineral content[2] (mg/mm)					
	Proximal 20%[3]		Mid-bone 50%[4]		Distal 20%[5]	
	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]
Diet[8]			Total[9]			
PC	29.0[a]	0.70	23.3[a]	0.50	29.2[a,b]	0.68
NC	26.5[b]	0.54	21.6[b]	0.34	27.5[b]	0.47
NC + 300	28.1[a,b]	0.70	22.2[a,b]	0.53	28.5[a,b]	0.85
NC + 600	30.3[a]	0.74	23.9[a]	0.55	30.6[a]	0.72
NC + 1,200	29.3[a]	0.66	22.8[a,b]	0.40	29.0[a,b]	0.62
Age (wk)						
42	27.3[c]	0.41	21.5[b]	0.25	27.7[b]	0.40
54	27.6[b,c]	0.48	22.3[a,b]	0.40	28.3[a,b]	0.62
64	29.3[a,b]	0.66	23.2[a]	0.43	29.7[a,b]	0.67
74	30.4[a]	0.77	24.0[a]	0.55	30.3[a]	0.70
Source of variation			Prob > F			
Diet	0.002		0.008		0.012	
Age	0.002		0.003		0.006	
Diet x age	0.366		0.258		0.184	
Body weight[10]	<0.001		<0.001		<0.001	
Diet[8]			Cortical[11]			
PC	18.4[a,b]	0.70	17.1[a,b]	0.57	18.7[a,b]	0.58
NC	16.5[b]	0.31	15.7[b]	0.25	17.1[b]	0.23
NC + 300	18.1[a,b]	0.62	16.9[a,b]	0.54	18.7[a,b]	0.80
NC + 600	19.9[a]	0.90	18.1[a]	0.73	20.2[a]	0.92
NC + 1,200	18.4[a]	0.55	17.1[a,b]	0.42	18.6[a,b]	0.51
Age (wk)						
42	17.4	0.33	16.3	0.24	17.7	0.31
54	17.7	0.46	16.7	0.35	18.4	0.54
64	18.6	0.52	17.1	0.40	18.8	0.57
74	19.4	0.86	17.8	0.74	19.7	0.81
Source of variation			Prob > F			
Diet	0.002		0.010		0.003	
Age	0.086		0.124		0.100	
Diet x age	0.734		0.234		0.525	
Body weight[10]	<0.001		<0.001		<0.001	
Diet[8]			Trabecular + medullary[12]			
PC	8.82	0.35	5.46	0.28	8.60	0.40
NC	8.89	0.28	5.40	0.24	8.93	0.22
NC + 300	8.32	0.28	4.66	0.28	7.87	0.27
NC + 600	8.33	0.43	5.19	0.26	8.33	0.44
NC + 1,200	8.65	0.44	4.80	0.31	8.16	0.40
Age (wk)						
42	8.05	0.28	4.50[b]	0.23	7.93	0.28
54	8.42	0.32	5.10[a,b]	0.27	8.17	0.33
64	8.72	0.25	4.91[b]	0.20	8.63	0.27
74	9.23	0.42	5.91[a]	0.28	8.77	0.38

Table 5. 4: *Continued.*

	Bone mineral content[2] (mg/mm)					
	Proximal 20%[3]		Mid-bone 50%[4]		Distal 20%[5]	
	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]
Source of variation	Prob > F					
Diet	0.606		0.197		0.069	
Age	0.120		0.003		0.210	
Diet x age	0.980		0.821		0.842	
Body weight[10]	0.039		0.471		0.311	

[1]Means of 8 replicate hens for each diet at each age.
[2]Bone mineral content was calculated as bone mineral density multiplied by the bone cross-sectional area, and is the amount of bone mineral contained in a 1 mm linear section of the scanned region of the bone.
[3]A single 1 mm-thick slice taken at a point 20% along the length of the femur from the proximal end.
[4]A single 1 mm-thick slice taken at a point 50% along the length of the femur from the proximal end.
[5]A single 1 mm-thick slice taken at a point 80% along the length of the femur from the proximal end.
[6]LSM = least squares mean.
[7]SEM = standard error of the mean.
[8]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.
[9]Total term was the weighted average of both the cortical and trabecular + medullary bone.
[10]Body weight was used as a covariate.
[11]Cortical bone was the outer part of the bone, and was defined as having a density of > 500 mg/cm^3
[12]Trabecular + medullary bone represents bone within the trabecular space, and is assumed to contain unknown proportions of trabecular and medullary bone.
[a-c]Means within column and within main effect for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).

5.7 FIGURES

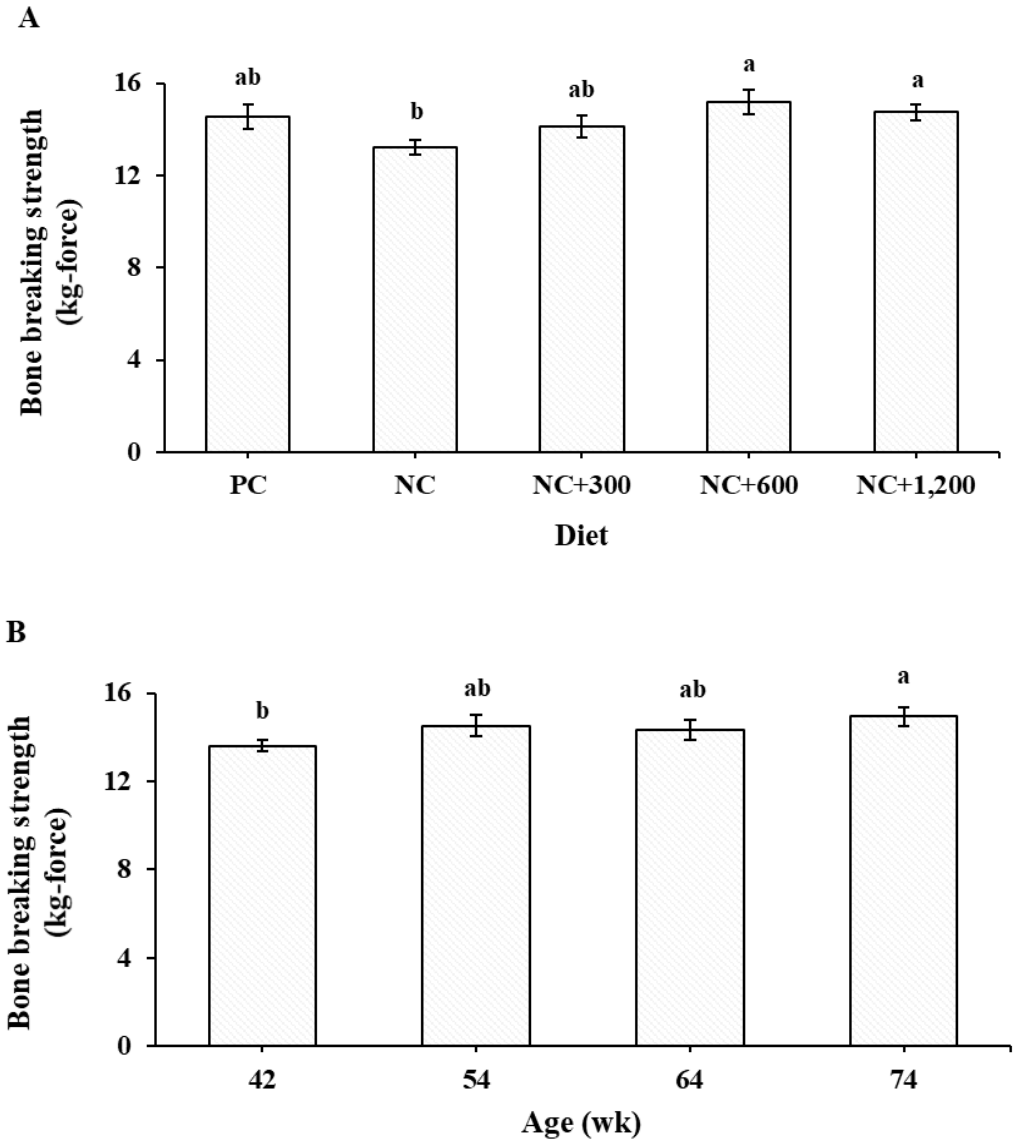

Figure 5. 1: Bone breaking strength of laying hens fed different dietary aP and Ca levels, and phytase supplementation (A; $P = 0.014$, n = 160) and bone breaking strength at each age (B; $P = 0.042$, n = 160). PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively. Body weight was used as a covariate. Data are presented as least squares means (LSM) with the respective standard errors of the mean. [a,b]Means with no common superscript differ significantly ($P \leq 0.05$). The interaction of diet and age was not significant.

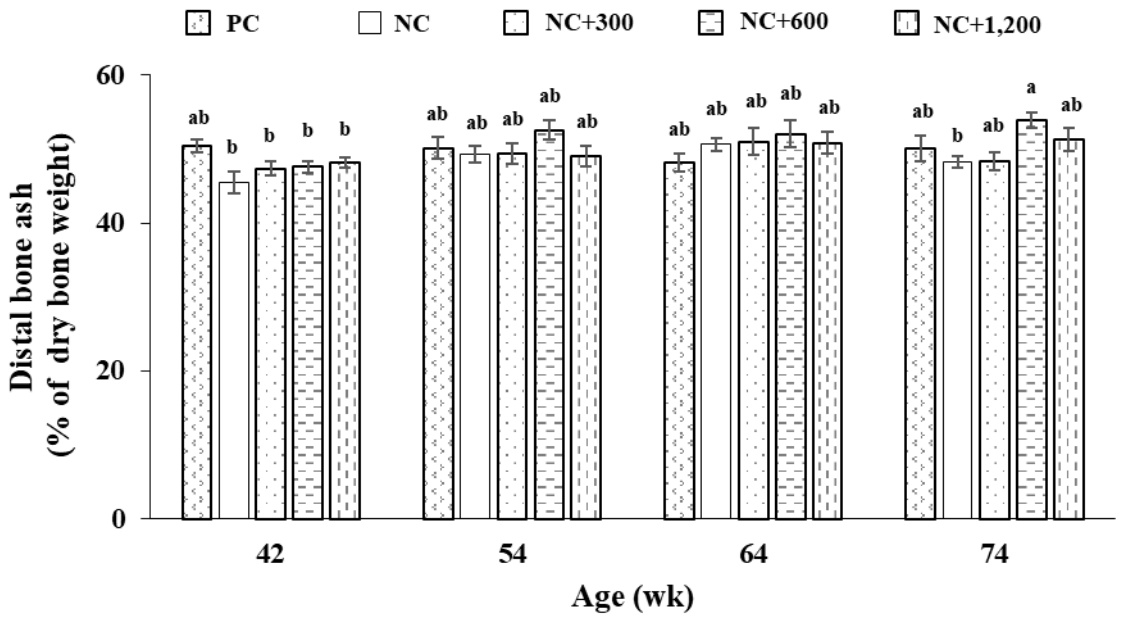

Figure 5. 2: Interaction between dietary treatment and age on distal bone ash of laying hens fed different dietary aP and Ca levels, and phytase supplementation ($P = 0.013$, n = 160). PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively. Body weight was used as a covariate. Data are presented as least squares means (LSM) with the respective standard errors of the mean. [a,b]Means with no common superscript differ significantly ($P \leq 0.05$).

6. RESEARCH SYNTHESIS

6.1 OVERVIEW

Phytate is a phosphorus storage form in plant-based feed ingredients (Hirvonen et al., 2019). Phytate is not well utilized by chickens as a source of P because of limited endogenous phytase in the digestive tract (Maenz and Classen, 1998). Low availability of P in feedstuffs leads to the need to supplement inorganic P such as mono- or di-calcium phosphate in poultry diets. However, inorganic P is an expensive ingredient and is a non-renewable resource (Naves et al., 2016; Kazempour and Jahanian, 2017). Dietary supplementation of inorganic P not only increases diet costs (Ponnuvel et al., 2014) but also increases P excretion (Abudabos, 2012), consequently causes P pollution in the environment (Selle and Ravindran, 2007; Lei et al., 2011; Zyla et al., 2012). In comparison to broilers, layers require high dietary Ca during the laying period to support eggshell formation. The level of Ca in layer feedstuffs is low, therefore a high amount of supplemental Ca is used in layer diets to meet the requirement for Ca. Sources of Ca such as limestone and oyster shell are relatively cheap, and considerable safety margins for Ca levels are used for commercial layer diets (Bedford and Rousseau, 2017). However, excess dietary Ca levels increase formation of Ca-phytate complexes in the small intestine (Hamdi et al., 2015; Sommerfeld et al., 2018b), which decreases Ca and P availability and absorption, and subsequently increase Ca and P excretion. Phytase supplementation has become a standard practice for laying hen diets. Phytase in laying hen diets allows for reduced aP and Ca levels, without negative impacts on body weight (**BW**), egg production, eggshell quality, bone breaking strength (**BBS**) and bone ash (Boling et al., 2000b; Lim et al., 2003; Panda et al., 2005; Hughes et al., 2008). It acts through increasing apparent ileal digestibility (**AID**; Gao et al., 2013; Bello and Korver, 2019; Pongmanee et al., 2020), and retention (Um and Paik, 1999; Keshavarz and

Austic, 2004; Panda et al., 2005; Abudabos, 2012; Wang et al., 2013) of P and Ca. Phytase usage also allows the poultry industry to decrease the use of inorganic P and to increase the use of high-phytate feedstuffs such as canola meal (Bello and Korver, 2019; Pongmanee et al., 2020) or the use of low-quality feed ingredients such in corn-soy-based diets .

Traditional bone quality analyses (e.g. bone ash and BBS) may not fully explain bone metabolism in laying hens due to some limitations. Bone ash represents the total amount of minerals present in the bone but does not distinguish among bone tissue compartments (structural or medullary bone tissue). Bone ash also does not provide information on bone integrity or resistance to breaking. Therefore, bone ash alone is not sufficient to draw a solid conclusion in bone quality specifically during the laying period when medullary is present (Whitehead, 2004). A three-point bending test is commonly used to determine BBS at the mid-shaft of the long bone, which is concentrated with cortical and medullary bone (Regmi et al., 2015; Bello and Korver, 2019). However, three-point bending BBS does not provide the breaking strength at the proximal and distal metaphyses, which have a high concentration of trabecular bone and are more susceptible to fracture than the mid-bone (Reich and Gefen, 2006). Although bone ash and BBS provide useful information on bone mineralization, they are still not sufficient to provide information on bone fracture resistance in laying hens. Therefore, quantitative computed tomography (**QCT**) assessment of bone mineralization provides complementary and more detailed information than that determined using traditional bone measures, because QCT provides information on bone mineral density (**BMD**) and cross-sectional area of total, cortical and trabecular space bone tissues (Korver et al., 2004; Kerschnitzki et al., 2014; Bello and Korver, 2019). Bone mineral content (**BMC**) of each bone tissues fraction can also be calculated using QCT (Saunders-Blades et al., 2009).

Although long-term studies on phytase supplementation in laying hens have been investigated (Boling et al., 2000b; Hughes et al., 2008; Bello and Korver, 2019), there is a very scant publication on phytase use from hatch to the end of the production cycle. To our knowledge, there is only one publication on phytase use in layers beginning with pullet rearing and extending well into production (Punna and Roland, 1999). Also, the majority of experimental diets used in phytase studies in layers were reduced only in dietary available P (**aP**) level, but with adequate dietary Ca (Punna and Roland, 1999; Boling et al., 2000a; Boling et al., 2000b; Hughes et al., 2008; 2009). Therefore, the first objective of this Ph.D. thesis was to determine the short-term effects of phytase supplementation in a diet with moderate reductions of aP and Ca on eggshell and bone quality, and AID of P and Ca of laying hens during peak production. The second objective was to investigate the long-term effects of phytase supplementation in diets with marginal reductions of aP and Ca on pullet performance and hen productivity, eggshell quality, bone mineralization, and P and Ca retention from 1-d-old to 74 wk of age.

The moderate reductions of aP and Ca were tested in the short-term study because we expected that hens would develop signs of P or Ca deficiencies during the 12-wk trial. The negative control (**NC**) diet contained 0.22% aP, 3.00% Ca and Na 0.13% from 25 to 28 wk of age and 0.19% aP, 3.02% Ca and 0.13% Na from 29 to 37 wk of age whereas the positive control (**PC**) diet contained 0.45% aP, 3.70% Ca and 0.16% Na from 25 to 28 wk of age, and 0.38% aP, 3.73% Ca and 0.15% Na from 29 to 37 wk of age (Lohmann Tierzucht, 2012). However, the less severe marginal reductions of aP and Ca were used in the long-term study to avoid the possible cumulative negative effects over the 74-week life of the birds. The PC diet with a sequence of 0.48-0.45-0.37-0.45-0.43-0.40% aP, 1.05-1.00-0.90-2.00-3.71-3.73% Ca, and 0.18-0.17-0.16-

0.16-0.17-0.16% Na for Starter-Grower-Developer-Pre-lay-19 to 54-55 to 74 wk periods, respectively, was formulated (H & N International, 2012). The NC diet, was similar to the PC but reduced in aP, Ca, and Na by 0.15%, 0.16%, and 0.035% of the diet in each phase, respectively.

6.1.1 Review of thesis hypotheses

Hypothesis 1: moderate reductions of aP and Ca in laying hen diets from 25 to 37 wk of age would decrease eggshell and bone quality, and phytase supplementation would alleviate the negative effects. This hypothesis was partially accepted and is discussed in Chapter 2.

Hypothesis 2: marginal reductions of aP and Ca in the NC diet during pullet rearing would decrease pullet performance and bone quality, and phytase supplementation would restore performance and bone quality to the same level of pullets fed aP- and Ca-adequate diet (the PC diet). This hypothesis was partially accepted and is discussed in Chapter 3.

Hypothesis 3: marginal reductions of dietary aP and Ca in the long-term would decrease the quality of femur cortical, trabecular, and medullary bone tissues, and phytase supplementation would increase P and Ca availability to maintain bone quality at the same level of hens fed aP- and Ca-adequate diets. This hypothesis was accepted and is discussed in Chapter 5.

Hypothesis 4: marginal reductions of dietary aP and Ca in the long-term would also consequently decrease eggshell quality, and phytase supplementation would restore the adverse effects of eggshell quality to a similar level of hens fed aP- and Ca-adequate diets. This hypothesis was rejected and is discussed in Chapter 4.

6.2 FINDINGS AND ANALYSES

Long-term reduction of dietary aP by 0.15% and Ca by 0.16% of the diet during pullet rearing (Chapter 3) and up to 74 wk of age (Chapters 4 and 5) did not decrease layer performance and hen productivity. Modern laying hens have high egg production and persistency, and are able to physiologically adapt to the reductions of aP and Ca in the diet depending on the degree of mineral reduction or deficiency (Nie et al., 2013; Geraldo et al., 2014; Bello et al., 2020) and varying by strain (Hughes et al., 2009). This thesis clearly showed that the NC hens were able to meet their P and Ca requirements for growth and egg production. Surprisingly, hens fed marginal reductions in dietary aP and Ca in the long-term maintained eggshell quality as good as the PC hens. However, the birds fed the NC diets from hatch through the end of the production cycle had some subtle cumulative effects on BW and bone characteristics, suggesting that the NC diets were only marginally deficient for these measures. This indicates that the aP and Ca requirements for egg production and eggshell quality differ from aP and Ca needs for bone metabolism. Likewise, moderate reductions of aP and Ca in the NC diet in the short-term did not decrease hen performance, egg production, nor eggshell quality. However, the NC diet decreased BBS at 6 wk of age but this adverse effect was no longer observed at 15 and 18 wk of age, suggesting that pullets may be more sensitive to the levels of dietary aP and Ca at a younger age than at later ages. The NC diet also decreased cortical and trabecular + medullary BMD, and total BMC at 37 wk of age (Pongmanee et al., 2020). Both the short- and long-term trials clearly indicate that the current recommendations for aP and Ca provided by the primary breeder guide have considerable safety margins, and are likely substantially higher than actually required by birds. Hens were able to physiologically adapt to the short- and long-term reductions of dietary aP and Ca at expense Ca bone reserves to

maintain egg production and eggshell formation without symptoms of P and Ca deficiencies. In regards to this thesis, modern laying hens would have responded to marginal and moderate reductions of aP and Ca in the short- and long-term. First, the NC hens were able to overcome the subtle effects of marginal reductions by increasing the efficiency of intestinal P and Ca absorption coupled with reabsorption of these minerals at the kidney during short-term adaptation (Chapter 4; Pongmanee et al., 2020). Secondly, the NC hens progressively mobilized structural bone and sacrificed bone Ca reserves to support egg production along with maintaining eggshell quality in the long term (Bello et al., 2020; Pongmanee et al., 2020). We speculate that at some point beyond 74 wk of age, a depletion of structural bone would occur and hens would develop osteoporosis, resulting in less Ca available for the eggshell formation and poor eggshell quality. Based on the findings in Chapters 2 and 4, good eggshell quality did not guarantee bone health of laying hens. Hens can maintain good eggshell quality but they may experience bone depletion or osteoporosis. Therefore, monitoring only eggshell quality may not sufficient to determine bone health status. This thesis shows that the degree of aP and Ca reductions and the duration the hens were on the experimental diets affected hen response. Either short-term moderate or long-term marginal reductions of aP and Ca in the diets caused marginal deficiencies and impaired bone quality in laying hens.

In the past, a depletion of structural bone was observed from 50 to 70 wk of age in laying hens (Fleming et al., 1998b). However, modern laying hens maintained cortical bone tissue up to 70 wk of age (Bello et al., 2019) or to 74 wk of age (Chapter 5) in this thesis. This indicates that current genetics of laying hens may be able to maintain skeletal health throughout the end of production cycle compared to hens in the last two decades. Modern genetics may be more resistant to osteoporosis than older genetics (Toscano, 2018) because genetic selection has been

focused on longer laying cycles as well as increased resistance to bone loss (Harlander-Matauschek et al., 2015; Stratmann et al., 2016; Hardin et al., 2019). Other factors such as nutrition, age, and housing systems also contribute to bone quality (Riczu et al., 2004; Hughes et al., 2009; Candelotto et al., 2017). The lack of the decrease in cortical bone measures up to 74 wk of age, along with the accretion of medullary bone tissue, resulted in greater BBS at 74 wk than at 42 wk of age (Chapter 5), which is surprising. Fundamentally, medullary bone is weaker than cortical and trabecular bone tissues, but it still provides some fracture resistance (Fleming et al., 1998a; Whitehead, 2004), which might explain the increase in BBS with hen age.

Exogenous phytase has been used in layer diets to liberate P from phytate in feed ingredients. Not only P, but proteins, starch, and minerals such as Ca also liberated by phytase. Supplementation of phytase alleviates negative effects of reduced dietary aP and Ca by increasing the availability of P and Ca and maintaining performance, and eggshell and bone quality of laying hens. Commercially, phytase is usually supplemented in layer diets at 300 phytase unit (**FTU**)/kg. In order to observe the effects of increasing exogenous phytase dose in aP- and Ca-reduced diets, varying doses of phytase ranging from 150 to 2,400 FTU/kg were used in this thesis. Overall, the effects of phytase supplementation at any level were not pronounced for performance, egg production, and eggshell quality (Pongmanee et al., 2020; Chapter 4), and thus the efficacy of phytase could not be validated for these measures. This does not mean the inclusion of phytase was not effective. The lack of the deficiency signs in the NC groups indicates that the levels of aP and Ca suggested by primary management guides include a considerable safety margin. Also, hens were able to physiologically adapt to aP- and Ca-reduced diets. It is therefore not surprising that phytase had no opportunity to affect performance and productivity, because the P and Ca liberated from phytate were not needed by the bird. However,

an only marginal deficiency was observed in the bone measures. This scenario allowed us to observe a phytase response. Marginal reductions of dietary aP and Ca in the long term decreased proximal and distal total BMD, and proximal and mid-shaft BMC in the NC hens. Phytase at 600 and 1,200 FTU/kg restored BMD and BMC and maintained the cortical bone cross-sectional area of the long bones during the laying period (Chapter 5). Long-term phytase supplementation maintained bone health through the end of the production cycle.

Phytase use in laying hen diets is a common practice. However, it is likely that phytase has been supplemented in layer diets for only the laying period without consideration during pullet rearing because the economic impact is less clear during the pullet period and there is no easily measurable return from egg production. It is clear that dietary phytase supplementation during the laying period maintains structural bone and also supports the metabolism of medullary bone, which is important for eggshell formation (Chapter 5). Phytase also increased P digestibility and retention during the laying period (Chapters 2 and 4). However, phytase supplementation at 2,400 FTU/kg increased P retention at 6 wk of age (Chapter 3), which would subsequently reduce P excretion to the environment. Phytase supplementation at 300, 600, 1,200, and 2,400 restored BBS of pullets at 6 wk of age (Chapter 3). Since deposition of cortical and trabecular bone tissues ceases when pullets approach sexual maturity (Fleming et al., 1998b; Whitehead, 2004), maximizing in structural bone health before sexual maturity would be important to the laying period. Supplementation of phytase in layer pullet diets reduced in Ca and aP would enhance and maximize structural bone growth and development during the pullet rearing. Enhanced skeletal structure at the onset of lay could reduce the occurrence of poor eggshell quality in the late production cycle.

The optimal level of phytase used depends on feed ingredients and compositions. For example, 300 FTU phytase/kg may be suitable for corn-soybean based diets whereas phytase levels at greater than 300 FTU/kg may be suitable for corn-soybean-canola-based diets or other high phytate based diets. Phytase more readily degrades phytate in corn and soybean meal than in canola meal and rice bran (Selle and Ravindran, 2007). This could be related to high levels of phytate contained in the two latter feed ingredients. High phytate as a substrate in the diets may require greater levels of phytase activity for optimal P liberation. The marginal or moderate reductions of aP and Ca in layer diets in this research had little or only subtle effects on BW and bone traits, and phytase had the potential to overcome these adverse effects. This suggests that dietary aP and Ca can be reduced to a greater degree than in our studies with the inclusion of exogenous phytase. This practice would increase the efficacy of phytase and also allow producers to use low quality of feedstuffs with high phytate, which would reduce diet costs overall.

In this thesis, bone QCT measures were used in conjugation with traditional bone analytical methods to obtain a more complete picture of bone mineralization in laying hens. The specific regions of the long bone were investigated because different locations have different bone structure. The proximal and distal regions of the long bones consist of cortical and trabecular bone tissues (Fleming et al., 1998b; Loveridge, 1999; Barak, 2010; Shipov et al., 2010). However, the mid-diaphysis contains primarily of cortical and medullary bone tissues (Fleming et al., 1998b; Shahnazari et al., 2006; Kerschnitzki et al., 2014; Bello and Korver, 2019). Based on bone ash content and bone QCT data in this research, the distal region of the long bone is the most responsive location to dietary treatment, potentially because the distal region is further from the centre of gravity of hens, and this location is likely to support more

body weight and total loading from all activities compared to the proximal or mid-shaft region, as reported in rats (Iwamoto et al., 1999; Iwamoto et al., 2004; Iwamoto et al., 2005). This may explain the apparently greater responsiveness to dietary treatment at the distal than proximal or mid-bone region. Therefore, the distal region of the long bone would be considered to represent the long bone mineralization in laying hens, rather than assessment of bone ash and QCT all three regions or the whole bone. This can reduce laboratory work and costs and may increase the sensitivity of bone quality measurement.

6.3 RECOMMENDATIONS FOR FUTURE RESEARCH

The reductions of aP and Ca levels in the NC diets had no effects on performance (Chapters 2, 3, and 4), egg production, and eggshell quality (Chapters 2 and 4). This could be because the levels of aP and Ca in the NC diets were above the actual requirements of birds. The PC diets in this thesis were not based on the NRC (1994) because this might not be suitable for modern laying hens, but were formulated to meet or exceed the commercial nutrient recommendations (H & N International, 2012; Lohmann Tierzucht, 2012), which include high safety margins for aP and Ca. Hence, further reductions of aP and Ca in the NC diets might still be possible, particularly with the inclusion of phytase. Currently, the levels of aP and Ca in commercial layer diets recommended by the primary breeder management guide have considerable safety margins. It would be useful to determine the actual requirements of aP and Ca in modern layers, both in the pullet rearing and the laying periods in the future studies. When the requirements for aP and Ca are defined, it then would be possible to determine the phytase efficacy at different levels; recommendation, high, and super-dosing levels in layers.

At the moderate reduction levels of aP and Ca in the NC diet in the short-term (Chapter 2), performance, egg production, and eggshell quality were not different between the PC and NC

hens from 25 to 37 wk of age. However, the reductions of these minerals had a minor impact on bone mineralization as shown in the tarsometatarsus QCT; hens had begun to mobilize bone mineral. Therefore, it would be interesting to extend the experimental period beyond 37 wk of age to observe the clinically negative effect on bone quality and subsequently affect performance and productivity to older ages. Although marginal reductions of aP and Ca in the long-term (from hatch to 74 wk of age; Chapters 3, 4, and 5) had negative effects on bone quality, but overall performance and productivity were not affected. Therefore, it would be useful to extend the study beyond 74 wk of age as the laying cycle of commercial flocks is moving towards up to 100 wk of age (Bain et al., 2016; Ugalde, 2019). This might make it more likely to observe the adverse effects in the NC hens and also to determine the efficacy of phytase.

Neither marginal nor moderate reductions of aP and Ca caused major negative effects on hen performance and productivity because hens are able to maintain egg production. Bone depletion and poor eggshell quality develop when hens were fed severely reduced dietary aP and Ca (Bello et al., 2020), but that short-term trial was investigated in the late production cycle. Punna and Roland (1999) reported that when 0.1% dietary aP was fed from 1 day of age, hens had only 79% egg production at peak at 26 wk of age and declined thereafter to 33% at 36 wk of age, with severe mortality (55%) by the end of wk 38. Therefore, further reductions of aP and Ca relative to those used in this thesis may be possible to observe the adverse effect on performance, productivity, and bone quality as well as the efficacy of phytase. Further investigation on phytase use and bone mineralization in pullets may be required because phytase increased BBS up to 6 wk of age in this thesis. Maximizing structural bone in pullets before the onset of sexual maturity using phytase supplementation would likely be important to long-term production of laying hens. Monitoring bone changes in each location of the long bone over time in modern laying hens

would also be interesting. To our knowledge, no information has been reported as the most responsive location of the long bone in poultry. It will be significant to investigate the region-specific responses of the long bones to phytase supplementation or age in modern laying hens.

6.4 STUDY LIMITATIONS

The analyzed Ca levels in dietary treatments were 5 to 24% higher from 25 to 34 wk of age (Chapter 2), and were 2 to 11% higher from 19 to 74 wk of age (Chapter 4), than the calculated. High Ca levels in the diets may have caused inconsistent results to hen performance, productivity, and eggshell quality. Therefore, analyzing dietary Ca before feeding it to laying hens would have reduced variability, and would have made the experiment more consistent.

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APPENDICES

Appendix 2. 1: The effect of dietary P and Ca levels, and phytase supplementation on egg weight, specific gravity and eggshell thickness of laying hens[1].

	Egg weight[2] (g)		Specific gravity[2] (g/cm^3)		Eggshell thickness[3] (mm)	
	LSM[4]	SEM[5]	LSM[4]	SEM[5]	LSM[4]	SEM[5]
Diet[6]						
PC	59.2	0.78	1.090[a,b]	<0.01	0.37	<0.01
NC	61.5	0.67	1.088[b]	<0.01	0.36	<0.01
NC + 150	60.3	0.92	1.090[a,b]	<0.01	0.37	<0.01
NC + 300	61.9	0.70	1.088[a,b]	<0.01	0.36	<0.01
NC + 600	61.5	0.78	1.089[a,b]	<0.01	0.36	<0.01
NC + 1,200	60.4	0.54	1.090[a]	<0.01	0.38	<0.01
PC + 1,200	60.4	0.83	1.088[a,b]	<0.01	0.36	<0.01
Age (wk)						
29	59.3[b]	0.48	1.088[b]	<0.01	0.36	<0.01
33	61.5[a]	0.48	1.090[a]	<0.01	0.36	<0.01
37	61.5[a]	0.52	1.089[b]	<0.01	0.37	<0.01
Source of variation			Prob > F			
Diet	0.153		0.004		0.105	
Age	0.002		0.001		0.072	
Diet x age	0.997		0.916		0.948	

[1]Eggs were collected from the last day of each period of 4-wk intervals.
[2]Means of 12 replicate eggs for each diet.
[3]Means of 6 replicate eggs for each diet.
[4]LSM = least squares mean.
[5]SEM = standard error of the mean.
[6]PC, the positive control diet, nutritionally complete diet containing 0.45% aP, 3.70% Ca and 0.16% Na (25 to 28 wk), 0.38% aP, 3.73% Ca and 0.15% Na (29 to 37 wk); NC, the negative control diet, similar to the PC diet but having 0.22% aP, 3.00% Ca and 0.13% Na (25 to 28 wk), 0.19% aP, 3.02% Ca and 0.13% Na (29 to 37 wk); NC + 150, NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 150, 300, 600, or 1,200 FTU/kg, respectively; and PC + 1,200, the PC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 1,200 FTU/kg.
[a,b]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

Appendix 2. 2: The effect of dietary P and Ca levels, and phytase supplementation on dry femur weight, femur ash content and percent ash of laying hens at 37 wk of age[1].

	Proximal 25%[2]		Mid-bone 50%[3]		Distal 25%[4]		Total[5]	
	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]	LSM[6]	SEM[7]
Diet[8]	Dry bone weight (g)							
PC	1.18	0.03	1.46	0.03	1.48	0.04	4.12	0.09
NC	1.23	0.04	1.40	0.03	1.46	0.04	4.10	0.09
NC + 150	1.15	0.02	1.36	0.03	1.44	0.03	3.96	0.06
NC + 300	1.20	0.04	1.41	0.02	1.44	0.03	4.05	0.08
NC + 600	1.12	0.04	1.37	0.03	1.38	0.04	3.87	0.09
NC + 1,200	1.17	0.04	1.38	0.04	1.40	0.04	3.95	0.11
PC + 1,200	1.20	0.04	1.40	0.03	1.43	0.04	4.02	0.09
Source of variation	Prob > F							
Diet	0.493		0.401		0.676		0.514	
Body weight[9]	<0.001		<0.001		<0.001		<0.001	
Diet[8]	Bone ash content (g)							
PC	0.57	0.02	0.84	0.02	0.72	0.02	2.13	0.06
NC	0.56	0.02	0.79	0.02	0.71	0.02	2.06	0.06
NC + 150	0.55	0.02	0.79	0.01	0.71	0.01	2.05	0.03
NC + 300	0.56	0.02	0.80	0.01	0.70	0.02	2.06	0.04
NC + 600	0.54	0.03	0.78	0.03	0.68	0.02	2.00	0.07
NC + 1,200	0.56	0.02	0.79	0.03	0.68	0.03	2.02	0.07
PC + 1,200	0.57	0.02	0.80	0.02	0.70	0.02	2.07	0.06
Source of variation	Prob > F							
Diet	0.963		0.552		0.879		0.903	
Body weight[9]	0.002		<0.001		<0.001		<0.001	
Diet[8]	Percent bone ash (% of dry bone weight)							
PC	47.9	1.14	58.0	1.05	49.0	1.01	51.8	1.03
NC	45.6	1.83	56.5	0.81	48.6	0.65	50.3	1.05
NC + 150	47.6	0.88	58.0	0.76	49.2	0.75	51.8	0.73
NC + 300	47.1	0.65	56.5	0.53	48.6	0.49	50.9	0.49
NC + 600	48.0	0.94	57.2	0.81	49.1	0.76	51.6	0.77
NC + 1,200	47.3	0.56	57.1	0.84	48.5	0.79	51.2	0.71
PC + 1,200	47.2	0.90	56.9	0.64	49.4	0.89	51.3	0.74
Source of variation	Prob > F							
Diet	0.942		0.760		0.974		0.907	
Body weight[9]	0.119		0.418		0.789		0.653	

[1]Means of 12 replicate hens for each treatment.

[2]Proximal 25% represented the entire section of bone from the proximal tip of the femur to a point 25% of the length of the bone from the proximal end.

[3]Mid-bone 50% represented the remaining segment of bone between the proximal 25% and distal 25% sections.

[4]Distal 25% represented the entire section of bone from the distal tip of the femur to a point 25% of the length of the femur from the distal end.

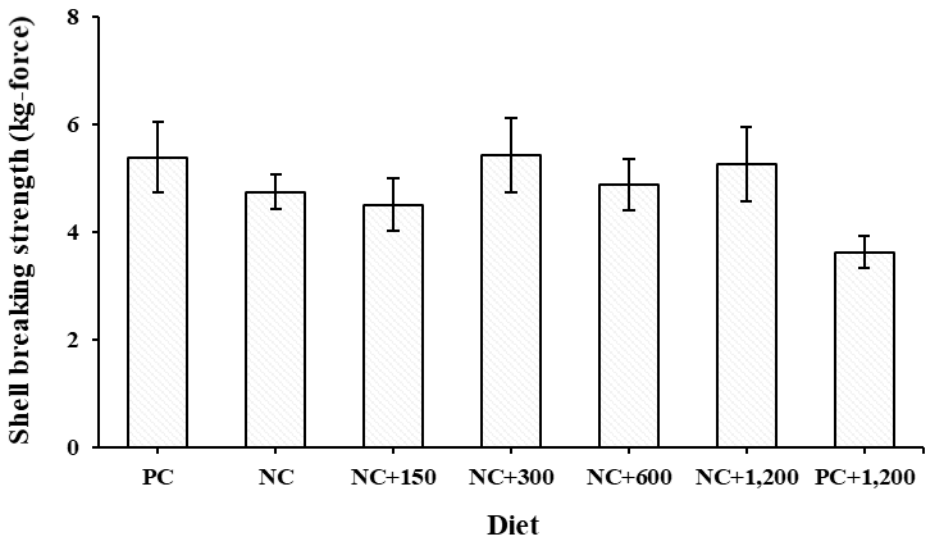
[5]Total term was calculated by summation of three sections of proximal 25%, mid-bone 50% and distal 25% femur.

[6]LSM = least squares mean.

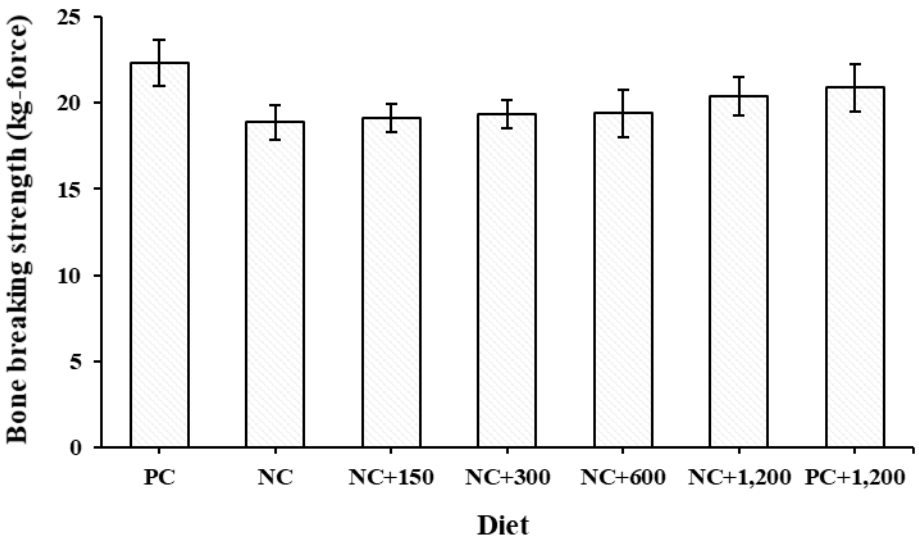
[7]SEM = standard error of the mean.

[8]PC, the positive control diet, nutritionally complete diet containing 0.45% aP, 3.70% Ca and 0.16% Na (25 to 28 wk), 0.38% aP, 3.73% Ca and 0.15% Na (29 to 37 wk); NC, the negative control diet, similar to the PC diet but having 0.22% aP, 3.00% Ca and 0.13% Na (25 to 28 wk), 0.19% aP, 3.02% Ca and 0.13% Na (29 to 37 wk); NC + 150, NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista,

Marlborough, UK) at 150, 300, 600, or 1,200 FTU/kg, respectively; and PC + 1,200, the PC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 1,200 FTU/kg.
[9]Body weight was used as a covariate.



Appendix 2. 3: Effect of dietary aP and Ca, and phytase supplementation on shell breaking strength (P = 0.077; n = 84) of laying hens at 37 wk of age. The positive control (PC) diet, nutritionally complete diet containing 0.45% aP, 3.70% Ca and 0.16% Na (25 to 28 wk), 0.38% aP, 3.73% Ca and 0.15% Na (29 to 37 wk); NC, the negative control diet, similar to the PC diet but having 0.22% aP, 3.00% Ca and 0.13% Na (25 to 28 wk), 0.19% aP, 3.02% Ca and 0.13% Na (29 to 37 wk); NC + 150, NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 150, 300, 600, or 1,200 FTU/kg, respectively; and PC + 1,200, the PC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 1,200 FTU/kg. Data are presented as least squares means with the respective standard errors of the mean.



Appendix 2. 4: Effect of dietary aP and Ca, and phytase supplementation on femur breaking strength (P = 0.446; n = 84) of laying hens at 37 wk of age. The positive control (PC) diet, nutritionally complete diet containing 0.45% aP, 3.70% Ca and 0.16% Na (25 to 28 wk), 0.38% aP, 3.73% Ca and 0.15% Na (29 to 37 wk); NC, the negative control diet, similar to the PC diet but having 0.22% aP, 3.00% Ca and 0.13% Na (25 to 28 wk), 0.19% aP, 3.02% Ca and 0.13% Na (29 to 37 wk); NC + 150, NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 150, 300, 600, or 1,200 FTU/kg, respectively; and PC + 1,200, the PC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 1,200 FTU/kg. Data are presented as least squares means with the respective standard errors of the mean.

Appendix 3. 1: Main effects of dietary treatment and age on pullet performance and uniformity.

	Body weight (g)		Body weight gain (g/d per bird)		Feed intake (g/d per bird)		Feed conversion ratio (g feed/g gain)		Uniformity[1] (%)	
	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]
Diet[4]										
PC	672[a,b]	3.89	8.2	0.12	46.6	0.60	6.62	0.16	77.7	2.13
NC	668[a,b]	3.81	8.3	0.13	46.9	0.51	6.90	0.33	83.9	1.95
NC + 300	660[b]	3.84	8.2	0.15	45.8	0.44	6.62	0.29	82.1	2.16
NC + 600	662[b]	3.11	8.2	0.09	46.7	0.35	6.30	0.13	85.7	2.49
NC + 1,200	679[a]	3.45	8.4	0.16	47.8	0.57	6.59	0.30	82.3	1.85
NC + 2,400	673[a,b]	3.66	8.4	0.17	47.2	0.44	6.60	0.33	82.4	2.10
Age										
1 d[5]	36[f]	0.12	-	-	-	-	-	-	-	-
3 wk[5]	189[e]	0.92	7.0[d]	0.04	15.5[e]	0.11	2.24[e]	0.02	76.0[b]	1.87
6 wk[5]	446[d]	2.04	12.2[a]	0.09	36.3[d]	0.19	2.98[d]	0.02	79.3[b]	1.88
12 wk[6]	973[c]	3.92	9.7[b]	0.10	62.5[b]	0.33	6.44[c]	0.05	88.7[a]	1.90
15 wk[6]	1,136[b]	4.10	7.8[c]	0.10	65.0[a]	0.44	8.35[b]	0.08	89.2[a]	1.61
18 wk[7]	1,234[a]	6.50	4.7[e]	0.23	55.0[c]	0.81	13.01[a]	0.54	78.6[b]	2.35
Source of variation					Prob > F					
Diet	0.002		0.812		0.132		0.562		0.224	
Age	<0.001		<0.001		<0.001		<0.001		<0.001	
Diet x age	0.018		0.002		0.637		0.008		0.245	

[1]Uniformity was calculated as percentage of pullets within 10 percent of the average body weight of each replicate cage.
[2]LSM = least squares mean.
[3]SEM = standard error of the mean.
[4]PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.
[5]Means of 10 replicates of 8 pullets for each treatment.
[6]Means of 10 replicates of 7 pullets for each treatment.
[7]Means of 10 replicates of 6 pullets for each treatment.
[a-f]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

Appendix 3. 2: Interaction of diet and age on uniformity of pullets fed different dietary aP and Ca, and phytase supplementation.

	Diet[1]											
	PC		NC		NC + 300		NC + 600		NC + 1,200		NC + 2,400	
	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]
Age (wk)	Uniformity[4] (%)											
3[5]	67.0	5.42	72.5	3.63	70.0	6.51	83.8	4.19	76.3	2.92	86.2	3.93
6[5]	67.1	4.59	81.2	3.84	77.5	6.12	87.5	5.59	80.9	2.90	81.2	3.84
12[6]	90.0	3.72	88.3	4.69	90.0	3.72	85.7	7.06	94.0	3.24	84.3	4.49
15[6]	82.9	4.66	91.2	3.86	92.9	2.38	91.4	3.81	89.5	4.53	87.1	3.96
18[7]	81.3	5.24	86.3	5.48	80.0	4.16	80.0	6.48	70.7	6.14	73.3	6.67
Source of variation	Prob > F											
Diet	0.224											
Age	<0.001											
Diet x age	0.245											

[1]PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.
[2]LSM = least squares mean.
[3]SEM = standard error of the mean.
[4]Uniformity was calculated as percentage of pullets within 10 percent of the average body weight of each replicate cage.
[5]Means of 10 replicates of 8 pullets for each treatment.
[6]Means of 10 replicates of 7 pullets for each treatment.
[7]Means of 10 replicates of 6 pullets for each treatment.

Appendix 3. 3: The effect of dietary P and Ca levels, and phytase supplementation on femur dry bone weight of pullets at 6, 15, and 18 wk of age.

	Proximal 25%[1]		Mid-bone 50%[2]		Distal 25%[3]		Total[4]	
	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]
Diet[7]	Dry bone weight (g)							
PC	1.11	0.02	1.13	0.02	0.92	0.02	3.16	0.05
NC	1.06	0.02	1.07	0.02	0.90	0.01	3.04	0.04
NC + 300	1.12	0.02	1.09	0.01	0.94	0.02	3.15	0.05
NC + 600	1.11	0.02	1.10	0.02	0.93	0.02	3.14	0.05
NC + 1,200	1.10	0.02	1.09	0.02	0.89	0.02	3.08	0.05
NC + 2,400	1.10	0.02	1.07	0.02	0.91	0.02	3.09	0.05
Age (wk)								
6[8]	0.94[c]	0.04	1.02[b]	0.04	0.84[b]	0.04	2.82[b]	0.09
15[8]	1.27[a]	0.02	1.31[a]	0.02	1.05[a]	0.02	3.63[a]	0.05
18[9]	1.10[b]	0.03	0.93[b]	0.03	0.86[b]	0.03	2.88[b]	0.08
Source of variation	Prob > F							
Diet	0.340		0.351		0.410		0.337	
Age	<0.001		<0.001		<0.001		<0.001	
Diet x age	0.788		0.405		0.260		0.366	
Body weight[10]	<0.001		<0.001		<0.001		<0.001	

[1]Proximal 25% represented all bone tissue from the proximal tip of the femur to 25% of the length of the bone from the proximal end.
[2]Mid-bone 50% represented the remaining segment of bone between the proximal 25% and distal 25% sections.
[3]Distal 25% represented all bone tissue from the distal tip of the femur to 25% of the length of the femur from the distal end.
[4]Total term was calculated by summation of three sections of proximal 25%, mid-bone 50% and distal 25% femur.
[5]LSM = least squares mean.
[6]SEM = standard error of the mean.
[7]PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.
[8]Means of 10 replicate birds for each treatment.
[9]Means of 8 replicate birds for each treatment.
[10]Body weight was used as a covariate.
[a-c]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

Appendix 3. 4: The effect of dietary P and Ca levels, and phytase supplementation on femur ash content and percent ash of pullets at 6, 15, and 18 wk of age.

	Proximal 25%[1]		Mid-bone 50%[2]		Distal 25%[3]		Total[4]	
	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]
Diet[7]				Bone ash content (g)				
PC	0.35	0.01	0.48	0.01	0.31	0.01	1.14	0.02
NC	0.33	0.01	0.46	0.01	0.30	0.01	1.09	0.01
NC + 300	0.35	0.01	0.46	0.01	0.31	0.01	1.12	0.02
NC + 600	0.34	0.01	0.45	0.01	0.30	0.01	1.09	0.02
NC + 1,200	0.34	0.01	0.45	0.01	0.29	0.01	1.08	0.01
NC + 2,400	0.34	0.01	0.46	0.01	0.30	0.01	1.10	0.01
Age (wk)								
6[8]	0.27[b]	0.01	0.42[b]	0.02	0.26[c]	0.01	0.94[b]	0.03
15[8]	0.38[a]	0.01	0.49[a]	0.01	0.31[b]	0.01	1.17[a]	0.02
18[9]	0.39[a]	0.01	0.46[b]	0.01	0.33[a]	0.01	1.18[a]	0.03
Source of variation				Prob > F				
Diet	0.113		0.238		0.159		0.183	
Age	<0.001		<0.001		0.005		<0.001	
Diet x age	0.446		0.113		0.437		0.237	
Body weight[10]	<0.001		<0.001		<0.001		<0.001	
Diet[7]				Percent bone ash (% of dry bone weight)				
PC	30.8	0.34	43.1	0.53	32.9	0.50	36.0	0.41
NC	30.6	0.37	43.6	0.48	32.2	0.30	35.8	0.34
NC + 300	30.3	0.30	42.9	0.46	32.3	0.52	35.6	0.44
NC + 600	29.9	0.40	42.3	0.55	31.8	0.54	35.1	0.45
NC + 1,200	30.3	0.36	42.8	0.44	31.7	0.32	35.4	0.34
NC + 2,400	30.3	0.36	43.8	0.54	32.6	0.35	35.8	0.35
Age (wk)								
6[8]	27.6[b]	0.92	42.9[a]	1.30	29.4[b]	0.91	33.9[b]	0.91
15[8]	29.6[b]	0.47	38.2[b]	0.64	30.2[b]	0.46	33.1[b]	0.45
18[9]	33.9[a]	0.72	48.1[a]	1.01	37.2[a]	0.77	39.9[a]	0.74
Source of variation				Prob > F				
Diet	0.653		0.424		0.334		0.683	
Age	<0.001		<0.001		<0.001		<0.001	
Diet x age	0.422		0.323		0.460		0.304	
Body weight[10]	0.995		0.093		0.811		0.188	

[1]Proximal 25% represented all bone tissue from the proximal tip of the femur to 25% of the length of the bone from the proximal end.
[2]Mid-bone 50% represented the remaining segment of bone between the proximal 25% and distal 25% sections.
[3]Distal 25% represented all bone tissue from the distal tip of the femur to 25% of the length of the femur from the distal end.
[4]Total term was calculated by summation of three sections of proximal 25%, mid-bone 50% and distal 25% femur.
[5]LSM = least squares mean.
[6]SEM = standard error of the mean.
[7]PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.

[8]Means of 10 replicate birds for each treatment.
[9]Means of 8 replicate birds for each treatment.
[10]Body weight was used as a covariate.
[a-c]Means within column and within main effect for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).

Appendix 3. 5: The effect of dietary P and Ca levels, and phytase supplementation on femur bone mineral density of pullets at 6, 15, and 18 wk of age.

	Bone mineral density (mg/cm^3)					
	Proximal 20%[1]		Mid-bone 50%[2]		Distal 20%[3]	
	LSM[4]	SEM[5]	LSM[4]	SEM[5]	LSM[4]	SEM[5]
Diet[7]			Total[6]			
PC	316	6.70	454	8.54	339	5.10
NC	315	4.21	455	5.44	338	3.77
NC + 300	318	6.92	453	7.12	338	6.02
NC + 600	313	6.66	450	7.15	332	5.75
NC + 1,200	312	4.19	454	5.20	331	4.13
NC + 2,400	331	18.49	468	14.75	349	18.03
Age (wk)						
6[8]	317[a,b]	14.31	543[a]	16.15	329	13.04
15[8]	303[b]	7.57	407[b]	8.39	331	6.90
18[9]	332[a]	14.56	417[b]	14.29	353	13.50
Source of variation			Prob > F			
Diet	0.908		0.928		0.686	
Age	0.023		<0.001		0.097	
Diet x age	0.429		0.809		0.680	
Body weight[10]	0.879		0.128		0.599	
Diet[7]			Cortical[11]			
PC	874	4.44	1,071	5.25	893	3.08
NC	874	5.02	1,080	3.27	889	3.79
NC + 300	869	3.22	1,076	3.81	885	3.20
NC + 600	865	4.07	1,074	3.54	876	3.85
NC + 1,200	866	3.62	1,072	3.65	885	4.00
NC + 2,400	873	4.13	1,075	5.69	884	4.04
Age (wk)						
6[8]	803[b]	10.02	987[b]	9.64	810[b]	8.95
15[8]	906[a]	5.37	1,117[a]	5.13	921[a]	4.62
18[9]	902[a]	7.91	1,120[a]	7.89	925[a]	7.06
Source of variation			Prob > F			
Diet	0.397		0.621		0.051	
Age	<0.001		<0.001		<0.001	
Diet x age	0.340		0.481		0.776	
Body weight[10]	0.843		0.424		0.600	
Diet[7]			Trabecular[12]			
PC	74.1	2.76	55.3	4.07	76.4	2.98
NC	77.2	2.21	53.3	3.46	83.8	1.61
NC + 300	73.1	2.48	42.8	3.16	76.5	2.48
NC + 600	70.4	3.50	45.9	4.31	75.7	3.80
NC + 1,200	75.8	3.47	52.1	3.80	79.1	2.50
NC + 2,400	80.5	2.84	53.7	2.98	83.8	2.62
Age (wk)						
6[8]	87.7[a]	7.21	71.7[a]	8.14	93.5[a]	5.87
15[8]	51.6[b]	3.93	30.9[b]	3.56	59.1[b]	3.29
18[9]	86.3[a]	5.54	49.0[a]	6.13	85.1[a]	4.46

Appendix 3. 5: *Continued.*

	Bone mineral density (mg/cm^3)					
	Proximal 20%[1]		Mid-bone 50%[2]		Distal 20%[3]	
	LSM[4]	SEM[5]	LSM[4]	SEM[5]	LSM[4]	SEM[5]
Source of variation			Prob > F			
Diet	0.241		0.116		0.052	
Age	<0.001		<0.001		<0.001	
Diet x age	0.763		0.569		0.379	
Body weight[10]	0.206		0.066		0.073	

[1]A single 1 mm-thick slice taken at a point 20% along the length of the femur from the proximal end.
[2]A single 1 mm-thick slice taken at a point 50% along the length of the femur from the proximal end.
[3]A single 1 mm-thick slice taken at a point 80% along the length of the femur from the proximal end.
[4]LSM = least squares mean.
[5]SEM = standard error of the mean.
[6]Total term was the weighted average of both the cortical and trabecular bone measures.
[7]PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg.
[8]Means of 10 replicate birds for each treatment.
[9]Means of 8 replicate birds for each treatment.
[10]Body weight was used as a covariate.
[11]Cortical bone was the outer shell of the bone, and was defined as having a density of > 500 mg/cm^3
[12]Bone in the trabecular space was defined as having a density of $\leq$ 400 mg/cm^3
[a,b]Means within column and within main effect for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).

Appendix 3. 6: The effect of dietary P and Ca levels, and phytase supplementation on femur bone cross-sectional area of pullets at 6, 15, and 18 wk of age.

	Bone cross-sectional area (mm^2)					
	Proximal 20%[1]		Mid-bone 50%[2]		Distal 20%[3]	
	LSM[4]	SEM[5]	LSM[4]	LSM[4]	SEM[5]	LSM[4]
Diet[7]			Total[6]			
PC	50.2	0.79	32.3	0.47	47.8	0.70
NC	48.8	0.64	30.7	0.39	46.4	0.51
NC + 300	49.3	0.57	31.2	0.43	47.0	0.59
NC + 600	48.7	0.56	31.1	0.44	47.4	0.67
NC + 1,200	48.7	0.94	31.2	0.55	47.1	0.98
NC + 2,400	49.3	0.91	31.5	0.46	47.0	0.96
Age (wk)						
6[8]	45.6	1.65	28.0[b]	0.96	46.6	1.65
15[8]	50.8	0.92	33.0[a]	0.56	47.0	0.92
18[9]	51.1	1.41	33.1[a]	0.80	47.8	1.42
Source of variation			Prob > F			
Diet	0.729		0.252		0.681	
Age	0.223		0.022		0.712	
Diet x age	0.232		0.968		0.404	
Body weight[10]	<0.001		<0.001		<0.001	
Diet[7]			Cortical[11]			
PC	14.3	0.29	12.3	0.23	14.4	0.23
NC	13.7	0.28	11.6	0.14	13.8	0.17
NC + 300	14.3	0.34	11.9	0.14	14.2	0.23
NC + 600	14.0	0.30	11.7	0.17	14.2	0.17
NC + 1,200	13.8	0.20	11.8	0.12	13.8	0.22
NC + 2,400	14.7	1.07	12.4	0.55	14.9	1.01
Age (wk)						
6[8]	13.8	0.52	13.4[a]	0.42	13.8	0.51
15[8]	14.2	0.29	11.3[b]	0.22	14.2	0.27
18[9]	14.4	0.71	11.2[b]	0.42	14.7	0.65
Source of variation			Prob > F			
Diet	0.521		0.141		0.278	
Age	0.889		0.035		0.689	
Diet x age	0.559		0.873		0.664	
Body weight[10]	<0.001		<0.001		<0.001	
Diet[7]			Trabecular[12]			
PC	34.4	0.92	19.6	0.45	36.6	1.03
NC	33.7	0.54	18.8	0.32	37.0	0.46
NC + 300	33.6	0.74	19.0	0.44	36.8	0.70
NC + 600	33.3	0.66	19.0	0.41	37.3	0.70
NC + 1,200	33.6	0.78	19.1	0.55	37.5	0.94
NC + 2,400	33.1	1.74	18.5	0.77	36.1	1.88
Age (wk)						
6[8]	29.8	1.72	15.3[b]	0.91	33.4[b]	1.54
15[8]	35.5	0.94	20.9[a]	0.52	47.8[a]	0.88
18[9]	35.5	1.66	20.8[a]	0.85	29.5[b]	1.63

Appendix 3. 6: *Continued.*

	Bone cross-sectional area (mm^2)					
	Proximal 20%[1]		Mid-bone 50%[2]		Distal 20%[3]	
	LSM[4]	SEM[5]	LSM[4]	LSM[4]	SEM[5]	LSM[4]
Source of variation			Prob > F			
Diet	0.960		0.765		0.973	
Age	0.163		0.002		<0.001	
Diet x age	0.346		0.922		0.642	
Body weight[10]	<0.001		<0.001		<0.001	

[1]A single 1 mm-thick slice taken at a point 20% along the length of the femur from the proximal end.
[2]A single 1 mm-thick slice taken at a point 50% along the length of the femur from the proximal end.
[3]A single 1 mm-thick slice taken at a point 80% along the length of the femur from the proximal end.
[4]LSM = least squares mean.
[5]SEM = standard error of the mean.
[6]Total term was the weighted average of both the cortical and trabecular bone measures.
[7]PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.
[8]Means of 10 replicate birds for each treatment.
[9]Means of 8 replicate birds for each treatment.
[10]Body weight was used as a covariate.
[11]Cortical bone was the outer part of the bone, and was defined as having a density of > 500 mg/cm^3
[12]Bone in the trabecular space was defined as having a density of $\leq$ 400 mg/cm^3
[a,b]Means within column and within main effect for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).

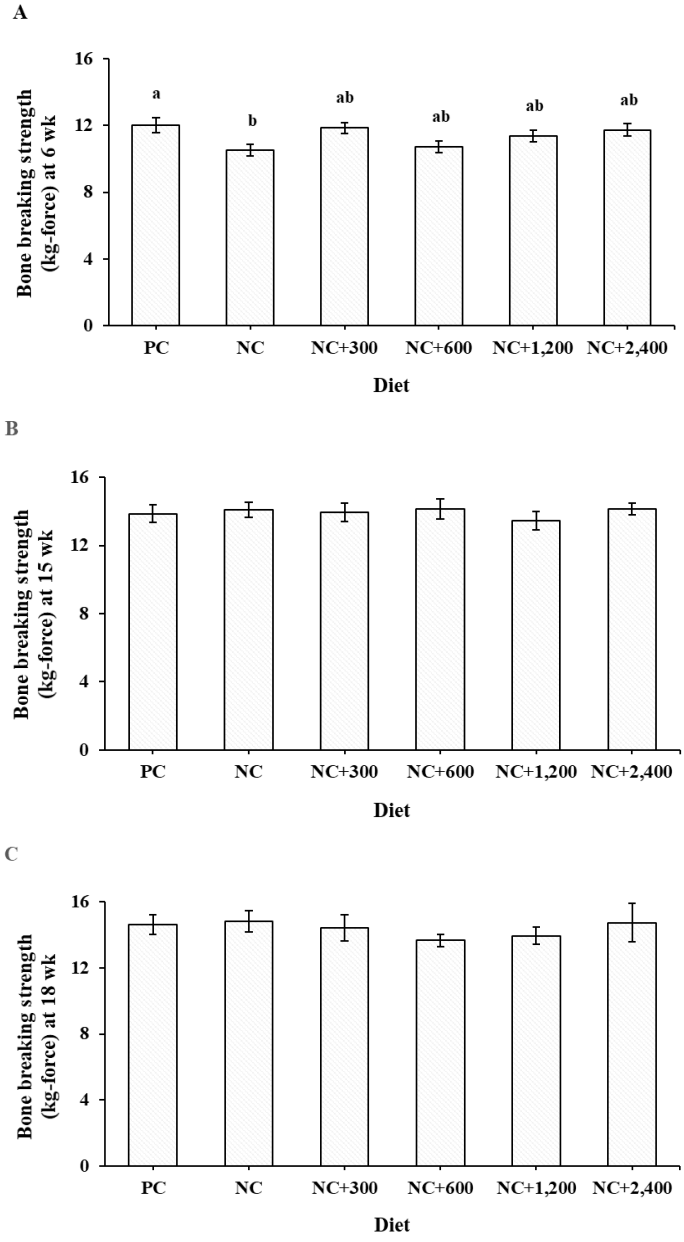
Appendix 3. 7: The effect of dietary P and Ca levels, and phytase supplementation on femur bone mineral content of pullets at 6, 15, and 18 wk of age.

	Bone mineral content[1] (mg/mm)					
	Proximal 20%[2]		Mid-bone 50%[3]		Distal 20%[4]	
	LSM[5]	SEM[6]	LSM[5]	LSM[6]	SEM[5]	LSM[6]
Diet[8]			Total[7]			
PC	15.8	0.28	14.4	0.27	16.2	0.26
NC	15.7	0.24	13.6	0.20	15.7	0.21
NC + 300	15.6	0.31	13.8	0.16	15.8	0.22
NC + 600	15.2	0.30	13.5	0.21	15.7	0.22
NC + 1,200	15.2	0.24	13.8	0.13	15.6	0.24
NC + 2,400	16.3	0.95	14.5	0.58	16.6	0.87
Age (wk)						
6[9]	14.6[b]	0.57	14.2	0.43	14.9[b]	0.58
15[9]	15.2[a,b]	0.31	13.5	0.25	15.6[a,b]	0.32
18[10]	17.0[a]	0.67	14.1	0.45	17.3[a]	0.63
Source of variation			Prob > F			
Diet	0.493		0.228		0.468	
Age	0.020		0.145		0.018	
Diet x age	0.479		0.831		0.452	
Body weight[11]	<0.001		<0.001		<0.001	
Diet[8]			Cortical[12]			
PC	12.7	0.24	13.3	0.21	13.0	0.20
NC	12.2	0.21	12.6	0.15	12.5	0.15
NC + 300	12.6	0.32	12.9	0.16	12.8	0.22
NC + 600	12.3	0.24	12.6	0.18	12.6	0.16
NC + 1,200	12.1	0.14	12.7	0.14	12.3	0.18
NC + 2,400	13.1	1.08	13.4	0.58	13.4	0.99
Age (wk)						
6[9]	11.2	0.47	13.6	0.46	11.5	0.44
15[9]	13.0	0.25	12.6	0.25	13.1	0.24
18[10]	13.3	0.68	12.6	0.46	13.7	0.61
Source of variation			Prob > F			
Diet	0.339		0.131		0.191	
Age	0.138		0.536		0.142	
Diet x age	0.434		0.807		0.668	
Body weight[11]	<0.001		<0.001		<0.001	
Diet[8]			Trabecular[13]			
PC	2.55	0.08	1.08	0.07	2.39	0.13
NC	2.63	0.08	1.00	0.08	2.65	0.08
NC + 300	2.41	0.10	0.82	0.08	2.38	0.09
NC + 600	2.32	0.11	0.87	0.09	2.39	0.14
NC + 1,200	2.56	0.14	1.03	0.10	2.56	0.11
NC + 2,400	2.59	0.17	1.00	0.08	2.59	0.17
Age (wk)						
6[9]	2.81[a]	0.24	1.05[a,b]	0.17	2.71[a,b]	0.24
15[9]	1.65[b]	0.14	0.68[b]	0.08	1.85[b]	0.13
18[10]	3.07[a]	0.21	1.16[a]	0.14	2.92[a]	0.22

Appendix 3. 7: *Continued.*

	Bone mineral content[1] (mg/mm)					
	Proximal 20%[2]		Mid-bone 50%[3]		Distal 20%[4]	
	LSM[5]	SEM[6]	LSM[5]	LSM[6]	SEM[5]	LSM[6]
Source of variation			Prob > F			
Diet	0.324		0.271		0.318	
Age	<0.001		<0.001		<0.001	
Diet x age	0.565		0.715		0.569	
Body weight[11]	<0.001		0.010		<0.001	

[1]Bone mineral content was calculated as bone mineral density multiplied by the bone cross-sectional area, and is the amount of bone mineral contained in a 1 mm linear section of the scanned region of the bone.
[2]A single 1 mm-thick slice taken at a point 20% along the length of the femur from the proximal end.
[3]A single 1 mm-thick slice taken at a point 50% along the length of the femur from the proximal end.
[4]A single 1 mm-thick slice taken at a point 80% along the length of the femur from the proximal end.
[5]LSM = least squares mean.
[6]SEM = standard error of the mean.
[7]Total term was the weighted average of both the cortical and trabecular bone measures.
[8]PC, the positive control diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively.
[9]Means of 10 replicate birds for each treatment.
[10]Means of 8 replicate birds for each treatment.
[11]Body weight was used as a covariate.
[12]Cortical bone was the outer part of the bone, and was defined as having a density of > 500 mg/cm^3
[13]Bone in the trabecular space was defined as having a density of $\leq$ 400 mg/cm^3
[a,b]Means within column and within main effect for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).



Appendix 3. 8: Effect of dietary P and Ca levels, and phytase supplementation on femur bone breaking strength of pullets at (A) 6 wk ($P = 0.043$; n = 60), (B) 15 wk ($P = 0.946$; n = 60), and (C) 18 wk of age ($P = 0.621$; n = 48). The positive control (PC) diet, nutritionally complete diet containing 0.48% aP, 1.05% Ca and 0.18% Na (0 to 3 wk), 0.45% aP, 1.00% Ca and 0.17% Na (4 to 12 wk), 0.37% aP, 0.90% Ca and 0.16% Na (13 to 15 wk), 0.45% aP, 2.00% Ca and 0.16% Na (16 to 18 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.33% aP, 0.89% Ca and 0.14% Na (0 to 3 wk), 0.30% aP, 0.84% Ca and 0.13% Na (4 to 12 wk), 0.22% aP, 0.74% Ca and 0.12% Na (13 to 15 wk), and 0.30% aP, 1.84% Ca and 0.12% Na (16 to 18 wk); NC + 300, NC + 600, NC + 1,200, and NC + 2,400, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, 1,200, and 2,400 FTU/kg, respectively. Data are presented as least squares means with the respective standard errors of the mean. [a,b]Means with no common superscript differ significantly ($P \leq 0.05$).

Appendix 4. 1: Interaction of diet and age on body weight in hens fed different dietary P and Ca levels, and phytase supplementation from 19 to 74 wk of age.

	Diet[1]									
	PC		NC		NC + 300		NC + 600		NC + 1,200	
	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]	LSM[2]	SEM[3]
Age (wk)	Body weight (kg)									
19[4]	1.36	0.02	1.34	0.02	1.32	0.02	1.33	0.02	1.40	0.02
22[4]	1.54	0.02	1.51	0.01	1.53	0.01	1.50	0.01	1.56	0.02
26[4]	1.57	0.02	1.52	0.01	1.55	0.01	1.53	0.02	1.58	0.02
30[4]	1.61	0.02	1.56	0.02	1.59	0.01	1.56	0.01	1.61	0.02
34[5]	1.64	0.02	1.61	0.02	1.62	0.02	1.58	0.02	1.61	0.02
38[5]	1.68	0.02	1.61	0.02	1.64	0.01	1.62	0.02	1.68	0.02
42[5]	1.67	0.02	1.61	0.02	1.64	0.02	1.62	0.02	1.68	0.02
46[6]	1.70	0.03	1.59	0.02	1.65	0.02	1.62	0.02	1.68	0.03
50[6]	1.71	0.03	1.62	0.02	1.66	0.02	1.63	0.02	1.69	0.03
54[6]	1.72	0.03	1.62	0.02	1.66	0.02	1.64	0.02	1.71	0.03
59[7]	1.71	0.03	1.65	0.03	1.66	0.02	1.65	0.03	1.72	0.03
64[8]	1.65	0.03	1.60	0.03	1.58	0.03	1.56	0.03	1.63	0.03
69[9]	1.67	0.04	1.62	0.04	1.61	0.03	1.60	0.03	1.66	0.04
74[9]	1.65	0.04	1.62	0.05	1.60	0.03	1.59	0.03	1.67	0.04
Source of variation	Prob > F									
Diet	<0.001									
Age	<0.001									
Diet x age	0.999									

[1]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.
[2]LSM = least squares mean.
[3]SEM = standard error of the mean.
[4]Means of 51 replicate hens (PC and NC), 52 replicates (NC + 300 and NC + 600), and 50 replicates (NC + 1,200).
[5]Means of 50 replicate hens (PC), 51 replicates (NC), 52 replicates (NC + 300 and NC + 600), and 50 replicates (NC + 1,200).
[6]Means of 42 replicate hens (PC), 43 replicates (NC), 44 replicates (NC + 300 and NC + 600), and 42 replicates (NC + 1,200).
[7]Means of 34 replicate hens (PC), 33 replicates (NC), 36 replicates (NC + 300 and NC + 600), and 33 replicates (NC + 1,200).
[8]Means of 33 replicate hens (PC and NC), 36 replicates (NC + 300 and NC + 600), and 33 replicates (NC + 1,200).
[9]Means of 24 replicate hens (PC), 25 replicates (NC), 27 replicates (NC + 300 and NC + 600), and 25 replicates (NC + 1,200).

Appendix 4. 2: Main effects of diet and age on body weight and egg production in hens fed different dietary P and Ca levels, and phytase supplementation from 19 to 74 wk of age.

	Body weight (kg)		Hen-day egg production[1] (%)	
	LSM[2]	SEM[3]	LSM[2]	SEM[3]
Diet[4]				
PC	1.63[a]	0.01	92.2	0.38
NC	1.58[b]	0.01	92.9	0.34
NC + 300	1.59[b]	0.01	92.7	0.33
NC + 600	1.57[b]	0.01	92.9	0.33
NC + 1,200	1.64[a]	0.01	93.1	0.40
Age (wk)				
19[5]	1.35[f]	0.01	-	-
22[5]	1.53[e]	0.01	56.8[i]	1.35
26[5]	1.55[d,e]	0.01	98.2[a,b]	0.30
30[5]	1.59[c,d]	0.01	98.6[a,b]	0.37
34[6]	1.62[b,c]	0.01	98.7[a]	0.18
38[6]	1.65[a,b]	0.01	98.2[a,b]	0.21
42[6]	1.64[a,b]	0.01	97.4[b,c]	0.29
46[7]	1.65[a,b]	0.01	97.1[b,c,d]	0.32
50[7]	1.66[a,b]	0.01	96.6[c,d,e]	0.33
54[7]	1.67[a]	0.01	95.9[d,e]	0.36
59[8]	1.68[a]	0.01	95.2[e,f]	0.44
64[9]	1.61[b,c]	0.01	92.2[g]	0.65
69[10]	1.63[a,b,c]	0.02	88.2[h]	0.86
74[10]	1.63[a,b,c]	0.02	92.7[f,g]	0.67
Source of variation	Prob > F			
Diet	<0.001		0.545	
Age	<0.001		<0.001	
Diet x age	0.999		0.957	

[1]calculated on 4-wk and 5-wk intervals from 19 to 54 and 55 to 74 wk of age, respectively.
[2]LSM = least squares mean.
[3]SEM = standard error of the mean.
[4]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg.
[5]Means of 51 replicate hens (PC and NC), 52 replicates (NC + 300 and NC + 600), and 50 replicates (NC + 1,200).
[6]Means of 50 replicate hens (PC), 51 replicates (NC), 52 replicates (NC + 300 and NC + 600), and 50 replicates (NC + 1,200).
[7]Means of 42 replicate hens (PC), 43 replicates (NC), 44 replicates (NC + 300 and NC + 600), and 42 replicates (NC + 1,200).
[8]Means of 34 replicate hens (PC), 33 replicates (NC), 36 replicates (NC + 300 and NC + 600), and 33 replicates (NC + 1,200).
[9]Means of 33 replicate hens (PC and NC), 36 replicates (NC + 300 and NC + 600), and 33 replicates (NC + 1,200).
[10]Means of 24 replicate hens (PC), 25 replicates (NC), 27 replicates (NC + 300 and NC + 600), and 25 replicates (NC + 1,200).
[a-i]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

Appendix 4. 3: Interaction of diet and age on P retention in hens fed different dietary P and Ca levels, and phytase supplementation from 19 to 74 wk of age[1].

	Diet[2]									
	PC		NC		NC + 300		NC + 600		NC + 1,200	
	LSM[3]	SEM[4]	LSM[3]	SEM[4]	LSM[3]	SEM[4]	LSM[3]	SEM[4]	LSM[3]	SEM[4]
Age (wk)					P retention (%)					
42	18.51[a]	2.38	17.60[a]	2.03	28.32[a]	3.98	15.38[a]	1.55	18.36[a,b]	4.21
54	19.95[a,b]	3.39	13.13[a,b]	3.38	21.15[a]	2.35	12.85[a,b]	4.07	22.76[a,b]	4.33
74	17.04[a,b]	2.55	11.46[a,b]	3.72	6.42[b]	1.99	27.88[a]	4.99	22.63[a,b]	5.08
Source of variation					Prob > F					
Diet					0.169					
Age					0.480					
Diet x age					<0.001					

[1]Means of 8 replicate hens for each dietary treatment group.

[2]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.

[3]LSM = least squares mean.

[4]SEM = standard error of the mean.

[a,b]Means within row with no common superscript differ significantly ($P \leq 0.05$).

Appendix 4. 4: Main effects of diet and age on Ca retention in hens fed different dietary P and Ca levels, and phytase supplementation from 19 to 74 wk of age[1].

	Ca retention (%)	
	LSM[2]	SEM[3]
Diet[4]		
PC	41.16[b,c]	1.88
NC	51.33[a]	2.16
NC + 300	50.02[a,b]	2.81
NC + 600	38.09[c]	2.50
NC + 1,200	39.54[c]	2.46
Age (wk)		
42	50.86[a]	1.94
54	37.57[b]	2.12
74	43.66[b]	1.41
Source of variation	Prob > F	
Diet	<0.001	
Age	<0.001	
Diet x age	0.129	

[1]Means of 8 replicate hens for each dietary treatment group.
[2]LSM = least squares mean.
[3]SEM = standard error of the mean.
[4]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.
[a-c]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

Appendix 5. 1: Main effects of diet and age on bone breaking strength of laying hens fed different levels of dietary aP and Ca, and phytase supplementation from 19 to 74 wk of age[1].

	Bone breaking strength (kg-force)	
	LSM[2]	SEM[3]
Diet[4]		
PC	14.6[a,b]	0.54
NC	13.2[b]	0.34
NC + 300	14.1[a,b]	0.48
NC + 600	15.2[a]	0.52
NC + 1,200	14.7[a]	0.35
Age (wk)		
42	13.6[b]	0.24
54	14.5[a,b]	0.46
64	14.4[a,b]	0.46
74	14.9[a]	0.43
Source of variation	Prob > F	
Diet	0.014	
Age	0.042	
Diet x age	0.824	
Body weight[5]	<0.001	

[1]Means of 8 replicate hens for each diet at each age.
[2]LSM = least squares mean.
[3]SEM = standard error of the mean.
[4]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.
[5]Body weight was used as a covariate.
[a,b]Means within column and within main effect with no common superscript differ significantly ($P \leq 0.05$).

Appendix 5. 2: Main effects of diet and age on bone cross-sectional area of laying hens fed different levels of dietary aP and Ca, and phytase supplementation from 19 to 74 wk of age[1].

	Bone cross-sectional area (mm^2)					
	Proximal 20%[2]		Mid-bone 50%[3]		Distal 20%[4]	
	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]
Diet[7]			Total[8]			
PC	63.0	0.83	40.6	0.71	59.6	0.70
NC	62.0	0.88	40.1	0.53	60.2	0.90
NC + 300	62.6	0.95	40.2	0.37	59.6	0.73
NC + 600	62.4	0.71	39.4	0.53	59.5	0.72
NC + 1,200	62.8	0.79	39.1	0.38	59.1	0.58
Age (wk)						
42	63.0	0.72	39.8	0.37	59.7	0.66
54	61.6	0.75	39.7	0.66	59.2	0.72
64	62.3	0.75	39.8	0.31	60.0	0.65
74	63.4	0.78	40.2	0.45	59.5	0.57
Source of variation			Prob > F			
Diet	0.923		0.182		0.895	
Age	0.345		0.912		0.894	
Diet x age	0.190		0.412		0.310	
Body weight[9]	<0.001		<0.001		<0.001	
Diet[7]			Cortical[10]			
PC	20.3[a,b]	0.97	15.6[a,b]	0.79	20.2[a,b]	0.78
NC	17.8[b]	0.35	13.9[b]	0.28	18.4[b]	0.24
NC + 300	19.8[a,b]	0.83	15.3[a,b]	0.70	20.3[a,b]	1.03
NC + 600	22.2[a]	1.18	16.8[a]	0.91	22.1[a]	1.22
NC + 1,200	20.4[a]	0.81	15.5[a,b]	0.59	20.2[a,b]	0.74
Age (wk)						
42	19.2	0.46	14.7	0.34	19.3	0.41
54	19.5	0.66	15.2	0.51	20.1	0.78
64	20.3	0.68	15.4	0.47	20.2	0.69
74	21.4	1.14	16.5	0.96	21.4	1.08
Source of variation			Prob > F			
Diet	0.001		0.011		0.006	
Age	0.267		0.350		0.281	
Diet x age	0.639		0.263		0.370	
Body weight[9]	<0.001		0.006		<0.001	
Diet[7]			Trabecular + medullary[11]			
PC	38.3[a,b]	1.49	22.7[a,b]	1.06	35.6[a,b]	1.01
NC	41.4[a]	0.86	25.0[a]	0.74	38.6[a]	0.91
NC + 300	38.9[a,b]	1.22	23.2[a,b]	0.88	35.1[a,b]	1.26
NC + 600	36.0[b]	1.55	20.1[b]	1.15	34.8[b]	0.96
NC + 1,200	37.0[a,b]	1.44	21.3[b]	1.00	33.6[b]	1.42
Age (wk)						
42	39.4	1.01	23.2	0.73	35.8	1.02
54	38.6	1.22	22.9	0.71	36.0	1.03
64	37.3	0.96	21.7	0.79	34.9	1.06
74	38.1	1.51	21.9	1.18	35.7	0.92

Appendix 5. 2: *Continued.*

	Bone cross-sectional area (mm^2)					
	Proximal 20%[2]		Mid-bone 50%[3]		Distal 20%[4]	
	LSM[5]	SEM[6]	LSM[5]	SEM[6]	LSM[5]	SEM[6]
Source of variation	Prob > F					
Diet	0.020		0.008		0.021	
Age	0.532		0.501		0.894	
Diet x age	0.603		0.741		0.817	
Body weight[9]	0.792		0.732		0.274	

[1]Means of 8 replicate hens for each diet at each age.
[2]A single 1 mm-thick slice taken at a point 20% along the length of the femur from the proximal end.
[3]A single 1 mm-thick slice taken at a point 50% along the length of the femur from the proximal end.
[4]A single 1 mm-thick slice taken at a point 80% along the length of the femur from the proximal end.
[5]LSM = least squares mean.
[6]SEM = standard error of the mean.
[7]PC, the positive control diet, nutritionally complete diet containing 0.43% aP, 3.71% Ca and 0.17% Na (19 to 54 wk), 0.40% aP, 3.73% Ca and 0.16% Na (55 to 74 wk); NC, the negative control diet, similar to the PC diet but reduced to 0.28% aP, 3.55% Ca and 0.14% Na (19 to 54 wk), 0.25% aP, 3.57% Ca and 0.13% Na (55 to 74 wk); NC + 300, NC + 600, and NC + 1,200, the NC diet supplemented with Quantum Blue phytase (AB Vista, Marlborough, UK) at 300, 600, and 1,200 FTU/kg, respectively.
[8]Total term was the weighted average of both the cortical and trabecular + medullary bone.
[9]Body weight was used as a covariate.
[10]Cortical bone was the outer part of the bone, and was defined as havinga density of > 500 mg/cm^3
[11]Trabecular + medullary bone represents bone within the trabecular space, and is assumed to contain unknown proportions of trabecular and medullary bone.
[a,b]Means within column and within main effect for each dependent variable with no common superscript differ significantly ($P \leq 0.05$).