Evaluation of dietary calcium level effects on the productivity, eggshell quality, and bone traits of laying hens

by

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Abstract

Given the significance of calcium **(Ca)** in the metabolism of laying hens, the purpose of this thesis was to contribute to existing knowledge regarding Ca metabolism and requirements in these highly productive birds.

Phosvitin (PV), is an egg yolk phospholipid protein that increases Ca bioavailability. Experiment 1 evaluated the effects of dietary phosvitin **(PV)** as a potential strategy for protecting the shell and bone quality of end-of-cycle laying hens fed a Ca-reduced diet. A total of eight experimental diets were used including a positive control **(PC),** negative control (**NC**; 21% less Ca than the PC) and six NC-based diets that contained either PV (37.4% purity), dephosphorylated phosvitin (DPV; 39.5% purity) or phosvitin peptides (PVP; 39.8% purity), each fed at a 1% or 0.01% of the diet. It was hypothesized that dietary PV products would protect laying hen bone quality via increased Ca digestibility, while maintaining productivity and shell quality in end-of-cycle laying hens. Overall, there was no effect of the NC diet on egg production and egg mass over the duration of the experiment. Additionally, bone and shell quality remained unaffected by dietary treatment. This suggests that commercial end-of-cycle laying hens can maintain egg production, shell quality and bone quality under substantial reductions in dietary Ca, at least in the short term.

The impressive performance of hens fed substantially reduced Ca prompted investigating Ca requirements in laying hens via a meta-analysis performed with publications using varying levels of dietary Ca. Dietary Ca level was defined as the independent variable of interest and dependant variables included: egg production (%), Ca intake (g/hen/day), phosphorus (**P**) intake (g/hen/day), egg mass (g), feed intake (**FI**; g/hen/day), feed conversion (kg feed: kg eggs), feed conversion (kg feed: dozen eggs), egg weight (g), shell weight (%), eggshell thickness (mm), egg specific gravity, egg breaking strength (N), bone breaking strength (**BBS**; N), bone Ca (%), bone

P (%) and bone ash (%). Regression models for each dependant variable were created with the inclusion of moderator (random) variables that specifically improved each model as reflected by the Bayesian information criterion. Ultimately a data set containing 792 observations was compiled from 57 published papers between 1981 and 2020. Seven moderator variables (Record No., Year, Strain, Molted, Ca Particle Size, Ca Source and Heat Stress) were considered for inclusion in each regression model.

Ca intake (1.04 g/hen/day per 1% increase in dietary Ca) and FI (1.54 g/hen/day per 1% increase in dietary Ca) were each significantly affected by dietary Ca levels. Additionally, each of the feed conversion parameters (feed:eggs and feed:dozen eggs) decreased with increasing levels of dietary Ca (-0.022 feed:egg ratio and -0.051 feed:dozen per 1% increase in dietary Ca). Shell weight (0.24 % per 1% increase in dietary Ca), eggshell thickness (0.0075 mm per 1% increase in dietary Ca), specific gravity (0.0015 per 1% unit increase in dietary Ca) and egg breaking strength (1.23 N per 1% unit increase in dietary Ca) each increased with increasing dietary Ca, which agrees with others that show that adequate Ca is essential for shell quality. The positive relationship between dietary Ca levels and bone ash (1.03 % per 1% unit increase in dietary Ca) was unexpected given the small amount of research that supports this. Alternatively, BBS significantly increased with increasing dietary Ca levels (12.92 N per 1% unti increase in Ca) which supports the idea that increasing dietary Ca levels prevent hens from depleting skeletal Ca when adequate dietary Ca is available. The presence of *Year* as a significant random variable in this relationship suggests the relationship between dietary Ca and bone quality is changing over time. Using the NRC (1994) Ca requirements as a reference, there do not seem to be negative consequences associated with over-supplementation of Ca within the Ca ranges studied as illustrated by the lack of plateaus or decreases in the response variables tested.

Preface

This thesis is an original work by Daniella Batres. In total, one trial and one meta-analysis were conducted and are presented in Chapters 3 and 4 of this thesis, respectively. The barn work (Chapter 3), analysis and writing for each of the experiments were conducted between 10/2019 to 04/ 2022. No part of this thesis has been previously published.

The first experiment was a pilot study conducted with approval of the University of Alberta Animal Care and Use Committee for Livestock (protocol AUP00003100). All animal handling followed principles established by the Canadian Council on Animal Care guidelines and policies (Canadian Council on Animal Care, 2009). Funding for the project described in Chapter 2 was provided by Alberta Agriculture and Forestry, with in-kind support provided by the University of Alberta. This experiment was designed with support of co-authors D.R. Korver and J. Wu. The MSc student was responsible for conducting the experiment, data collection, analysis, laboratory work and thesis writing. E. Opoku, M. Oryschak, A, Ruiz-Sanchez and K. Thorsteinson provided technical assistance with management of the experiment, laboratory work and analysis. Part of the data generated from this work was presented at the 2021 Poultry Science Association Annual scientific meeting (Batres et al., 2020).

The meta-analysis designed with support of co-authors D.R. Korver and M. J. Zuidhof who provided critical review and manuscript edits. The MSc student was responsible for conducting the experiment, data collection, analysis and thesis writing. M. J. Zuidhof contributed to the design of statistical analyses. This experiment involved no bird work and therefore did not require ethics approval. Statistical analysis for Chapter 4 was provided with in-kind support from Dr. M. J. Zuidhof.

Dedication

This thesis is dedicated to Maria Rosario Batres, Maria Eugenia Levia and my parents who gave me the first generation urge to heal, collect degrees and build generational wealth while drinking cafecito.

Acknowledgements
I want to thank the University of Alberta for reminding me that large and powerful institutions insist you use the proper channels to report inappropriate behaviour because they control the proper channels and are confident it will not work.

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List of Symbols and Abbreviations

μ = micro

1,25-$(OH)_2$-D_3 = vitamin D

2D = 2-dimensional

AIDCa = apparent ileal digestibility of calcium

avP = available phosphorus

BBS = bone breaking strength

BIC = Bayesian Information Criterion

BMC = bone mineral content

BMD = bone mineral density

BW = Body weight

ºC = degrees centigrade

Ca = calcium

d = day

DEXA = dual-energy X-ray absorptiometry

DPV = dephosphorylated phosvitin

FCR = feed conversion ratio

FI = feed intake

g = gram

h = hour

IU = international units

kcal = kilo calorie

kg = kilogram

kgF = kilograms of force

ME = metabolizable energy

mL = milliliter

mm = millimeter

N = Newtons

n = number of observations

Na = sodium

NC = negative control; the PC diet except with a 21% reduction in calcium (from 3.6% to 2.84% dietary Ca), achieved by reducing the amount of limestone added to the basal diet

NRC= National Research Council

P = phosphorus

PC = positive control; corn-soy-canola meal positive control diet providing 2,807 kcal ME/kg, 3.6% Ca, 0.33% available phosphorus, formulated to meet the first 6 limiting amino acids and no restriction on crude protein; with other nutrients according to the Lohmann LSL-Lite management guide

PTH = parathyroid hormone

PV = phosvitin

PVP = phosvitin peptides

SAS = Statistical Analysis System

SEM =standard error of the mean

woa = weeks of age

1.0 Chapter 1: Introduction

To satisfy the increasing global demand for table egg consumption over the past century, the industry has focused on increasing productivity through genetics, management, and nutrition. Recent estimates suggest that the global population of laying hens is between 6.1 billion and 7.6 billion, reflecting this demand (Fernyhough et al., 2020). While enhancing modern laying hens' productivity is essential to meet the rising demand, the effects on skeletal health must be considered. The skeleton of laying hens is important for structural support and locomotion and plays an essential role in eggshell calcification. Calcium **(Ca)** demands from the onset of egg production require that birds regularly mobilize and replace Ca from their bones, which makes managing skeletal health important in layers. Although genetic progress in reducing the risk of skeletal diseases has occurred, studying additional strategies to support skeletal health and eggshell quality is important due to physiological constraints which genetic selection cannot overcome. These constraints include inevitable age-related decreases in dietary Ca absorption (Al-Batshan et al., 1994), shell gland Ca transport (Bar et al., 1999) and the gradual depletion of Ca stores in structural bone over time, which cause bones to become weak (Casey-Trott et al., 2017).

The importance of nutritional strategies is evident when one considers the worldwide trend towards extending laying cycles from the traditional goal of 72 weeks to beyond 90 weeks (Bain et al., 2016) and consumers' demand for welfare-friendly housing (Von Massow et al., 2018). Hens have more time to develop skeletal issues over long laying cycles due to extended periods of egg production. Additionally, consumer demands for non-cage housing, such as free-run barns and aviary systems has also highlighted the need to support bone health due to an increased frequency of failed and crash landings, which results in deformations and fractures (Rodenburg et al., 2008;

Käppeli et al., 2011). Additionally, strategies for supporting skeletal health must do so without negatively affecting eggshell quality, which is required for profitable egg production. Consequently, this thesis aims to explore Ca metabolism, skeletal health, and eggshell quality in laying hens to understand how nutritional strategies and evaluating Ca requirements might support laying hen productivity, profitability, and skeletal health.

Chapter 2.1 provides an overview on dietary Ca sources and challenges, with emphasis on the relationship between dietary Ca and P (Chapter 2.1.2). Chapters 2.2 and 2.3 provides an in-depth review of Ca metabolism in laying hens, including intestinal absorption (Chapter 2.2.1) and how it relates to the laying hen skeleton (Chapter 2.3). This is followed by a review of the role of dietary and skeletal Ca during shell calcification (Chapter 2.4). A brief review of the importance of shell quality and the various ways to measure it is discussed in Chapter 2.5. Chapter 2.6 summarizes the potential use of phosvitin (**PV**) as a feed additive to support Ca metabolism in laying hens, while Chapter 2.6.6 describes the challenges of using PV. Chapter 2.7 review various publications assessing Ca requirements in modern laying hens followed by Chapter 2.8 which describes the usefulness of a meta-analytical approach to assessing Ca requirements in modern layers. The experiment in Chapters 3 evaluates the effects of dietary PV, dephosphorylated phosvitin (**DPV**) and phosvitin peptides (**PVP**) on their ability to protect productivity, shell and bone quality in aged laying hens fed a Ca-reduced diet for 4 weeks. The meta-analysis in Chapter 4 compiled the dose-response effects of varying levels of dietary Ca on performance, shell quality and bone quality parameters from various publications while accounting for random sources of variation. A synthesis and conclusions from the experiments is provided in Chapter 5.

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2.0 Chapter 2: Literature Review

2.1 Dietary Calcium Supply

Dietary calcium (**Ca**) is an essential mineral for laying hen skeletal health, eggshell formation and biochemical reactions. Common supplemental sources of Ca in laying hen diets include limestone, bone meal, dicalcium phosphate, monocalcium phosphate and oyster shell (Rennie et al., 1997; Fleming et al., 1998b; David et al., 2019). Absorption of dietary Ca allows for Ca to be used during shell calcification or incorporated into skeletal reserves to support shell calcification when needed, which requires 2 to 3 g of Ca per egg laid (Fleming, 2008; Ahmadi and Rahimi, 2011). In 1948, Driggers and Comar measured the partitioning of radioactive Ca fed to egg-laying hens and estimated that 60 to 75% of the Ca required for eggshell calcification was obtained directly from feed rather than from bone reserves.

2.1.1 Calcium Requirements

The National Research Council (**NRC**, 1994) recommends 3.25% dietary Ca in the diet, which corresponds to 3.25 g/day/hen for Single-Comb White Leghorn hens and 3.6 g/day/hen for Brown egg-laying hens due to differences in expected feed intake (FI). After the NRC (1994) guidelines were published, some researchers suggested that levels between and 3.34 to 5.57 g/hen/day increased shell quality (Ahmad et al., 2003; Hernández-Sánchez et al., 2006; Lichovnikova, 2007; Valdés Narváez et al., 2011; An et al., 2016; Kakhki et al., 2019), productivity (Narváez-Solarte et al., 2006; Araujo et al., 2011; Pelicia et al., 2011; Vieira et al., 2011) or a combination of those parameters (Keshavarz, 2003; Rao et al., 2003; Castillo et al., 2004; Safaa et al., 2008; Rodrigues et al., 2013; Attia et al., 2020). Presently, most commercial laying hen management guides seem to err on the side of caution, which has led to recommendations that range from 4 g/hen/d to 4.7 g/hen/d depending on the age (Table 2.1).

Additionally, it is typical for primary breeder management guides to recommend increases in Ca and decreases in available phosphorus (**avP**) as hens age. Discrepancies in Ca recommendations are likely due to experimental differences in the birds used (i.e., strain, age, production level), trial conditions (i.e., housing, duration of the trial, environmental temperatures, FI), or diets (i.e., nutrient specifications, range of Ca tested, feed particle size, and the supply of particulate Ca). Additionally, discrepancies between the NRC (1994) requirements and more recent literature are likely due changes brought about by genetic selection of birds by primary breeding companies. This idea was previously suggested by Bolden and Jensen (1985) when the NRC requirements for Ca increased from 2.25% to 3.5% between 1960 to 1977 (NRC, 1960; NRC, 1977). The lower Ca requirements from the older NRC document were likely due to the lower egg productive potential of birds. Alternatively, today's birds are not only highly productive but can also sustain production for a longer duration (Preisinger, 2018). This suggests that the Ca requirements of modern layers have also increased, however, recent research has shown that the relationship between higher productivity and Ca metabolism in modern layers may not be so simple. For example, historically, high egg production has been associated with skeletal health problems (Budgell and Silversides, 2004; Whitehead, 2004), but some have questioned this association (Eusemann et al., 2018; Jansen et al., 2020; Dunn et al., 2021). Jansen et al. (2020) to characterize the adaptation response of white and brown layers with differing performance lineages (high production vs. low production) to a severe dietary Ca reduction (1.09%). While it was hypothesized that egg production would be maintained at the expense of bone health in the high producing lines, this was not the case and rather both parameters declined significantly, with a greater effect in the white laying hen strains. Gordon and Roland (1998) reported a 7-fold difference in the incidence of bone fractures between two high-producing commercial strains with comparable egg weights and eggshell weights. It is

possible, therefore, that intense genetic selection has likely changed Ca utilization and, thus its requirements; however, a comprehensive meta-analysis exploring Ca requirements in modern layers has not been performed. Instead, recent research results on Ca requirements within the past ten years have a high degree of variation in defining optimum Ca recommendations in modern layers (Table 2.2).

2.1.2 Dietary Calcium:Phosphorus Ratio

Ca requirements are highly linked to phosphorus **(P)** requirements. Because of this, poultry nutritionists often consider Ca and P requirements together (Proszkowiec-Weglarz and Angel, 2013; Li et al., 2017) in a recommended Ca: avP dietary ratio between 1:1 and 2:1 as a guideline for growth, turnover and maintenance (Koutsos et al., 2001; de Matos, 2008; Fleming, 2008; Li et al., 2017; Whitehead, 2019). This is because P is vital in skeletal Ca deposition through the formation of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) with Ca in a 2:1 ratio (de Matos, 2008; Fleming, 2008; Adedokun and Adeola, 2013; Li et al., 2017). In mature laying hens the dietary Ca:P ratio increases to as high as 13:1 due to the high demand for Ca for egg production and optimal eggshell (Li et al., 2017).

The mobilization of Ca and P from bone to support eggshell formation means that both minerals' metabolism is highly coordinated (Li et al., 2016; Li et al., 2017). An improper dietary Ca:P ratio can induce physiological responses that can affect laying hen health, shell quality and productivity. For example, high dietary Ca can increase a bird's intestinal pH (Adedokun and Adeola, 2013) which causes the formation of insoluble Ca-phytate complexes (Rama Rao et al., 2006; Selle et al., 2009; Skřivan et al., 2016) and can reduce Ca and P absorption and retention (Proszkowiec-Weglarz and Angel, 2013; Li et al., 2016). Additionally, improper Ca:P ratios can reduce the efficacy of exogenous phytase (Selle et al., 2009), negatively affect bone mineralization

(Hamdi et al., 2015), trigger bone resorption (Rama Rao et al., 2006) and negatively affect shell quality (Kebreab and Vitti, 2010; Kakhki et al., 2019) and egg production (Jiang et al., 2013).

2.2 Ca metabolism

Ca is essential in laying hens because of the large requirement for eggshell production. For example, the equivalent of 10% of a hen's total bone Ca stores is needed per egg, which is a significant metabolic feat (Fleming, 2008; Ahmadi and Rahimi, 2011; Jonchère et al., 2012b). In fact, eggshell formation is the most rapid known biomineralization process (Arias et al., 1990) and laying hens have the most efficient dietary Ca absorption among vertebrates (Bar, 2009). To accommodate for the daily variations in Ca supply and demand, laying hens deposit and mobilize Ca to and from the skeleton daily (Kim et al., 2012). For these reasons, Ca metabolism is particularly unique in laying hens and essential in supporting productivity and bird health.

2.2.1 Intestinal Ca absorption

Hens typically absorb approximately between 60 to 70% of dietary Ca consumed (David et al., 2021). Before shell calcification begins, hens develop a specific appetite for Ca and, if available free choice, they can select Ca out of the mixed feed. Increases in Ca intake increase the amount of soluble Ca available for absorption from the intestines (Gilbert, 1983; de Matos, 2008; Jonchère et al., 2012b; Nys and Le Roy, 2018). Most Ca absorption occurs in the proximal small intestine (duodenum and upper jejunum) (Hurwitz and Bar, 1965; Hurwitz and Bar, 1972; Hurwitz et al., 1973; Nys and Mongin, 1980). During eggshell formation dietary Ca absorption increases 6-fold in the intestines (Al-Batshan et al., 1994). Conversely, in the absence of eggshell calcification, the rate of Ca absorption is reduced (Hurwitz, 1965). There are two processes of Ca absorption: the transcellular active (saturable) transport process and the paracellular passive diffusion (nonsaturable) process. Transcellular transport of Ca occurs in the proximal part of the

intestine when Ca levels are low or moderate and involves the use of epithelial Ca channels called transient receptor potential vanilloid Ca channels (TRPVs) (Bar, 2009; Jonchère et al., 2012b; Yi et al., 2015). Gene expression studies have revealed the presence of TRPV6 and TRPV2 in hens (Jonchère et al., 2012b; Brionne et al., 2014). Alternatively, the paracellular pathway typically happens with high levels of Ca and involves the paracellular space between adjoining enterocytes (Nys and Le Roy, 2018).

Once inside the intestinal cell, ionized Ca (Ca^{2+}) accumulates at the subapical brush border and is transported across the cell cytoplasm to the basal membrane by calbindin-D28K, a transcellular calcium-binding transport protein (Ebel et al., 1969; Lippiello and Wasserman, 1975; Jande et al., 1981; Jonchère et al., 2012b; Brionne et al., 2014). Vitamin D_3 (1,25-$(OH)_2$-D_3) supports the absorption of Ca by increasing the synthesis of calbindin-D28K (Wasserman et al., 1968; Bar and Hurwitz, 1979; Christakos et al., 1989; Hurwitz, 1989; Corradino and Fullmer, 1991). At the basal membrane, Ca^{2+} is moved into the blood via Ca^{2+}/Na^{+} exchangers and possibly two forms of plasma membrane Ca^{2+} ATPase pumps (PMCAs) (Melancon and De Luca, 1970; Davis et al., 1987; Centeno et al., 2004), ATP2B1 and ATP2B4 (Jonchère et al., 2012b).

2.3 Ca in the laying hen skeleton

In addition to intestinal absorption, Ca homeostasis also involves a steady balance of bone formation and mobilization (Renkema et al., 2008; Li et al., 2017). In laying hens, this rapid daily turnover of bone is uniquely synchronized with eggshell formation (Kerschnitzki et al., 2014; Li et al., 2017).

2.3.1 Bone biology in laying hens

Laying hens have three types of bone tissue; cortical, trabecular and medullary (Shahnazari et al., 2006; Kim et al., 2007b). While cortical and trabecular bone provide structural integrity,

medullary bone serves as a metabolic Ca reserve to support eggshell formation (Van De Velde et al., 1985; Thorp, 1994; Cransberg et al., 2001).

2.3.2 Development of medullary bone

Cortical bone is an organized and compact bone tissue that forms the outer shell of the bone (Kim et al., 2012). Trabecular (also known as cancellous bone) is a less organized and less dense bone tissue that forms within each bone as struts which provide additional support (Kim et al., 2012). During the rearing phase, pullets develop a thick layer of cortical bone and extensive trabecular bone in the medulla. Approximately 2 weeks before sexual maturity, non-structural medullary bone develops in the central cavity of many bones in response to increasing estrogen (Whitehead and Fleming, 2000; de Matos, 2008; Johnson, 2015). The onset of medullary bone formation happens concurrently to the cessation of cortical bone remodeling before the laying period (Whitehead and Fleming, 2000; Tamamoto et al., 2001; Yamamoto et al., 2001; Beck and Hansen, 2004).

In sexually mature laying hens, medullary bone acts as a labile mineral reserve, which in addition to dietary Ca, provides the remaining Ca needed for eggshell calcification via bone resorption (Whitehead, 2004). Bone resorption refers to the breakdown of bone mineral by osteoclasts, which release Ca and other minerals from bone into the blood (Kerschnitzki et al., 2014). During shell formation the percentage of active osteoclasts in medullary bone increases 7-fold, making it an incredibly active bone resorption site (Van de Velde et al., 1984). Upon contact with exposed mineralized bone, osteoclasts use ventral protrusions called podosomes that attach to the matrix of bone (Hurwitz, 1989; Kim et al., 2012). The high number of osteoclasts found in avian medullary bone (compared to structural bone) reflects their important role in mobilizing Ca during eggshell formation (Van de Velde et al., 1984). In addition to providing calcium for eggshell

calcification, bone resorption is also responsible for the homeostasis of blood serum Ca levels (Fleming et al., 1998a; Choi et al., 2005). Although medullary bone is a labile source of Ca, osteoclasts are not specific to medullary bone, which means hens mobilize structural bone as well. The non-specific nature of osteoclasts is problematic because although medullary bone is replenished daily, structural bone is not replaced if the hen remains in lay (Fleming et al., 1998a). Over time, the maintenance of medullary bone, which contributes little to bone strength (Fleming et al., 1998a) and the gradual erosion of structural bone can lead to susceptibility to bone fractures, especially in modern, high-producing birds (Candelotto et al., 2017; Yamada et al., 2021).

2.3.3 Hormonal control of medullary bone

Upon sexual maturity, hens are under the physiological control of estrogen, which mediates the use of skeletal Ca during eggshell formation. This hormonal control starts with a gradual increase in blood estrogen levels from 16 to 22 weeks of age (**woa**; Senior, 1974) and surges on a laying day from 4 to 6 h before ovulation during the cycle (Johnson and Van Tienhoven, 1980). This pre-ovulatory surge of estrogen initiates Ca storage in medullary bone in preparation for eggshell calcification (Etches, 1987). This is followed by a decline in estrogen that makes medullary bone more sensitive to osteoclastic activities initiated by parathyroid hormone (PTH; Etches, 1987).

While the hen is in lay, estrogen regulates estrogen receptors on osteoclasts and osteoblasts. For example, decreases in plasma estrogen concentration at the beginning of calcification downregulate estrogen receptors on osteoclasts, which triggers bone resorption (Beck and Hansen, 2004). Additionally, estrogen and parathyroid hormone (**PTH**) each trigger the production of active vitamin D_3 (**$1,25(OH)_2D_3$**) in the kidneys (Castillo et al., 1977; Schenck et al., 2006). In addition to stimulating intestinal absorption of Ca, $1,25(OH)_2D_3$ increases osteoclastic bone

resorption (Raisz et al., 1972). Both $1,25(OH)_2D_3$ and PTH target the kidneys to limit renal excretion of Ca during eggshell calcification (Wideman, 1987). There is also evidence suggesting that prostaglandins may also help facilitate bone resorption (de Matos, 2008). The rate of bone resorption increases 9-fold during shell calcification, which is the net result of the pro-resorption stimuli involved in promoting osteoclast activity (Van de Velde et al., 1984).

2.4 Shell Calcification

2.4.1 Dietary and bone calcium in eggshell formation

Ca homeostasis is a balance of supply and demand, which changes depending on the time of day and whether shell calcification is occurring. When a hen enters an active stage of shell formation, approximately 100 to 200 mg of Ca is removed from the blood per h (Etches, 1987). To accommodate the high demand for Ca during eggshell calcification, laying hens depend on a constant supply of Ca via a combination of absorption in the proximal intestine and from medullary bone resorption. During the day, when hens are consuming Ca, the intestinal supply of Ca enters the vascular system to support various physiological pathways, with an emphasis on shell formation, if it is occurring. If the demand for Ca by the shell gland exceeds that provided by intestinal Ca absorption, bone is resorbed to maintain circulating Ca levels. For shell deposited during the night, the relative contribution of Ca from bone increases because of a reduced supply of Ca being absorbed from the intestine (de Matos, 2008; Bar, 2009). The balance between dietary and medullary Ca was explored by (Tyler, 1954) by feeding rations containing radioactive Ca to distinguish between the deposition of dietary calcium (radioactive calcium) and bone Ca (non-radioactive Ca) deposited on the shell. When birds were fed in the morning and laid eggs in the afternoon, a layer of radioactive Ca on the outside of the shell corresponded to dietary calcium. Alternatively, when birds laid their eggs in the morning, the outer layer of non-radioactive Ca on

the outside corresponded to bone-derived Ca. Given that radioactive Ca corresponded to dietary Ca, this experiment showed that hens primarily use dietary Ca when eggshell calcification happens during the day when birds are eating, and skeletal Ca during the birds' nocturnal fast when dietary Ca is limited to what remains in the gizzard

2.4.2 Bone metabolism during shell calcification

On non-shell-forming days, the demand for calcium is low compared to shell-forming days. In laying hens, the egg laying (or oviposition) cycle occurs approximately every 24 to 26 hours and is synchronized with the mobilization of medullary bone (Etches, 1990). The cycle restarts after an egg is laid and the release of a follicle (ovulation) which travels down the oviduct to the shell gland. Up to 9 h after laying the preceding egg, blood Ca increases as birds consume feed rich in Ca in preparation for eggshell calcification. Estrogen receptors on the kidneys and shell gland (Hansen et al., 2003), and Ca channels such as TRPV6 (Weber et al., 2001) each promote Ca retention at this stage. The arrival of the developing egg in the shell gland signals the start of eggshell calcification (Nys et al., 1999) and triggers a drop in circulating estrogen (Taylor and Belanger, 1969; Beck and Hansen, 2004). This reduction in estrogen enhances bone resorption via a downregulation of antiresorptive effects on osteoclasts (Väänänen and Härkönen, 1996). During this time, PTH is released and stimulates bone Ca mobilization via an increase in active osteoclast numbers (Van de Velde et al., 1984; Kim et al., 2012), size (de Matos, 2008), spreading area (Miller et al., 1984; de Matos, 2008), and changes in morphology and ultrastructure such as the development of ruffled borders adjacent to bone surfaces (Miller et al., 1984; de Matos, 2008). The formation of a highly acidic area at the bone surface by osteoclasts results in rapid skeletal mobilization as the bone starts to dissolve (Schenck et al., 2006; Kerschnitzki et al., 2014). Once

Ca is released into the blood, it is transported through the uterine glandular cells and ultimately to the uterine fluid to participate in eggshell calcification.

2.4.3 Post-oviposition

Once eggshell calcification is complete, the egg exits the shell gland. At this time plasma Ca levels are high as the demand for Ca for shell calcification will start to decrease slowly (Dacke et al., 1972). In response to high plasma Ca levels, calcitonin is released from the ultimobranchial glands (Lasmoles et al., 1985; Hurwitz, 1989). Whereas PTH stimulates bone resorption, calcitonin causes the disappearance of the osteoclasts' ruffled borders, which is where bone resorption occurs (Sugiyama et al., 1993), thus inhibiting osteoclast activity (Lasmoles et al., 1985; Sugiyama and Kusuhara, 1993). During this time, estrogen acts as an antiresorptive hormone via upregulation of its receptors on osteoclasts (Beck and Hansen, 2004). In this way, calcitonin, and estrogen work collaboratively against PTH by preventing bone resorption. Once the egg has been laid, dietary consumption of Ca by the hen replenishes medullary Ca reserves in preparation for the next oviposition cycle.

2.5 Shell Quality

Shell quality refers to a shell's strength and construction. Shell quality is an indicator of Ca metabolism in laying hens (Gordon and Roland, 1998); however, this relationship is complex. For example, although the purpose of medullary bone is to provide Ca storage for eggshell formation (Kim et al., 2012), shell quality declines as the production cycle progresses due to the inability for the hen to deposit additional Ca as egg size increases, even though there may be an extensive reservoir of medullary bone available (Whitehead, 2004). It is only after molting, which involves acute resorption of medullary bone (Kim et al., 2007a), that shell weight, percent,

thickness and microstructure (i.e. reduction in shell calcite crystals and increase in soluble organic matrix) are increased (Al-Batshan et al., 1994). Phenomena such as this illustrates the complexity of Ca and bone metabolism when it comes to shell quality.

2.5.1 Importance of shell quality

The longest phase of egg formation is spent in the shell gland (15 to 19 h), which reflects how metabolically demanding eggshell calcification is (Roberts, 2004; Hunton, 2005; Mazzuco and Bertechini, 2014; Ketta and Tůmová, 2016). Eggshells contain approximately 96 to 97% Ca carbonate which assembles on a matrix of proteins, glycoprotein, and proteoglycans. Although the shell makes up only 9 to 12% of the egg weight, it is important to the economic viability of the egg-laying industry (Ahmadi and Rahimi, 2011). Weak eggshells are prone to mechanical damage such as gross cracks, hairline cracks and star cracks, which prevent eggs from being collected, processed, packaged, transported, and sold (Mazzuco and Bertechini, 2014). Additionally, a compromised eggshell is a food safety issue since it makes eggs prone to contamination with bacteria (Nascimento et al., 1992; De Reu et al., 2006). A good quality shell acts as a physical and chemical barrier to pathogens (Gantois et al., 2009; Ishikawa et al., 2010). From an aesthetic point of view, consumers are interested in eggshell quality in terms of cleanliness, shape, texture, and soundness, making the eggshell an important marketing parameter (De Ketelaere et al., 2004). Consequently, the laying industry has focused on improving shell quality via genetic selection programs to reduce economic losses (Thiruvenkadan et al., 2019).

2.5.2 Measuring shell quality

Determining shell quality involves evaluating physical qualities such as eggshell strength, thickness, weight, and egg specific gravity. Physical qualities also include the shell's quality of construction, which refers to its ultra-structure (palisade layer width, mammillary knob layer

thickness, deposition of calcite columns) (Carnarius et al., 1996; Ketta and Tůmová, 2016), and micro-structure (crystal size and orientation). The ultra-structure and micro-structure are significant determinants of shell thickness and strength (Rodriguez-Navarro et al., 2002; Bain et al., 2009). Shell quality can be quite variable among individual birds, which is why an adequate sample size must be used to reflect the shell quality of a flock.

2.5.2.1 Shell breaking strength

Shell strength refers to the shell's ability to withstand an external force until failure or deflection and measures material strength (Solomon, 2010). Shell strength is quantified via shell breaking strength, stiffness, and shell thickness (De Ketelaere et al., 2002). Measuring shell breaking strength involves slowly compressing an egg vertically at a constant compression rate between flat parallel surfaces until an abrupt load drop occurs as the shell cracks (Bain, 2005). The maximum load achieved before the shell cracks is recorded as breaking strength and can be expressed as in Newtons (**N**) or kg of force (**kgF**). Breaking strength depends heavily on the compression speed (Voisey and Hunt, 1969); therefore, the use of automated machines that provide force at a steady speed, such as the Instron Materials tester, are important for research purposes. Unfortunately, due to its destructive nature, breaking strength can only be measured once per egg. Additionally, breaking strength is a local measurement and therefore not reflective of overall eggshell strength but still provides a useful starting point for assessing shell quality.

2.5.2.2 Shell Thickness

Shell thickness is related to shell strength, as demonstrated by a significant phenotypic correlation (0.77) between the two (Zhang et al., 2005). Shell thickness is also highly related to other important shell parameters such as egg weight (Zhang et al., 2005; Rozempolska-Rucińska et al., 2011). Eggshell thickness, however, is not uniform throughout the egg. Generally, the

eggshell is thickest on the pointed end and progressively thinner towards the blunt end (Sun et al., 2012). Because of these differences, shell thickness is often measured as a mean of different shell regions (different latitudes from the pointed or blunt end).

2.5.2.2 Micrometer shell thickness gauge

One of the most practical and reliable methods of measuring shell thickness is using an electronic or manual micrometer screw gauge (Voisey and Hunt, 1974). This measurement involves breaking the egg near the equator and using the micrometer's forceps to physically measure shell thickness at that location. Although this measurement is reliable, it has two disadvantages. Firstly, it destroys the entire egg, which does not allow for further shell quality measurements. Secondly, micrometer measurements may underestimate thickness values compared to other methods, such as ultrasounds technology, due to the crushing of egg membranes between the micrometer's forceps (Cordts et al., 2002; Kibala et al., 2015). Despite this, it is a reliable and cost-effective method for evaluating shell thickness.

2.5.2.3 Ultrasound technology

Measuring shell thickness using ultrasound technology is a direct and non-destructive way of assessing shell strength. This measurement involves reflecting ultrasonic beams from the eggshell to measure its thickness (Aboonajmi et al., 2010). First, a small dollop of ultrasound gel is placed at the egg equator, which acts as a transmission medium for the ultrasonic beams. The egg is then placed laterally on a plastic support with the gel contacting the ultrasonic device. A pulse of ultrasonic energy is delivered by the transducer, through the gel, and to the shell. Shell thickness is determined by measuring the amount of time it takes for the energy waves to travel from the transducer and echo back from the material. Studies have shown that there is no significant difference between ultrasonic measurements and electronic micrometer measurements

in the same area, making them comparable (Yan et al., 2013; Kibala et al., 2015). Voisey and Hamilton (1976) found a significant correlation between micrometer shell thickness and ultrasonic shell thickness readings. Since shell thickness measured via ultrasonic technology is non-destructive and accounts for the thickness of the outer and inner membranes, it is considered advantageous compared to micrometer measurements.

2.5.2.4 Egg specific gravity

Specific gravity is an indirect method of assessing shell thickness, porosity, and strength (Kibala et al., 2015). Unlike others, this measurement is unique because it reflects the entire egg rather than a specific shell area. The process involves immersing eggs sequentially in saline solutions of increasing salt concentrations or calculating the specific gravity using Archimedes' principle (Hamilton, 1982). Saline solutions typically range in density from 1.060 to 1.104 and increase in varying increments (i.e., 0.002, 0.004, 0.005) within a study. The higher the salt concentration of the solution the eggs float in, the greater the shell thickness (Hamilton, 1982). Although it is a shell thickness indicator, some suggest that specific gravity is a better indicator of the amount of shell relative to egg size (Roberts, 2004). Additionally, specific gravity can be altered by strain (Şekeroğlu et al., 2008), storage length due to an increase air cell size with moisture loss (Akyurek and Okur, 2009) and temperature due to a change in the density of the saline solutions (Voisey and Hamilton, 1977). Many researchers, however, use it as an inexpensive and valuable non-destructive measurement for assessing shell quality (Cordts et al., 2002).

It is important to note that shell thickness does not always correspond to a stiffer or stronger shell (Bain, 2005). For example, using three brown egg strains (Hubbard, Warren, and Tatum) and three white egg strains (Hyline, Dekalb and Babcock). Potts et al. (1974) found that although the

brown eggs had thinner shells, the majority had higher breaking strengths than white eggshells. In addition to thickness, shell strength comes from a combination of structural and material properties. Structural variables include egg size, shape, curvature, whereas material properties include organic components (membrane, cuticle, protein matrix) and inorganic components (ultrastructure and microstructure; Bain, 2005). Some have suggested that a more meaningful measurement of shell strength is effective thickness. Effective thickness refers to the eggshells ultrastructure and is the distance between the fusion of the palisade columns to the outer edge of the cuticle measured in micrometers (**µm**; Bain, 1990). Effective thickness has been reported to have a more significant contribution to shell strength than shell thickness (Van Toledo et al., 1982). The palisade layer is the main layer of the shell and takes up two-thirds of the eggshells cross-sectional length, which explains why effective thickness has such a significant effect on shell strength (Parsons, 1982; Ketta and Tůmová, 2016). Unfortunately, measuring effective thickness involves pre-scanning, washing, and drying of the eggshell and scanning electron microscopy, which makes it a time-consuming and expensive option.

2.5.3 Factors affecting shell quality

The factors that affect shell quality are internal (genetics, age, shell calcification process) and external (nutrition and environment).

2.5.3.1 Ca nutrition

A dietary Ca deficiency reduces shell quality due to a lack of mineral material for the formation of $CaCO_3$ (Jiang et al., 2013). For example, a dietary Ca deficiency causes eggshells to become thinner (Taylor and Moore, 1954; De Bernard et al., 1980; Narbaitz et al., 1987). Additionally, the upregulation of eggshell gland calbindin, a critical Ca-binding protein, is dependent on the formation of a Ca flux. This refers to the net movement of Ca across the shell

gland (serosa-to-mucosa minus the movement of Ca from the mucosa-to-serosa) which depends on sufficient Ca (Nys et al., 1992; Jonchère et al., 2012a). Vitamin D_3 also plays an important role in shell quality. As previously discussed, the transcellular movement of Ca after it accumulates at the subapical brush border is vitamin D_3-dependant, as the later increases synthesis of calbindin-D28K. Bar et al. (1999) found that hens that laid uncalcified shells synthesized less $1,25(OH)_2D_3$ compared to hens laying normal shells. Because intestinal Ca transport and calbindin synthesis depend on $1,25(OH)_2D_3$, it makes sense that a deficiency of this vitamin would reduce shell quality (Bar et al., 1999). Thus, adequate dietary Ca and vitamin D_3 (exogenous supplementation or endogenous vitamin D synthesis via exposure to ultraviolet B light) is essential in promoting shell quality.

Ca particle size also influences shell quality. In the 1970s, researchers discovered that oyster shell was more effective in supporting shell quality than the same amount of Ca fed in a finely ground or pulverized form. This was demonstrated by Scott et al. (1971) who found that birds receiving Ca partially in the form of oyster shell (2/3 oyster shell and 1/3 pulverized limestone) had 17% higher blood Ca levels overnight and laid eggs with increased breaking strengths compared to hens only fed pulverized limestone. These changes were likely because birds fed large particle Ca (i.e., oyster shell; >1 mm) can retain Ca particles in the gizzard for 12 h after the end of feeding, which allows for the slow release of Ca into the blood, particularly at night when shell calcification is occurring, but birds are not eating (Etches, 1987; Rao et al., 1992; Fleming, 2008). In addition to oyster shell, large particle Ca can be supplied in the form of limestone. Ideally, large particulate Ca (ranging between 2 mm and 5 mm) should make up two-thirds of the Ca provided to be most effective in supporting shell quality (Lichovnikova, 2007).

2.5.3.2 Genetics

Genetic selection is a desirable strategy for enhancing eggshell quality because it improves the shell productivity of bird producers work with. Modern laying hens are primarily selected for egg production (Preisinger, 2018), which puts pressure on the bird's skeleton and may negatively affect shell quality (Cordts et al., 2002; Wistedt et al., 2019). Consequently, genetic selection must focus on creating robust hens with a high productive potential while balancing other underlying processes (i.e., skeletal health and eggshell quality). Balancing these needs can be challenging, since bone quality is negatively associated with egg production and shell quality. For example, (Kim et al., 2005) found that shell weight, percent shell, and shell thickness were also negatively correlated with various bone quality indicators. Fortunately, however, shell quality traits such as shell strength, specific gravity, shell thickness and deformation have heritabilities ranging between 0.12 and 0.53 (Cordts et al., 2002). Additionally, the heritability of eggshell microstructural characteristics (i.e., calcite crystal size and orientation), which contributes to mechanical strength, has been estimated at 0.6 and 0.35, respectively (Dunn et al., 2012). In general, shell strength increases when crystals become smaller and are more randomly oriented (Rodriguez-Navarro et al., 2002; Ahmed et al., 2005) thus selection for these characteristics contributes to increasing shell quality. Selection for multiple goals (i.e., productivity, shell quality and liveability) can be difficult, however, it is possible to achieve (Bain et al., 2016). For example, selection for longer flock cycles to increase productivity and promote efficient use of resources requires the decline in shell quality that hens exhibit with age to be simultaneously considered. Consequently, in selecting for persistency of lay, breeding companies also focus on egg number to 60 woa, liveability, and feed conversion (O'Sullivan, 2009; Bain et al., 2016).

2.5.3.3 Age

As birds age, shell quality deteriorates (Rodriguez-Navarro et al., 2002; Hansen et al., 2003; Saunders-Blades et al., 2009), reflected in an increased number of soft-shelled and thin shelled eggs laid, in addition to an increased incidence of cracks (Bennett, 1993). This loss of shell quality is due to increased egg size, reduced ability to absorb Ca, and the less random orientation of calcite crystals in mineral layer of the eggshell (i.e., parallel to the palisade layers) which results in the hen's reduced ability to form sound shell quality over time (Al-Batshan et al., 1994; Rodriguez-Navarro et al., 2002; Roberts, 2004; Wistedt et al., 2019). Although older birds develop a greater bone Ca reservoir as medullary bone, they deposit a constant amount of eggshell material after peak production (Roland et al., 1975; Roland, 1979). This constraint means that increasing egg size is not matched by a proportional increase in shell deposition, resulting in larger, thinner shelled eggs over time that are more prone to breakage (Carnarius et al., 1996; De Ketelaere et al., 2002). Age also affects a hen's ability to utilize Ca due to a reduced ability to absorb Ca from the intestine (Al-Batshan et al., 1994). Intestinal absorption of Ca is $1,25(OH)_2$ vitamin D_3-dependent and, therefore, deterioration of vitamin D_3 metabolism may lead to physiological Ca deficiencies in older hens, which inevitably leads to poor shell quality over time (Elaroussi et al., 1994). It is interesting to note that reduced shell quality is not due to a lack of medullary bone reserves. In fact, older hens tend to have greater bone ash (Whitehead and Fleming, 2000) and medullary bone specifically (Fleming et al., 1998b), at the end of production, suggesting that the lack of shell quality is not due to a lack of skeletal Ca reserves. For these reasons, the use of genetic selection as a tool to increase shell quality through via selection for egg size and shell strength is important.

2.6 Phosvitin

Although genetic selection can be an effective solution to solving skeletal health and shell quality issues in laying hens, there are other physiological means by which these issues can be managed. Management is important due to age-related decreases in dietary Ca absorption (Al-Batshan et al., 1994), decreases in shell gland Ca transport (Bar et al., 1999), decreases in shell quality (Cordts et al., 2002; Rodriguez-Navarro et al., 2002), and depletion of structural bone which leads to weakness (Cransberg et al., 2001). Consequently, exploring other strategies in addition to genetic selection, such as functional feed additives are especially helpful as laying industries worldwide start pursuing the extension of production cycles.

One proposed nutritional strategy for supporting skeletal health is phosvitin (**PV**), a phospholipid protein present in the egg yolks of birds, reptiles, and fish (Allerton and Perlmann, 1965; Hegenauer et al., 1979; Ho et al., 1980; Losso et al., 1993). PV plays a role in embryogenesis, more specifically in bone formation of the chicken embryo (Li et al., 2014). As the name suggests, PV is highly phosphorylated, containing approximately 10% P. In addition to being a source of P, PV's amino acid composition consists of more than 50% serine, most of which are phosphorylated (Samaraweera et al., 2011). Due to its highly phosphorylated nature and serine content, PV has a very stable conformation that is resistant to proteolytic actions by proteases such as pepsin, trypsin, and α-chymotrypsin (Mecham and Olcott, 1949; Goulas et al., 1996; Choi et al., 2005). Dephosphorylation of PV (i.e., the removal of phosphate groups) enhances PV susceptibility to proteolytic action (Jiang and Mine, 2000; Volk et al., 2012), which results in two large peptides and several smaller peptides, called PV phosphopeptides (Choi et al., 2005). The smaller peptides increase absorption of Ca in vivo (Choi et al., 2005; Samaraweera et al., 2014) and enhance bone Ca incorporation in vivo and in vitro (Choi et al., 2005; Chakrabarti et al., 2020), however, its

biological activity in vivo is unclear due to limited information on the digestibility of such peptides. Additionally, previous research using casein phosphopeptides has shown that it is the presence of phosphopeptides that enhances passive absorption of soluble Ca and that they play no direct role in the active transport process (Kitts and Yuan, 1992). Furthermore, it is difficult to test PV's effect on bone remodeling in any in vivo animal model due to continuous cycles of bone turnover, making it difficult to assess if any impact is on bone formation or resorption (Liu et al., 2013).

2.6.1 Metal binding capacity

The high number of phosphorylated serine residues in PV is responsible for its ability to act as one of the strongest metal chelators (Grizzuti and Perlmann, 1973; Hegenauer et al., 1979), which refers to its ability to form complexes with metal ions in a stable, water-soluble complex. The intense binding capacity of PV with Ca and iron is an example of this property (Taborsky, 1963; Grizzuti and Perlmann, 1973; Castellani et al., 2004). For example, 95% of an egg yolk's iron is bound in a stable conformation with PV (Greengard et al., 1964; Sattar Khan et al., 2000; Lee et al., 2002; Wang et al., 2011). PV's chelating ability, however, is particularly relevant in skeletal metabolism, when it comes to its effect on enhancing Ca bioavailability and absorption from the intestine (Jiang and Mine, 2000; Jiang and Mine, 2001; Cui et al., 2019).

2.6.2 Enhanced Calcium bioavailability

PV and its peptides can form stable complexes with Ca, thus preventing the formation of insoluble Ca phosphate complexes in the intestine (Grizzuti and Perlmann, 1973; Jiang and Mine, 2000; Jiang and Mine, 2001; Choi et al., 2005; Cui et al., 2019). It has been suggested that phosvitin peptides (PVP) enhance Ca bioavailability due to their strong Ca binding capacity, which prevents the formation of insoluble Ca-phosphate complexes which increase Ca absorption (Li et al., 2018; Cui et al., 2019; Zhang et al., 2019). For example, Choi et al. (2005) found that less Ca was

excreted in the feces of rats fed PV-supplemented feed, suggesting higher intestinal Ca absorption. Others have also demonstrated that PVP enhance Ca absorption (Zhong et al., 2016). In laying hens, enhanced bioavailability of Ca is beneficial due to age-related decreases in Ca absorption over time (Al-Batshan et al., 1994).

2.6.3 Effect on bone metabolism

Since PV and its derivatives increase the bioavailability of Ca, their effect on Ca incorporation and bone formation is also of interest. In mice and rats, PV enhances bone Ca incorporation in vitro and in vivo (Choi et al., 2005; Jie et al., 2018), decreases PTH-induced bone resorption (Liu et al., 2013), and even mimics the role of ascorbate in facilitating new osteoid and bone synthesis (Liu et al., 2013).

2.6.4 Bone Calcium incorporation

The effects of PV on bone Ca incorporation have been demonstrated through in vivo and ex vivo trials (Choi et al., 2005; Onuma, 2005; Liu et al., 2013; Zhong et al., 2016). In vitro studies show that PV enhances the nucleation and growth of hydroxyapatite on collagen (Onuma, 2005), and increases bone Ca incorporation. Using weanling rats, Choi et al. (2005) fed varying levels of PV peptides and saw higher bone ash, Ca content, and Ca/bone and Ca/bone ash ratios in the femurs and tibias of rats fed PVP within 4 weeks. These same effects, including increases in bone mineral density (BMD) and bone mineral content (BMC), have also been observed in vivo after feeding PVP to rats (Zhong et al., 2016); however, Choi et al. (2005) found that increases in BMC occurred in a dose-dependent nature. Using bone culture calvaria, Liu et al. (2013) found that cell layers of bone treated with PV had Ca levels that were 4 to 5 times higher than that of the controls, which provide further evidence of PV's role in promoting bone Ca incorporation.

2.6.5 Bone formation and resorption

Osteoid refers to the unmineralized organic matrix of protein and polysaccharides that is the scaffold of bone. Upon mineralization, osteoid becomes bone. Pre-osteoblast cells, such as MC3T3-E1, differentiate into osteoblasts (Quarles et al., 1992; Franceschi et al., 1994), which form bone tissue, and also express inflammatory chemokines, which promote bone resorption (Graves et al., 1999). Water-soluble yolk proteins, such as PV, can stimulate the activity of MC3T3-E1 cells, which are models for studying in vitro osteoblast differentiation (Kim et al., 2008; Jie et al., 2018; Ren et al., 2019). High dephosphorylation levels (up to 60% dephosphorylation) increases the proliferation of MC3T3-E1 cells (Jie et al., 2018). After proliferation, osteoblastic differentiation of MC3T3-E1 cells into mature osteoblasts typically occurs in the presence of ascorbic acid (i.e., Vitamin C; Franceschi et al., 1994), which is needed for the synthesis and stability of collagen (Franceschi, 1992). Surprisingly, however, PV can facilitate MC3T3-E1 osteoblastic differentiation in the absence of ascorbate. For example, Liu et al. (2013) observed that when ascorbic acid was replaced by PV, rat calvaria showed significant osteoblast differentiation and clusters, collagen synthesis, and hydroxyproline formation on bone surface cultures exposed to PV. Additionally, others have found that PV and ascorbate target the same genes associated with biomineralization and osteoblast differentiation. For example, PV upregulates Runt-related transcription factor 2, which is the master transcription factor responsible for osteoblast differentiation (Ren, 2019) and is responsible for the upregulation of other osteogenic gene markers, collagen type I and osteocalcin, like ascorbic acid. Additionally, PV can enhance bone calvaria Ca uptake by up to 10 times (Liu et al., 2013) compared to control media, and increased alkaline phosphatase (ALP) activity by 145%, which is a marker for osteoblast differentiation (Jie et al., 2018). Overall, it appears that osteoblasts respond in a similar

physiological and biochemical manner to PV as they do to do ascorbate (Liu et al., 2013; Liu et al., 2017; Jie et al., 2018), which is likely the key to understanding PV's effects on bone formation.

In addition to bone formation, PV also has an inhibiting effect on bone resorption. PTH is responsible for inducing bone resorption, thus initiating Ca release into the blood (Taylor and Belanger, 1969). Lui et al. (2013) found that the PTH-induced release of Ca from live mouse bone cultures was inhibited by 60% in cultures treated with PV. This inhibition was due to reduced osteoclast differentiation, which is typically induced by PTH.

2.6.6 Challenges with Phosvitin

The main challenge in using bioactive peptides such as PV in commercial animal nutrition as a feed additive is the extraction process itself. The PV extraction process requires precise and tedious procedures that often involve non-food-grade chemicals such as organic solvents, magnesium sulfate, and high concentrations of salt. Under laboratory conditions, extraction recovery rates range between 39% to 82.7%, depending on the extraction method and scale (Mecham and Olcott, 1949; Heald and McLachlan, 1963; Ko et al., 2011; Ren and Wu, 2014; Ren, 2019). Due to the complexity of the process, scale-up extraction of PV will reduce recovery rates; however, scale-up extraction is necessary for future industrial applications. PV extraction is also challenging due to the high costs of specialized equipment and trained staff necessary to complete the process. Additionally, the quantity of PV as an additive in small rodent models is much smaller than the quantity that would be needed for commercial use in laying hens, which adds to the cost. Furthermore, while the biological effects of PV sound promising in mouse and rat models, laying hens physiology is drastically different. Laying hens experience a huge amount of bone mobilization and re-deposition daily which is associated with daily egg production. Thus, determining whether any positive effects of PV on skeletal health would be at the expense of shell

quality is essential. A feed additive that is beneficial to skeletal health but reduces shell quality would negatively affect the laying industry's economic viability. Consequently, PV's potential in other animal models, such as laying hens, needs to be determined.

2.7 Calcium requirements in modern laying hens

While Ca indubitably plays a significant role in the metabolism of laying hens, discrepancies over the optimal dietary levels of this minerals are also significant. Currently, the NRC (1994) states a dietary requirement of 3.25% Ca per day for white-egg laying hens and 3.4% to 3.6% for brown-egg laying hens. Some have suggested that no clear explanation exists for the given recommendations (Safaa et al., 2008), while others state that it is based on a theoretical net absorption of 70 to 75% of daily Ca intake by hens aged approximately 57 to 71 woa laying approximately 0.9 egg/d (Bar et al., 2002). In reality the NRC values are based on published papers. The lack of clarity surrounding this, however, even for those studying the topic, illustrates the uncertainty surrounding this area of research. Even so, many have suggested that 3.25% Ca for laying hens is insufficient. For example, both Clunies et al. (1992) and Wallner-Pendleton et al. (1996) recommend that a minimum of 3.75 g Ca/hen/d were necessary for optimal shell and bone quality. Additionally, Scott et al., (1999) compared two dietary levels of Ca (3.7% and 4.0%), each of which were well above the NRC (1994) requirements. Surprisingly, despite the small difference between the levels, 4.0% Ca increased egg production and egg weight, which supports the industry's practice of providing more Ca than the NRC requirements at the time (Scott et al., 1999). In additional to questions regarding the suitability of the NRC (1994) requirements, others have criticized the recommendations set in primary breeder management guides. For example, Roland et al. (1996) asserted that the upper limit of 3.85% Ca/day/hen stated in some Hy-Line W-36 management guides at the time were insufficient, instead recommending a minimum of 4.25% to

4.5% Ca for optimizing profit, shell quality and skeletal strength. While all these recommendations are higher than the NRC (1994) guidelines, they are still lower than recommendations made for optimal shell quality almost a decade prior to the NRC (1994) recommendation. For example, the NRC (1984) Ca recommendations themselves were much higher at 3.85 g/hen/day than the more recent NRC (1994) recommendations. Additionally, Roush et al (1986) recommended that 4.73% was optimal for shell quality at the time.

The lowering Ca recommendations is important to consider in the context of genetic selection. While breeding companies do not select directly for Ca utilization, genetic selection over the last few decades has focused on increased persistency of production, livability, and shell quality (Van de Baak, T., Nijkerk, Netherlands, personal communication). All these selection pressures have likely changed Ca utilization in each generation of extremely productive modern layers, and, therefore, the Ca requirement (Shafey et al., 1990; Hurwitz et al., 1995; McDevitt et al., 2006). For example, reduced resorption of structural bone tissues in modern layers is the result of genetic selection for skeletal health, which has altered Ca metabolism in a manner that protect against structural bone loss (Fleming et al., 2006; Raymond et al., 2018). Increased Ca utilization by modern layers may explain why research, suggests that only minor adjustments to the NRC (1994) Ca recommendation are needed and that increases above 3.6 to 3.9% are not beneficial to shell quality (Leeson et al., 1993; Bar et al., 2002; Keshavarz, 2003; Valkonen et al., 2010). Given this, modern layers are likely able to maintain production, shell quality and skeletal health at lower dietary Ca levels than currently suggested by many management guides (Table 2.1).

If this is the case, nutritionists must consider the consequences of overfeeding Ca, which includes decreases in performance (Pelicia et al., 2009), bone development (Akbari Moghaddam

Kakhki et al., 2019), and phytase efficacy (Beutler, 2009), each of which are costly to producers. Despite the efforts of individual research groups in determining the optimal level of dietary Ca for laying hens, it is possible that combining data from various studies using a meta-analysis may provide a different perspective.

2.8 Meta-analyses

Often, in many academic disciplines, different researchers explore similar research questions, yet the data from multiple studies may never be combined to provide a new perspective and potential knowledge. A meta-analysis, however, is a research strategy that can be used to do this. Meta-analyses involve systematic reviews of literature exploring the same topic in a specific context and the use of statistical methods to combine and analyze the data from these various studies (Ahn and Kang, 2018; Akhter et al., 2019). The benefits of meta-analyses are that they can summarize large bodies of research, compare differences in results, improve the precision of measuring an effect, develop new research questions and save on resources (Bergstrom and Taylor, 2006; Walker et al., 2008; Ahn and Kang, 2018; Pigott and Polanin, 2019).

Meta-analyses involving laying hens have covered topics such as housing systems (Freire and Cowling, 2013; Weeks et al., 2016; Schuck-Paim et al., 2021), limestone particle size (Hervo et al., 2021), feather pecking and damage (van Staaveren et al., 2021), heat stress (Mignon-Grasteau et al., 2015), non-phytate P and phytase (Ahmadi and Rodehutscord, 2012) and garlic supplementation (Ogbuewu et al., 2021). Meta-analyses can include many studies, but at the minimum must involve at least two studies (Ahn and Kang, 2018). Meta-analyses account for moderator variables, which are factors that affect the relationship between independent and dependent variables (Field and Gillett, 2010). In the case of Ca requirement studies, this would include P and phytase inclusion (Gordon and Roland, 1997; Gordon and Roland, 1998), level of

Vitamin D_3 (Wen et al., 2019), Ca source and particle size (Saunders-Blades et al., 2009), phase feeding regime (Keshavarz and Nakajima, 1993), proportion of large particle Ca, to name a few. Ahn and Kang (2018) outline the processes involved in completing a meta-analysis, which initially involves the same steps required of a systematic review. A systematic review collects available published data related to a question and reviews and analyzes the results to find answers to a specific question. A systematic review turns into a meta-analysis when the results of these different publications are combined and statistical methods to derive a conclusion. This allows researchers to develop a conclusion based on higher statistical power and accuracy compared to individual studies (Ahn and Kang, 2018).

While meta-analyses are a valuable research tool, there are limitations that must be considered. There are a variety of biases that affect whether a data set is included in a meta-analysis. Examples include publication bias, the English-language bias, citation bias, and search bias (Ferrer, 1998). Publication bias refers to when the outcome of an experiment affects if it is published or not. This is difficult to avoid because most researchers only publish favorable results (Murad et al., 2018). The publication of peer-reviewed studies showing a lack of statistically significant differences or results contrary to what was expected would help with this issue. The English-language bias is an extension of publication bias, as studies with non-statistically significant differences are less likely to be published in English-language journals compared to studies with statistically significant differences studies. In this case, access to non-English language journals and translation services would help. Citation bias refers to the tendency for studies with statistically significant differences to be more easily identified and therefore included in a meta-analysis. To avoid this, one must avoid only using the reference lists from publications as a method of identifying other potential studies. Search bias is another concern which is affected

by the key words selected and the search engines used to look for publications. To avoid this, developing a consistent set of key words and search strategy is essential (Felson, 1992). To avoid many of these biases, researchers must follow a strict set of criteria in selecting published data sets. This may include an objective assessment of the paper's objectives, experimental design, sample size, types of treatments, etc. (Pigott and Polanin, 2019). In addition to preventing biases, this also reduces dissimilarities among papers, which affect can affect the final conclusions drawn. This can be particularly difficult to avoid in the context of laying hen research if moderator variables (i.e., housing, management, details on experimental diets or designs) are not reported on. In summary, for the conclusions of a meta-analysis to be reliable, several conditions must be followed to avoid bias and successfully contribute to gaps in research.

2.9 Research application

In an effort feed a growing world population, promote sustainability, and create more potential cost savings for producers, each generation of modern laying hens is becoming more productive and efficient. In addition to changing genetics, producers must prepare for changes in management (i.e., longer laying cycles) and consumer demands (i.e., non-cage housing systems) which make managing these birds more difficult. These changes must not be done at the expense of hen health and welfare (i.e., skeletal health) to protect the table egg industry's social license and economic viability. Consequently, exploring innovative strategies such as the potential beneficial effects of dietary PV on changes in bone mineralization to prevent skeletal issues in laying hens is important. There are limited studies addressing PV's mechanism of action and use in animals and this thesis includes the first study in hens.

The lack of effect of a dietary Ca reduction used in assessing the effects of PV led to exploring Ca requirements in laying hens. Despite this nutritional challenge, the impressive performance of the birds in the phosvitin pilot study reflected the robustness of modern-day laying hens. While breeding companies do not select directly for Ca utilization (Teun van de Baak, Schothorst Feed Research, Nijkerk, Netherlands, personal communication), it is likely that selection for other traits has likely changed Ca utilization, which prompted an exploration of Ca requirements in modern laying hens via a meta-analysis. Consequently, in addition to this thesis' focus on the effect of PV supplementation in Ca-reduced diets on productivity, shell quality and bone quality, there was also an additional assessment on Ca requirements and recommendations using publications from the past four decades.

2.10 Objectives and hypothesis

With the fast-changing genetics of laying hens, longer laying cycles and increasing consumer demands for welfare friendly housing, that of which requires good skeletal health, exploring nutritional strategies that support laying hen health and welfare are more important than ever. Thus, research is needed to evaluate new nutritional strategies and the nuances of Ca requirements as it related to important economic parameters, such as productivity, shell quality and skeletal health.

2.10 .1 Objectives

The specific objectives of this thesis were as follows:

1. To evaluate the effects of dietary PV, dephosphorylated phosvitin (DPV) and phosvitin peptides (PVP) on their ability to protect productivity, shell and bone quality in aged laying hens fed a Ca-reduced diet for 4 weeks (Chapter 3)
2. To compile the dose-response effects of varying levels of dietary Ca on performance, shell quality and bone quality parameters from various publications while accounting for random sources of variation. (Chapter 4)

2.10 .2 Hypothesis

It was hypothesized that:

1. Dietary PV would protect laying hen bone quality via increased dietary Ca absorption and maintenance of skeletal mineral reserves while preserving productivity and shell quality in end-of-cycle laying hens.
2. Genetic selection for increasing productivity by primary breeding companies in recent years has made Ca utilization more efficient, thus reducing the Ca requirements of modern commercial laying hens expressed as a percentage of the diet.

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2.12 Tables

Table 2.1 Management guide Ca recommendations

Breeding Company	Strain	Ca Recommendations (g/hen/day)	Age (weeks)	Source
Lohmann	LSL-Classic	4.10	19	(Lohmann Tierzucht, 2021c)
		4.40	50-70	
		4.50	>70	
	Brown Classic	4.10	19	(Lohmann Tierzucht, 2021b)
		4.40	50-70	
		4.50	>70	
	LSL-Lite	4.10	19	(Lohmann Tierzucht, 2021d)
		4.40	50-70	
		4.50	>70	
	Brown-Lite	4.10	19	(Lohmann Tierzucht, 2021a)
		4.40	50-70	
		4.50	>70	
Hendrix Genetics	Babcock	4.1 – 4.2	16/17-38	(Hendrix Genetics, 2021a)
		4.2 – 4.3	39 – 51	
		4.3 – 4.4	52- 64	
		4.4 – 4.5	65 – 77	
		4.6 – 4.7	>78	
	Bovans Brown[3]	4.0 – 4.2	17 – 28	(Hendrix Genetics, 2021b)
		4.2 – 4.4	29 – 50	
		4.5 – 4.7	>50	
	Bovans White	4.0 – 4.2	17-28	(Hendrix Genetics, 2021c)
		4.2 – 4.4	29-50	
		4.5 – 4.7	>50	
	Dekalb White	4.1 – 4.2	16/17-38	(Hendrix Genetics, 2021d)
		4.2 – 4.3	39-51	
		4.3 – 4.4	52-64	
		4.4 – 4.5	65-77	
		4.6 – 4.7	>78	

	ISA Brown	4.0 – 4.2	17-28	(Hendrix Genetics, 2021e)
		4.2 – 4.4	29-50	
		4.5 – 4.7	>50	
	Shaver White	4.1 – 4.2	16/17-38	(Hendrix Genetics, 2021f)
		4.2 – 4.3	39-51	
		4.3 – 4.4	52-64	
		4.4 – 4.5	65-77	
		4.6 – 4.7	>78	
Hy-line	W-36	4	18-32	(Hy-Line International, 2020)
		4.15	33-55	
		4.3	56-72	
		4.45	73-85	
		4.6	>86	
	W-80	4	18-32	(Hy-Line International, 2019)
		4.15	33-55	
		4.3	56-72	
		4.45	73-85	
		4.6	>86	
	Brown (Economical Performance)	4	18-33	(Hy-Line International, 2022)
		4.2	34-48	
		4.4	49-62	
		4.6	63-76	
		4.7	>77	
	Brown (Economical Performance)	4	18-33	(Hy-Line International, 2022)
		4.2	34-48	
		4.4	49-62	
		4.6	63-76	
		4.7	>77	
	Brown (Optimal Performance)	4	18-33	(Hy-Line International, 2022)
		4.2	34-48	
		4.4	49-62	
		4.6	63-76	
		4.7	>77	

Table 2.2. Dietary calcium recommendations for laying hens in production compiled from publications (n=38) used in the meta-analysis

Year Range	Year	Dietary calcium recommendation (g/hen/day)[a]	Initial Age (weeks)	Final Age (weeks)	Basis of recommendation	Strain	White or Brown	Source
1980- 1989	1985	4.35	20	30	---	Hy-line W-36	White	(Roland et al., 1985)
1990 – 1999	1993	3.75	20	36	Shell formation	---	---	(Keshavarz and Nakajima, 1993)
	1993	3.4	19	23	---	ISA Brown	Brown	(Leeson et al., 1993)
	1996	3.4 – 3.8	21	32	---	Hy-line W-36	White	(Roland et al., 1996)
2000 – 2009	2002	3.6	57	65	Production Shell quality	*Various*[b]	---	(Bar et al., 2002)
	2003	3.51	28	48	---	---	White	(Rao et al., 2003)
	2003	5.57	57	58	Egg specific gravity	Bovanes	Brown	(Ahmad et al., 2003)
	2004	3.34	23	71	Production, Shell quality	Hy-line W-98	White	(Castillo et al., 2004)
	2006	4	46	62	----	Lohmann	White	(Narváez-Solarte et al., 2006)
	2006	3.17 – 4.02	79	87	Egg mass, shell thickness and economic optimum level	Hy-line W-98	White	(Hernández-Sánchez et al., 2006)
	2007	4.51	56	57	Eggshell quality	---	---	(Lichovnikova, 2007)
	2008	4.08	58	73	---	Lohmann	Brown	(Safaa et al., 2008)

2010- 2020	2011	5.00	23	35	Egg production Blood Ca	ISA Brown	Brown	(Pelicia et al., 2011)
	2011	4.25	25	49	Performance	Dekalb	White	(Araujo et al., 2011)
	2011	3.70	40	44	Feed intake, feed conversion	Hisex Brown	Brown	(Vieira et al., 2011)
	2011	3.83	20	36	Shell quality	Hy-line W-36	White	(Valdés Narváez et al., 2011)
	2012	3.51	42	58	---	Hy-line W-36	White	(Pastore et al., 2012)
	2013	3.57	16	18	Egg weight Shell quality	Hisex	Brown	(Rodrigues et al., 2013)
	2016	5.57	70	80	Shell quality	Hy-Line	Brown	(An et al., 2016)
	2019	4.1 – 4.2	72	81	Shell quality	Lohmann	White	(Kakhki et al., 2019)
	2020	4.16	60	72	Performance, Shell quality Haugh units, physiological and immunological status	H&N	Brown	(Attia et al., 2020)

[a]All dietary calcium recommendations given in percentages (%) were converted to g/hen/day based on the reported feed intake of the birds in that publication fed that given percentage of dietary calcium. Papers that proposed calcium recommendations based on regression models or as a dietary percentage without a corresponding feed intake, were not included.
[b] Publication used a variety of strains.

3.0 Chapter 3: Phosvitin

Calcium (**Ca)** metabolism and skeletal health are important for egg production and welfare in laying hens. Dietary Ca is important for optimizing skeletal development in pullets and in mature laying hens to support long-term productivity and eggshell calcification (Jiang and Mine, 2000; Hester et al., 2004; Khanal et al., 2019; Bello et al., 2020). Alternatively, excess dietary Ca can lead to metabolic disorders and impaired Ca metabolism (Siller, 1981; Wideman and Cowen, 1987; Wideman et al., 1993; Guo et al., 2005; Julian, 2005; Guo et al., 2008; Saif et al., 2008), reduced shell quality (Kakhki et al., 2019), and interfere with phytase activity (McCuaig et al., 1972; Van der Klis et al., 1997; Beutler, 2009; Bello and Korver, 2019). Consequently, Ca homeostasis involves a delicate balance of intestinal Ca absorption, bone mineralization and renal excretion to allow maximum performance. Dietary Ca is the most important source of Ca for eggshell formation, while medullary bone is a hen's primary metabolic mineral reserve to support shell calcification and is mobilized and replenished daily throughout the laying cycle. Despite this, structural bone will also be resorbed (Whitehead and Fleming, 2000) over the course of a laying cycle, which is problematic since structural bone formation ceases at the onset of sexual maturity (Whitehead, 2004). This inevitably leads to the gradual loss of structural bone over time (Wilson et al., 1992; Hudson et al., 1993; Wilson et al., 1998; Cransberg et al., 2001; Khanal et al., 2019), unless hens are molted (Yosefi et al., 2003; Kim et al., 2007b). Aging leaves laying hens susceptible to skeletal health problems such as bone fractures, brittleness, weakness, and osteoporosis at the end of subsequent cycles (Rennie et al., 1997; Cransberg et al., 2001; Fleming, 2008).

Phosvitin (**PV**) is an egg yolk phosphoprotein, which in some rodent models may increase dietary Ca availability, absorption, and efficiency of Ca incorporation into bone (Jiang and Mine,

2000; Choi et al., 2005; Liu et al., 2013; Zhong et al., 2016). PV is a strong metal chelator (Taborsky, 1963; Grizzuti and Perlmann, 1973; Grizzuti and Perlmann, 1975; Hegenauer et al., 1979; Castellani et al., 2004), which makes the protein highly resistant to proteolytic enzymes (Goulas et al., 1996; Khan et al., 1999). However, dephosphorylation of PV enhances its susceptibility to proteolysis in vitro, which can result in the formation of bioactive phosphopeptides (Goulas et al., 1996; Jiang and Mine, 2000; Volk et al., 2012; Jie et al., 2018). Native PV and its bioactive phosphopeptides increased the solubility of Ca ions in an in vitro ileum model (Choi et al., 2005), and inhibited the formation of insoluble Ca phosphate in an in vitro intestinal Ca precipitation model (Jiang and Mine, 2000). Additionally, they promoted higher rates of intestinal Ca absorption (Jiang and Mine, 2000) and enhanced efficiency of bone Ca incorporation (i.e., higher bone ash, Ca content, bone mineral density (bone mineral density (**BMD**) and bone mineral content (**BMC**) ratios of Ca/bone weight and Ca/bone ash) in in vivo rodent models (Choi et al., 2005). Despite PV's role in enhancing Ca bioavailability, absorption, and skeletal health in rodent models, the use of PV in laying hens has not been explored to the best of our knowledge. Given the inevitable age-related decreases in dietary Ca absorption and shell quality (Al-Batshan et al., 1994; Cordts et al., 2002; Hansen et al., 2003) and depletion of structural bone in laying hens over time (Cransberg et al., 2001; Fleming, 2008), strategies that support skeletal health are necessary. This is especially true as laying operations worldwide start pursuing longer flock cycles (Bain et al., 2016).

The objective of this study was to evaluate the effects of dietary PV, dephosphorylated phosvitin (**DPV**), and phosvitin peptides (**PVP**) on their ability to protect productivity, shell and bone quality in aged laying hens fed a Ca-reduced diet for 4 weeks. We hypothesized that dietary PV would protect laying hen bone quality via increased dietary Ca absorption and maintenance of

skeletal mineral reserves while preserving productivity and shell quality in end-of-cycle laying hens.

3.1 Materials and Methods

This trial was approved by the Animal Care and Use Committee: Livestock of the University of Alberta, following the Canadian Council on Animal Care guidelines (Canadian Council on Animal Care, 2009). The acclimation and experimental periods were conducted in an environmentally controlled barn at the University of Alberta Poultry Research Centre in Edmonton, Alberta, Canada.

3.1.1 Animals and Housing

One hundred seventy-five healthy Lohmann LSL-Lite laying hens aged 85 woa were selected from the University of Alberta flock. The individual hens were selected based on consistent egg production, body weight (**BW**), and an evident lack of unhealed broken bones. Upon selection, hens were randomly housed individually in a two-tier battery cage system (1,839 cm^2/bird). Feed was provided to each cage using an individual feeder; water was available ad libitum via two nipple drinkers per cage. Water was available ad libitum via two nipple drinkers per cage. During this period, birds were acclimatized to these cages for 5 weeks (from 85 to 90 woa). The hens were fed a corn-soy-canola meal Positive Control diet (PC; 2,807 kcal ME/kg, 3.6% Ca, 0.33% available phosphorus **(avP**), formulated to meet the first 6 limiting amino acids and no restriction on crude protein; with other nutrients according to the Lohmann LSL-Lite management guide (Table 1; Lohmann Tierzucht, 2019) as a mash. During the adaptation period, egg production was recorded daily, and hens were monitored closely for signs of molting (i.e., reduced feed consumption and egg production and a loss of plumage); however, no birds were removed due to molting. Of the original 175 hens, one bird died of unknown causes during the

acclimation period. Before starting the experiment, the 6 hens producing the fewest egg among the remaining birds were removed from the group, leaving 168 hens. Eight birds were then randomly selected and euthanized via cervical dislocation for collection of baseline femur samples. Of the remaining 160 hens, 16 birds per treatment were selected and included throughout the entire duration of the study. The average BW and egg production of all hens at the start of the experiment (90 woa) was 1.67 $\pm$ 0.12 kg and 94.4$\pm$6.54%, respectively. Birds were managed as per the primary breeder management guidelines (Lohmann Tierzucht, 2019). The barn temperature was maintained between 18 and 21°C, and a photoperiod of 16:8D was used. Egg collection and health checks were conducted twice daily.

3.1.3 Experimental Diets

At 90 woa, the selected hens were randomly allotted to one of eight dietary treatments with 16 replicate hens per treatment in a completely randomized design. The PC diet, as described previously, was made in a separate batch with 3.6% Ca (Table 3.1). The negative control (NC) diet was identical to the PC diet except with a 21% reduction in Ca (from 3.6% to 2.84% calculated dietary Ca), achieved by reducing the small and large particle limestone added to the NC basal diet by the same proportion. The amounts of other ingredients were not adjusted, resulting in an approximately 2% greater density of nutrients other than Ca relative to the PC. The Ca reduction in the NC diet was intended to induce a Ca deficiency to test our hypothesis but was limited to 4 weeks to protect bird welfare. The basal NC diet was made separately from the PC basal diet and subdivided to make the different NC-PV product diets containing either 1% or 0.01% (final product concentration) of either PV (PV; 37.4% purity), DPV (DPV;39.5% purity), or PVP (PVP; 39.8%). Different amounts of each PV product were added to account for differences in purity. Feed was provided daily as needed in 100 g increments to each cage's external feeder to minimize

feed wastage due to sorting. At the end of the trial, total feed disappearance was used to calculate average daily feed intake (**FI**; g/d/hen) and feed conversion ratio (**FCR**) (g egg mass/g feed) per hen over the study period. Titanium dioxide (Sachtleben Chemie GmbH., Duisburg, Germany) was added at 0.5% of the experimental diets from day 17 to day 28 of the experiment as an indigestible marker for subsequent determination of Ca and P digestibility. Feed samples were collected at the time of mixing, ground, and stored at -20°C until analysis.

3.2.4 Laying Performance

Individual BW data were measured at the beginning (90 woa) and end of the experiment (28 d; 94 woa). Eggs were collected twice daily, at 9:00 AM and 3:30 PM and hen-day egg production was calculated weekly for each hen and over the entire 28-day period (weeks 1 through 4). Fresh egg weights were measured daily and used to calculate weekly average egg weight per hen. Egg mass was calculated by dividing the total egg mass over the duration of the trial by the total number of days. Average daily FI per hen was calculated by taking the total feed disappearance (total feed added (g) minus total feed removed over the duration of the trial) and dividing it by 28 (the number of days of the experiment). FCR was calculated by dividing the total amount of FI by the total egg mass produced per hen.

3.2.5 Eggshell Quality

Before the experiment (-1 d) and on the final day (28 d) of the experiment, eggs were collected from each hen for the determination of eggshell thickness, specific gravity, and eggshell breaking strength. Shell thickness was assessed using a non-destructive ultrasonic eggshell thickness gauge (ESTG-1, ORKA Food Technology Ltd, West Bountiful, UT) as described by Mwaniki et al. (2018). Eggs were then stored at room temperature overnight (18 h) prior to determination of egg specific gravity and eggshell breaking strength the following day. Specific

gravity (g/cm^3) of each egg produced was measured on the day following collection (0 d and 29 d) via the flotation method (Hamilton, 1982) by immersing eggs in 11 sequential saline solutions ranging from 1.060 to 1.090 specific gravity in increments of 0.002 (Holder and Bradford, 1979). The saline solutions were calibrated before each test. Eggshell breaking strength (kgF**)** was determined using an Instron Materials Tester (Model 4411, Instron Corp., Canton, MA) with Automated Materials Test System software (Version 8.09) as described by Bello and Korver (2019).

3.2.6 Bone Characteristics

Before the experiment (90 woa), eight birds were euthanized for baseline bone quality measurements. At 94 woa, all remaining birds were euthanized via injection with T-61 (0.35 ml/kg BW; Merck & Co., Inc. Kirkland, QC, Canada). All soft tissues were removed from the right and left femurs and the bones were then kept at -20°C for subsequent determination of bone-breaking strength (**BBS**), bone densitometry via dual-energy X-ray absorptiometry (**DEXA**) and ash content.

3.2.6.1 Bone Breaking Strength

Right femurs were thawed at 4°C for a minimum of 24 h before BBS analysis was conducted using the Instron Materials Tester previously used to determine egg breaking strength using the method described by Pongmanee et al. (2020). The compression force that resulted in bone breakage was recorded as BBS (kgf).

3.2.6.2 Bone Ash

Right femurs used in BBS measurements were then cut at 25 and 75% from the proximal epiphysis of the bone's length using a Dremel tool (Model 200, Racine, WI). This divided the bone

into 3 sections: the proximal end (25%), mid-diaphysis (50%), and the distal end (25%). The weight of each bone segment was recorded, then oven-dried at 100°C for 24 h (Despatch Oven Co., Minneapolis, MN), cooled in a desiccator for a minimum of 24 h and weighed again before being ashed in a muffle furnace (30400 Thermolyne Furnace, Dubuque, IA) at 500°C for 48 h as described by Pongmanee et al. (2020). Bone ash content for each segment was calculated as a percentage of the dry bone weight (ash (g)/ dry weight (g)*100).

3.2.6.3 Bone Densiometry.

Left femurs were thawed at 4°C for a minimum of 24 h before determination of bone densitometry using DEXA. Bone samples were then scanned in two groups using the Lunar Prodigy DEXA scanner (GE Healthcare, Madison, WI USA). The densitometer was calibrated according to manufacturer specifications. Bones were then placed directly onto the scanner bed such that the 2-dimensional images of all bones were taken on the frontal plane. The image of each individual bone was circumscribed as discrete regions of interest and were analyzed as using enCORE 2011 software v13.60.033 (GE Healthcare, Madison, WI USA). Radiographic bone mineral density was determined by the same software and is reported in g/cm^2.

3.2.7 Mineral Digestibility

Titanium-supplemented diets and digesta samples were analyzed to calculate apparent ileal digestibility of Ca (**AIDCa**; %) and phosphorus (AIDP; %). At 94 woa, digesta from the distal ileum was collected from each bird euthanized for femur collection. This was done by gently squeezing the distal part of the ileum (posterior two-thirds of the section between Meckel's diverticulum and 2 cm anterior to the ileocecal junction). Samples were then immediately frozen at -20°C. Prior to analyses, digesta were freeze dried for 5 d to remove any moisture. For digesta samples only, two samples from the same treatments were pooled before the grinding to ensure

enough sample to analyze the titanium marker and minerals. Digesta and feed samples were ground using a 1-mm screen in a centrifugal mill (model ZM200; Retsch, Haan, Germany). Feed samples and distal ileum digesta samples were analyzed for dry matter by drying samples at at 110° C for at least 2h (Method 930.15; AOAC, 2006). Titanium dioxide in the digesta and feed samples was analyzed by spectrophotometry at a wavelength of 408 nm with a modified method based on Myers et al. (2004). Samples were then homogenized and transferred into a 0.2 g microcentrifuge tube. Samples (0.07 g) were weighed into 55 mL teflon digestion vessels. Afterwards, 5 mL of Trace Metal Grade nitric acid was added to the sample, before being capped and left overnight. Samples were then digested using a MARS6 microwave digestion system where the temperature was increased over 5.5 minutes to 185 °C and held for 10 minutes. The samples were then diluted with 20 mL of 18.3 MΩ.cm milliQ water. The minerals in the feedstuff and digesta were analyzed via inductively coupled plasma-optical emission spectrometry (ICP-OES; iCAP6300, Thermo Fisher Corp., Cambridge, United Kingdom). For determination of AIDCa and AIDP, the following formula was used:

$$\text{AIDCa (\%)} = 100 - [100 \times (\frac{\%\ marker\ in\ diet\ x\ \%\ component\ in\ the\ digesta}{\%\ marker\ in\ the\ digesta\ X\ \%\ component\ in\ the\ diet})]$$

Calculated Ca uptake was calculated by multiplying Ca digestibility of the paired hen digesta samples by the average Ca intake of those two respective hens. Ca intake was calculated by multiplying the analyzed Ca for each diet by the FI per hen. Calculated Ca uptake was calculated by multiplying the Ca digestibility of the paired hen digesta samples by the average Ca intake of those two respective hens.

3.2.8 Statistical Analysis

The effect of diet was tested in a completely randomized design with 16 replicates (individually caged hen) per treatment, except for digestibility analysis (8 replicates per treatment). Data were analyzed as generalized linear mixed models using the GLIMMIX procedure of SAS 9.4 (SAS Institute Inc, Cary, NC). Models included the main effect of diet, age (i.e., week) and the diet x age interaction. Pairwise differences between means were determined using Tukey's HSD test. Outliers were defined as being more than three standard deviations from the mean and were removed from the data. Data were tested for normality using Shapiro-Wilk and Kolmogorov-Smirnov tests and homogeneity of variances was tested using Levene's test (Leveine, 1961). Variances for all variables were confirmed to be homogenous, however several variables were not normally distributed: hen-day egg production, egg mass, FCR, BBS, specific gravity, eggshell thickness, and apparent ileal digestibility of Ca and P. The preferred distribution assumed in the models for these variables (and the corresponding link function) among the normal, gamma, negative binomial, Poisson, and multinomial distribution (specific gravity only) was selected based on minimization of the Akaike Information Criterion. Models for egg production and egg mass assumed a Poisson distribution as is appropriate for variables consisting of discrete counts. Statistical significance was set at $P \leq 0.05$, while trends were reported where $0.05 < P \leq 0.10$.

3.3 Results

Following the 5-week adaptation period, all hens were confirmed to be in good health and laying well before being randomly allocated to treatments. Additionally, there were no significant differences in egg production, BW or egg quality parameters (shell breaking strength, specific gravity and eggshell thickness) between experimental groups at the beginning of the experiment at 90 woa (data not shown).

3.3.1 Egg Production

During the experimental period, egg production, and egg mass (Table 3.2) were not affected by the diet x age interaction. Additionally, during the adaptation period, egg production for each treatment group (Table 3.2) was above the expected egg production range of 76% to 79% for birds between 87 to 90 woa for Lohmann LSL-Lite layers (Lohmann Tierzucht, 2021d). At 91, 92 and 93 woa, egg production was significantly depressed for birds fed the NC + DPV 1% diet compared to those fed the PC (P=0.002; P<0.001; P=0.002, respectively; Table 3.2). All other groups had egg production that was intermediate to these treatments, and not different from the PC group. By 94 woa, the diet main effect was no longer significant (P=0.095). Over the course of the experiment egg production was significantly lower for the NC + DPV 1% birds compared to the PC, NC + PVP 1% and NC + DPV 0.01% diet-fed hens (P=0.014; Table 3.2). All other groups had egg productions that was intermediate to these treatments. Across dietary treatments, egg production was lower at 94 woa relative to the other weeks (P=0.002; Table 3.2).

3.3.2 Egg Mass

At 91 woa, there was a trend for egg mass to be depressed for birds fed the NC + DPV 1% diet relative to the NC + PVP 0.01% diet fed birds (P=0.084; Table 3.2), whereas egg mass for each of the other dietary treatments was intermediate to, and not different from these two diets. Additionally, over the duration of the experiment (90 to 94 woa), there was a trend for egg mass to be depressed for birds fed the NC + DPV 1% diet relative to the PC diet (P=0.064; Table 3.2). There was no dietary treatment effect on egg mass at 92, 93 nor 94 woa.

3.3.3 Body weight, Feed Intake, Feed Conversion Ratio

Body weight was not affected by dietary treatment, age, nor their interaction (Table 3.3). FI during the experiment was significantly increased for birds fed NC, NC + PVP 1%, NC + PV

0.01%, NC + DPV 0.01%, (ranging from 111.9 ± 2.91 to 116.3 ± 2.91 g/hen/day) compared to birds fed the PC diet (93.6 ± 2.91 g/hen/day; $P<0.001$; Table 3.3). The remaining NC-PV product diets had FI that were intermediate to these treatments. FCR was not affected by dietary treatments ($P=0.955$; Table 3.3).

3.3.4 Digestibility, calcium intake and calcium uptake

At 94 woa, AIDCa was lower (54.91 + 3.46%) in the PC-fed birds compared to hens fed each of the NC-based diets (P<0.001; Table 3.4). AIDP was the highest for the birds fed the NC + PV 0.01% and NC + PVP 0.01% diets (72.88 ± 1.83 and NC + PVP 0.01%, respectively) and lowest for birds fed the PC diet (45.48 ± 2.11%) compared to all other treatments except the hens fed the NC diet (P<0.001; Table 3.4). Calculated Ca intake was highest for the PC diet-fed hens and lowest for the NC + DPV 1% diet-fed birds (P<0.001; Table 3.4). The remaining NC-PV product diets had calculated Ca intakes that were intermediate to these treatments. Calculated Ca uptake was highest for the NC + PVP 0.01% diet fed hens and lowest for PC diet fed hens, while the remaining NC diets were intermediate to these treatments (P<0.001). This included a pattern of increasing calculated Ca uptake for hens fed the NC PV product diets compared to those fed the PC-diet fed hens, except for NC + DPV 1% fed hens.

3.3.5 Shell Quality and Bone Characteristics

Shell thickness, specific gravity and shell breaking strength were not affected by diet, however, there was a nearly significant trend for shell thickness where the baseline measurement had the highest thickness compared to all other treatments (P = 0.088; Table 3.5). Likewise, bone breaking strength, femur bone ash (distal, mid and proximal) and BMD were not affected by dietary treatment (Table 3.5).

3.4 Discussion

The hens in the present study were selected at 85 woa from the PC group (3.6% dietary Ca) of a previous study. The NC diets in the present study were formulated by reducing Ca (both fine and coarse limestone by the same proportion) from the original PC diet. This formulation strategy resulted in an NC diet with a 21% reduction in dietary Ca and small proportional increases in other nutrient levels (~2%; Table 3.1). However, in this pilot study, the much greater difference in dietary Ca levels between the PC and NC diets still allow us to draw valid conclusions about Ca nutrition in laying hens.

Before the start of the experiment at 90 woa, average hen BW was 1.66 $\pm$ 0.011 kg (Table 3.3), which is lower than the management guide range (between 1.70 kg and 1.80 kg) (Lohmann Tierzucht, 2019). There were no mortalities over the duration of the experiment, and all treatment groups, including the NC hens, maintained a consistent BW through the 4-week experiment (Table 3.3). Given that these were older hens, these results demonstrate the increased livability and persistency of lay of modern laying hens, which primary breeding companies have been selecting for with hen health and welfare in mind (Fulton, 2004). Still, it is important to note that we specifically selected healthy, high producing hens for this study, which likely contributed to the hen's livability and persistency of production.

At 94 woa, there was no significant dietary effect on egg production (P=0.095; Table 3.2) or egg mass (P=0.650; Table 2.2), which suggests that the birds were performing well at the end of the experiment. This performance is consistent with the lack of difference in egg production and egg mass observed between the PC and NC diet fed birds over the duration of the experiment (Table 3.2). More specifically, from 90 to 94 woa, egg production of the NC hens (93.6 + 2.50%) and average daily egg mass (58.4 + 1.97g) were each similar to the PC-fed hens and higher relative

to the management guide (73.6 % and 47.3 g, respectively; Lohmann Tierzucht, 2019), which suggests that the NC hens were still performing very well over the duration of the experiment in spite of the reduction in dietary Ca.

The only hens that struggled to maintain a comparable egg production to the PC diet-fed hens over the duration of the experiment were the NC + DPV 1% diet-fed hens, however they appeared to have nearly adapted by 94 woa (P=0.095; Table 3.2). Given that this was the only treatment that showed significantly depressed egg production during the trial at 91 woa (83.0 + 2.28%; P=0.002; Table 3.2) and overall (83.4 + 2.36%; P=0.014; Table 3.2), it is possible that this initial lower production level did not allow the NC + DPV 1% diet-fed birds to overcome the dietary Ca reduction to the same extent as the other NC-diet fed birds.

The NC + DPV 1% fed hens had the lowest FI compared to those fed any of the NC-PV product diets (P<0.001; Table 3.3), suggesting that the NC + DPV 1% birds did not respond to the Ca reduction as completely, via an increase in FI, as the other NC-PV product hens. Instead, an alternative stimulus, stronger than the appetite for Ca expressed by hens in the other NC treatments, led to a lower FI by the NC + DPV 1% hens. This ultimately led to a significantly lower Ca intake (P<0.001; Table 3.4), which negatively affected egg production overall (P=0.014; Table 3.2). Given the high degree of feed sorting noted in this group, it is possible that palatability, was an issue with this diet (Liu et al., 2018). When birds were observed to be sorting feed, as was often the case for the NC + DPV 1% hens, the feed was removed, weighed, and replaced with fresh feed. Since this is the first study feeding dietary PV to laying hens, understanding the effect of palatability is important since laying hens have a highly developed taste system (Ganchrow and Ganchrow, 1985; Kudo et al., 2008; Kudo et al., 2010). It is unclear why palatability was only an issue for the hens fed the NC + DPV 1% diets. In vivo trials in which PVP were added at 0.125 to

0.5% of the diet, offered ad libitum to mice did not result in issues with palatability (Choi et al., 2005). Additionally, intact PV as a natural antimicrobial compound in human diets showed no effect on sensory qualities such as color and odor compared to controls (Palomar et al., 2020) or no effect at all in some cases (Jung et al., 2012). Still, the NC + DPV +1% diet specifically contained DPV at the highest concentration (1% vs. 0.01%), consequently, exploring whether the dephosphorylation process has any effect on the palatability of its products might be a future area of interest.

Each of the other NC-diet fed hens consumed approximately 20 g/hen/day more feed than the PC hens over the 4-week duration of the experiment (P<0.001; Table 3.3). The increase in FI by the NC hens was likely in response to the dietary Ca reduction resulting in an increased appetite for Ca (Wilkinson et al., 2019). Consequently, the NC hens likely increased FI to normalize Ca intake. Still, this increase in FI did not completely compensate for Ca intake, as only the hens fed the NC + PV 0.01% and NC + DPV 0.01% diets were able to reach Ca intake levels similar to that of the PC diet fed hens (Table 3.4). This was likely due to the hen's inability to increase FI beyond the point of satisfying their energy needs (Forbes and Shariatmadari, 1994; Bouvarel et al., 2011). Still, this attempt to increase Ca intake contributed to the ability of the hens fed the NC-based diets (except for the NC + DPV 1% group) to maintain egg production and egg mass to levels equivalent to PC-fed birds (Table 2.2) Unfortunately, since FI was not recorded on a weekly basis, it is impossible to know at what timepoint the hens fed the NC-PV product diets increased FI. Interestingly, however, this increase in FI did not increase FCR for birds fed the NC-based diets, compared to PC-fed birds (P= 0.955; Table 3.3). This suggests that the birds were able to overcome the severe reduction in dietary Ca without sacrificing efficiency. Furthermore, the lack of a statistical difference in egg production between the PC-diet-fed hens and the NC-diet-fed hen may

be due to high variation in the population. With a larger sample size, it is possible that the egg production differences would be statistically significant, however, the present experiment was a pilot study, therefore, only a small number of birds were used.

Overall, with the exception of the NC + DPV 1% hens, production, shell quality and bone quality were largely unaffected by the substantial reduction in dietary Ca. Dietary Ca levels as low as 2.96% fed for three weeks to mid-production laying hens did not decrease eggshell quality compare to dietary Ca levels between 4.49% to 5.06% (Kaur et al., 2013). One contributing factor may relate to the initial bird selection of high-producing, healthy hens from the PC group in a previous experiment. This was intended to reduce variability in egg production during the trial and minimize the risk of attrition due to factors unrelated to treatment, and therefore, does not reflect a typical flock at 90 to 94 woa. Additionally, the purpose of the NC diet was to induce a reduction in performance to assess the ability of the various PV product treatments to prevent the reduction productivity. Consequently, starting with a group of birds with high performance meant that changes to egg production would likely have been attributed to the dietary treatment, rather than other factors, such as age. Ultimately, however, the NC the diets were apparently not clinically deficient in Ca over the 4-week duration of the trial.

Given the adverse effect of age on Ca metabolism (Pelicia et al., 2009), it was assumed that 2.84% dietary Ca (2.93% analyzed dietary Ca) for 4 weeks in the present study would elicit the start of a Ca deficiency and allow the determination of PV's effects. One possibility for the lack of NC-diet effect is that although the dietary Ca level was below published requirements, it still contained more Ca than formulated. Secondly, the duration of the trial was too short to elicit an adverse NC effect. Testing the effect of Ca deficiency in laying hens can require long periods of deprivation to create an overt deficiency, since bone Ca mobilization is one of the first lines of

defense in restoring blood Ca (Wilkinson et al., 2019). In the present study, however, the short trial duration of this pilot study was intended to protect welfare given the advanced age of the birds.

Although dietary Ca is the primary source of Ca utilized for eggshell calcification, skeletal stores act as a secondary source (Mueller et al., 1964; Buss and Guyer, 1984). With a Ca-reduced diet, skeletal Ca stores may be mobilized more extensively (Hester et al., 2004). Despite the early effect of reduced dietary Ca on the productivity of birds fed the NC+ DPV 1% diets, there was no overall dietary effect on BW (Table 3.3), bone breaking strength, bone ash or BMD (Table 3.4). Additionally, shell thickness, specific gravity, and shell breaking strength were not affected by dietary treatment (Table 3.5).

In the context of laying hen bone biology, medullary bone is replenished daily while structural (cortical and trabecular) bone is not, if the hen remains in lay (Fleming et al., 1998). Although medullary bone does not normally contribute substantially to bone strength in laying hens, the volume of medullary bone present has a direct relationship with humeral breaking strength (Fleming et al., 1998). Consequently, this could partially explain the lack of treatment effects on bone ash and breaking strength (Table 3.5). Older hens, such as the ones used in the present study, tend to have greater bone ash at the end of production than at the start due to the accretion of medullary bone, even as structural bone is lost (Whitehead et al., 2000). Therefore, the possible accretion of large amounts of medullary bone prior to the start of the current experiment may have masked changes in structural versus medullary bone reserves over the course of the experiment. Overall, given that the measures of bone quality remained unaffected, some other mechanism, such as increased digestibility of Ca in the NC and NC PV products-fed hens, likely prevented excessive bone Ca mobilization in the short term.

Interestingly, the calculated daily Ca intake of the NC hens (3.33 $\pm$ 0.081 g/hen/day; Table 3.3) was significantly lower than the calculated daily Ca intake of the PC hens (3.69 + 0.083 g/hen/day; Table 3.3). Still, the NC hens showed no visible symptoms of Ca deficiency over the 4 weeks of the experiment. The lack of an effect of the NC diet on shell quality and bone quality parameters also suggest that the reduced Ca level of the NC and NC-PV product diets stimulated the birds to compensate through other mechanisms to meet the requirements for maintenance of performance over the 4-week duration of the experiment. In addition to increased FI, the increase in Ca digestibility (P<0.001; Table 3.4) for the NC and each of the NC-PV product treatments compared to PC also played a role. This also resulted in a higher calculated Ca uptake with the exception of the hens fed the NC + DPV 1% diet (P<0.001; Table 3.4). This increased Ca digestibility appeared to be enough to prevent or delay any adverse effects or reduced dietary Ca on production (with the exception of NC + DPV 1% fed hens), shell quality and bone quality in the 4 weeks of the trial. However, the lack of difference in Ca digestibility between the NC hens and the hens in the NC-PV product groups suggests that dietary PV did not increase Ca digestibility. This makes sense considering that low Ca intake by laying hens itself is known to increase efficiency of Ca absorption (Hurwitz and Bar, 1969; Bar and Hurwitz, 1984; Clunies et al., 1992b), particularly via the transcellular active transport process. This absorption mechanism is saturable and occurs when Ca levels are low or moderate involving the use of epithelial transient receptor potential vanilloid Ca channels (TRPVs; Bar, 2009; Jonchère et al., 2012a; Yi et al., 2015).

Like Ca digestibility, the PC diet-fed hens had the lowest P digestibility compared to the NC and NC PV-product diets. Ca and P requirements are highly linked as the latter is a vital component in skeletal Ca deposition through the formation of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)

with Ca in an approximately 2:1 ratio (de Matos, 2008; Fleming, 2008; Adedokun and Adeola, 2013; Li et al., 2017). An improper dietary calcium:phosphorus ratio can induce physiological responses that reduce laying hen health, shell quality, and productivity (Rama Rao et al., 2006; Selle et al., 2009b; Kebreab and Vitti, 2010; Adedokun and Adeola, 2013; Jiang et al., 2013; Proszkowiec-Weglarz and Angel, 2013; Hamdi et al., 2015; Li et al., 2016; Skřivan et al., 2016; Kakhki et al., 2019). Additionally, high dietary Ca to total P ratios decrease phytase activity in the intestine (McCuaig et al., 1972; Qian et al., 1996; Qian et al., 1997). Consequently, the higher intestinal dietary Ca levels in PC-diet-fed hens, compared to NC-diet-fed hens may have decreased the utilization of P.

Each of the NC-PV product diets (except NC + PV 1%) resulted in increased P digestibility compared to the NC diet. PV itself is highly phosphorylated (~10% phosphorus; (**P**)) (Joubert and Cook, 1958), however, there is no research addressing the effects on P digestibility. Alternatively, PV's role in providing P during embryogenesis is well known. PV serves as the main source of P for bone mineralization during incubation (Richards, 1997) and contains approximately 80% of the yolk's P (Yilmaz and Agagunduz, 2020). During incubation, phosphate is released from PV via alkaline phosphatase-induced dephosphorylation, which initiates changes to the secondary structure of PV (Li et al., 2014). This results in increased P bioavailabilty for mineralization. In the present study it would be interesting to know if the P in PV was released from the NC-PV products diets. Interestingly, however, the NC-PV product diets with the highest P digestibility were two of the low-dose PV products: one with structual modifications of the protein (NC + PVP 0.01%) and one without structual modifications (NC + PV 0.01%). This suggests that high doses and structual modifications of PV may not be essential for the release of PV-derived P, compared

to other metals that PV has very strong bonds with such as Ca, Mg and Fe (Samaraweera et al., 2011).

Although these findings illustrate the resilience of modern layers to reduced dietary Ca, the lack of adverse effects of the NC diet did not allow us to assess the effect of PV on laying hens fed a reduced Ca diety. Dietary Ca typically provides 60 to 75% of the Ca required for eggshell mineralization, with the remaining provided through bone Ca mobilization (Mueller et al., 1964; Buss and Guyer, 1984). The mechanism of action in rodent models suggests that PV increases intestinal absorption of Ca (Choi et al., 2005), which, if also observed in hens, would reduce the need to mobilize bone Ca reserves. In the present study however, there was no difference in Ca digestibility between the NC and NC-PV product diets, which suggests that PV did not increase intestinal absorption in response to reduced dietary Ca, at least in the short term. Additionally, the lack of an NC effect on bone quality suggests that there was no difference in bone mobilization between the treatment groups, particularly the PC-fed hens and the NC-fed hens. The digestibility data and bone data together suggest that there was no need to increase Ca via these mechanisms in response to the reduced dietary Ca and precluded the opportunity to observe a PV effect. Consequently, future research in this field should use a dietary calcium reduction of greater than 21% or extend the duration of feeding the reduced Ca diet to elicit a Ca deficient state.

3.5 Conclusion

In conclusion, birds fed reduced dietary Ca increased FI to increase Ca intake and increased Ca digestibility to overcome the challenge in the short term. This suggests hens have an appetite for Ca and will increase their FI to maintain Ca intake. Considering the cumulative lack of effect of the NC diet on egg production (except for NC + DPV 1% fed hens), shell quality and bone quality, these diets were not deficient in Ca, at least in the short term. Additionally, the lack of

effect of reduced dietary Ca on bone breaking strength, bone ash and BMD indicates that skeletal stores played little to no role in maintaining productivity and shell quality over the 4-week trial duration. A more substantial decrease in dietary Ca or longer experimental period may have decreased performance, shell, and bone quality; however, this approach may compromise hen welfare. Overall, these results suggest that primary breeder feeding guidelines include large safety margins for Ca, and that laying hens can also tolerate large substantial dietary reduction in Ca in the short term. Given the lack of an NC diet effect, we were unable to fully assess the effects of dietary PV, however, increased P digestibility in the NC-PV products diets compared to the NC diet suggests that P was released from PV itself. To fully test the effects of PV in laying hens, future trials should include a longer trial duration or larger reduction in dietary Ca, with bird welfare in mind.

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3.7 Tables

Table 3.1. The ingredients and nutrient composition of positive control and negative control diets fed to laying hens from 90 to 95 weeks of age.

	90-94 wk	
	PC[1]	NC[2]
Ingredients (%)		
Corn	61.98	63.34
Soybean meal	6.21	6.34
Canola meal	10.00	10.22
Oat hulls	2.00	2.04
Wheat bran	4.91	5.02
Canola oil	2.57	2.63
Large particle limestone	5.73	4.40
Small particle limestone	2.87	2.20
Monocalcium phosphate	0.984	1.00
Salt	0.293	0.299
DL-Methionine	0.196	0.200
L-Lysine HCl	0.192	0.196
L-Tryptophan	0.025	0.026
L-Valine	0.025	0.026
HyD premix[3]	0.500	0.51
Vitamin-mineral premix[4]	0.500	0.51
Choline chloride premix[5]	0.500	0.51
Phosvitin products[6] (g/kg)	---	Variable
Calculated nutrient composition		
Crude protein (%)	13.08	13.37
ME (kcal/kg)	2,807	2,869
Dig. Lysine (%)	0.63	0.64
Dig. Methionine (%)	0.41	0.42
Dig. Met.+ Cys. (%)	0.57	0.58
Calcium (%)	3.60	2.84
Total phosphorus (%)	0.58	0.59
Available phosphorus (%)	0.33	0.34

Sodium (%)	0.15	0.15
Analyzed values (as fed)		
Calcium (%)	4.03	2.93
Total phosphorus (%)	0.61	0.54

[1]PC=positive control diet; PC diet was mixed as a single batch.

[2]NC = negative control diet; two batches of the NC diet were mixed for d0 to d23 (average of 3.00% dietary Ca) and one for d23 to d28 (average of 2.86% dietary Ca). The NC diets were subdivided into the NC and the various NC + phosvitin product treatments.

[3]DSM Nutritional Products Ltd., Parsippany, NJ; provided 25-hydroxyvitamin D_3 at 69 μg/kg feed.

[4]Vitamin–mineral premix provided (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.

[5]Provided 100 mg of choline per kilogram of mixed feed.

[6]Phosvitin (PV), dephosphorylated phosvitin (DPPV) and phosvitin peptides (PVH) were mixed into the complete NC diet at the following amounts: 2.67 g/100g for PV 1%, 2.53 g/100g for DPPV 1%, 2.51 g/100g for PVH 1%, 0.267 g/100g PV 0.01%, 0.253 g/100g DPPV 0.01% and 0.251 g/100g PVH 0.01%.

Table 3.2 Effect of dietary phosvitin, dephosphorylated phosvitin and phosvitin peptides on hen-day egg production and egg mass from 90 to 94 weeks of age

				Hen-day egg production (%)[1,2] ± SEM[3]			
Weeks of age		87 to 90	91	92	93	94	90 to 94
Diets[4]	n						
PC	16	96.13 ± 2.45	97.3 ± 2.47^{A}	99.1 ± 2.49^{A}	98.2 ± 2.48^{A}	93.0 ± 2.41	96.8 ± 2.46^{A}
NC	16	94.35 ± 2.43	92.0 ± 2.40^{AB}	94.6 ± 2.43^{A}	93.3 ± 2.49^{AB}	90.0 ± 2.45	93.6 ± 2.50^{AB}
NC + PV 1%	16	93.16 ± 2.41	94.6 ± 2.43^{A}	97.3 ± 2.47^{A}	92.0 ± 2.40^{AB}	89.8 ± 2.37	93.3 ± 2.42^{AB}
NC + DPV 1%	16	92.86 ± 2.40	83.0 ± 2.28^{B}	77.7 ± 2.20^{B}	83.9 ± 2.29^{B}	83.6 ± 2.29	83.4 ± 2.36^{B}
NC + PVP 1%	16	97.62 ± 2.47	94.6 ± 2.43^{A}	92.0 ± 2.40^{A}	92.9 ± 2.41^{AB}	89.8 ± 2.37	94.5 ± 2.51^{A}
NC + PV 0.01%	16	94.35 ± 2.42	96.4 ± 2.45^{A}	92.9 ± 2.41^{A}	92.0 ± 2.40^{AB}	84.4 ± 2.30	91.2 ± 2.39^{AB}
NC + DPV 0.01%	16	93.75 ± 2.42	94.6 ± 2.43^{A}	95.5 ± 2.44^{A}	98.2 ± 2.48^{A}	88.3 ± 2.35	94.0 ± 2.42^{A}
NC + PVP 0.01%	16	93.75 ± 2.42	92.9 ± 2.41^{AB}	93.7 ± 2.42^{A}	90.2 ± 2.37^{AB}	86.7 ± 2.33	90.7 ± 2.38^{AB}
	128	94.48 ± 1.17^{X}	93.5 ± 1.16^{X}	93.2 ± 1.16^{XY}	93.3 + 1.17^{XY}	88.7 + 1.14^{Y}	88.7 + 1.14^{Y}
Source of variation		----------	----------	*P-value*	----------	----------	----------
Diet		0.877	0.002	<0.001	0.002	0.095	0.014
Diet							<0.001
Age			-	-	-	-	0.002
Diet x Age	-		-	-	-	-	0.491
				Average daily egg mass (g/hen/day) [1,5]+SEM[3]			
Diets[4]	n						
PC	16	-	61.1 ± 1.95^{ab}	63.2 ± 1.99	61.7 ± 1.96	58.4 ± 1.91	61.0 ± 1.95^{a}
NC	16	-	59.2 ± 1.92^{ab}	59.6 ± 1.99	58.0 ± 1.97	56.7 ± 1.94	58.4 ± 1.97^{ab}
NC + PV 1%	16	-	58.8 ± 1.92^{ab}	62.6 ± 1.98	58.8 ± 1.91	59.1 ± 1.92	59.8 ± 1.93^{ab}

NC + DPV 1%	16	-	52.8 ± 1.88[b]	54.5 ± 1.97	54.1 ± 1.90	55.6 ± 1.86	52.2 ± 1.81[b]
NC + PVP 1%	16	-	59.2 ± 1.92[ab]	58.8 ± 1.98	58.1 ± 1.91	61.0 ± 2.02	59.3[ab] ± 1.99 [ab]
NC + PV 0.01%	16	-	58.7 ± 1.92[ab]	60.2 ± 2.00	61.0 ± 2.01	57.4 ± 1.96	57.7[ab] ± 1.90 [ab]
NC + DPV 0.01%	16	-	58.7 ± 1.91[ab]	59.0 ± 1.92	61.0 ± 1.95	58.1 ± 1.91	59.2[ab] ± 1.92[ab]
NC + PVP 0.01%	16	-	61.7 ± 2.03[a]	59.3 ± 1.93	57.9 ± 1.97	57.1 ± 2.02	56.8 ± 1.88 [ab]
	128		58.7 ± 0.73	59.6 ± 0.75	58.8 ± 0.74	57.9 ± 0.74	58.0 ± 0.73
Source of variation		----------			*P-value*		----------
					-		
Diet			0.084	0.108	0.152	0.650	0.064
Diet							<.0001
Age	-		-	-	-		0.4802
Diet x Age	-		-	-	-		0.9944

[A-B or X-Y] Treatment means with no common superscript within a column differ significantly (P≤0.05). Uppercase letters indicate P ≤ 0.01.

[1] Means of 16 replicates of 1 hen for each treatment.

[2] Arcsine transformation was used before statistical analysis; egg production data are original values in percent.

[3] SEM = standard error of the mean.

[4] PC= positive control with 3.60% Ca and 0.33% aP (with other nutrients according to the Lohmann LSL-Lite management guide); NC = negative control with 2.84% (the PC diet with a 21% reduction in Ca); NC + PV 1%, NC + DPV 1%, NC + PVP 1%, the NC diet supplemented with 1% phosvitin (PV), dephosphorylated phosvitin (DPPV) or phosvitin peptides (PVH); NC + PV 0.01%, NC + DPV 0.01%, NC + PVP 0.01%, the NC diet supplemented with 0.01% phosvitin (PV), dephosphorylated phosvitin (DPPV) or phosvitin peptides (PVH).

[5] Egg mass weights was calculated from 90 to 94 weeks.

Table 3.3 Effect of dietary phosvitin, dephosphorylated phosvitin and phosvitin peptides on BW, FI and FCR from 90 to 94 weeks of age

		Body weight[1] kg ± SEM[3]		Feed intake g/hen/day ± SEM[3]	FCR feed (g) : egg mass (g) ± SEM[3]
Diets[4]	n	90 woa	94 woa		
PC	16	1.63 ± 0.032	1.63 ± 0.032	93.6 ± 2.91^{C}	1.53 ± 0.310
NC	16	1.65 ± 0.032	1.62 ± 0.032	113.2 ± 2.91^{A}	2.18 ± 0.369
NC + PV 1%	16	1.67 ± 0.032	1.66 ± 0.032	108.3 ± 2.91^{AB}	1.81 ± 0.337
NC + DPV 1%	16	1.67 ± 0.032	1.66 ± 0.032	97.2 ± 2.91^{BC}	1.90 ± 0.345
NC + PVP 1%	16	1.69 ± 0.032	1.69 ± 0.032	111.9 ± 2.91^{A}	1.98 ± 0.352
NC + PV 0.01%	16	1.64 ± 0.032	1.64 ± 0.032	116.3 ± 2.91^{A}	2.03 ± 0.356
NC + DPV 0.01%	16	1.70 ± 0.032	1.70 ± 0.032	112.6 ± 2.91^{A}	1.91 ± 0.345
NC + PVP 0.01%	16	1.67 ± 0.032	1.63 ± 0.032	109.5 ± 2.91^{AB}	1.96 ± 0.350
	128	1.66 ± 0.011	1.65 ± 0.011		
Source of variation		----------*P-value*----------			
Diet		0.823	0.502	<0.001	0.955
Diet		0.246			
Age		0.547		---	---
Diet*Age		0.998		---	---

A-B Treatment means with no common superscript within a column differ significantly ($p<0.05$). Uppercase letters indicate $P \leq 0.01$.

1 BW was recorded at 90 and 94 weeks from all birds.

2 Calculated as calculated by multiplying Analyzed Ca (%) in the feed by feed disappearance.

3 SEM = standard error of the mean.

4 PC= positive control with 3.60% Ca and 0.33% aP (with other nutrients according to the Lohmann LSL-Lite management guide); NC = negative control with 2.84% (the PC diet with a 21% reduction in Ca); NC + PV 1%, NC + DPV 1%, NC + PVP 1%, the NC diet supplemented with 1% phosvitin (PV), dephosphorylated phosvitin (DPPV) or phosvitin peptides (PVH); NC + PV 0.01%, NC + DPV 0.01%, NC + PVP 0.01%, the NC diet supplemented with 0.01% phosvitin (PV), dephosphorylated phosvitin (DPPV) or phosvitin peptides (PVH).

Table 3.4 Effect of dietary phosvitin on apparent ileal digestibility of calcium (AIDCa) and phosphorus (AIDP) in laying hens at 94 weeks of age[1]

Diets[2]	AIDCa % ± SEM[4]	AIDP (%) % ± SEM[4]	Calculated Ca intake[3] g/hen/day ± SEM[4]	Calculated Ca uptake g/hen/day ± SEM[4]
PC	54.91 ± 3.46^{B}	45.48 ± 2.11^{E}	3.69 ± 0.083^{A}	2.02 ± 0.066^{D}
NC	80.24 ± 2.99^{A}	52.87 ± 1.83^{DE}	3.33 ± 0.081^{B}	2.67 ± 0.064^{AB}
NC + PV 1%	78.76 ± 2.99^{A}	59.96 ± 1.83^{CD}	3.09 ± 0.081^{BC}	2.43 ± 0.064^{BC}
NC + DPV 1%	80.99 ± 2.99^{A}	63.61 ± 1.83^{BC}	2.82 ± 0.083^{C}	2.29 ± 0.066^{CD}
NC + PVP 1%	84.34 ± 2.99^{A}	66.82 ± 1.83^{ABC}	3.32 ± 0.081^{B}	2.80 ± 0.064^{A}
NC + PV 0.01%	84.60 ± 2.99^{A}	72.88 ± 1.83^{A}	3.43 ± 0.081^{AB}	2.90 ± 0.064^{A}
NC + DPV 0.01%	81.82 ± 2.99^{A}	68.53 ± 1.83^{AB}	3.40 ± 0.081^{AB}	2.78 ± 0.064^{A}
NC + PVP 0.01%	86.76 ± 2.99^{A}	72.76 ± 1.83^{A}	3.17 ± 0.081^{BC}	2.75 ± 0.064^{A}
P-value	<0.001	<0.001	<0.001	<0.001

$^{A-B}$ Treatment means with no common superscript within a column differ significantly (P<0.05). Uppercase letters indicate P ≤ 0.01. [1]Each dietary treatment mean was calculated from 16 birds at 94 wk of age.

[2] PC= positive control formulated to contain 3.60% Ca and 0.33% aP (with other nutrients according to the Lohmann LSL-Lite management guide); NC = negative control (the PC diet with Ca reduced by 0.76% of the diet); NC + PV 1%, NC + DPV 1%, NC + PVP 1%, the NC diet supplemented with 1% phosvitin (PV), dephosphorylated phosvitin (DPPV) or phosvitin peptides (PVH); NC + PV 0.01%, NC + DPV 0.01%, NC + PVP 0.01%, the NC diet supplemented with 0.01% phosvitin (PV), dephosphorylated phosvitin (DPPV) or phosvitin peptides (PVH).

[3] Calculated Ca intake was calculated by multiplying the analyzed Ca for each diet by the feed intake per hen.

[4] Calculated Ca uptake was calculated by multiplying Ca digestibility of the second batch of NC-based diets of each pooled digesta sample by the average Ca intake of those two respective hens.

[4]SEM = standard error of the mean.

Table 3.5 Effect of dietary phosvitin, dephosphorylated phosvitin and phosvitin peptides on egg quality and bone quality parameters at 94 weeks of age

		---------Eggshell quality parameters[1]---------			----------Bone quality parameters[1]----------				
		Shell Thickness	Specific gravity	Shell Breaking Strength	Bone Breaking Strength	Distal Bone Ash[2]	Middle Bone Ash[2]	Proximal Bone Ash[2]	Bone Mineral Density[3]
		mm ± SEM[4]	g/cm^3 ± SEM[4]	----------kgf ± SEM[4]---------		----------%[2] ± SEM[4]----------			g/cm^2 ± SEM[4]
Diets[5]	n								
Baseline	8	0.35 ± 0.007	1.073 ± 0.0017	2.91 ± 0.53	18.6 ± 1.81	51.3 ± 1.92	65.0 ± 1.35	52.3 ± 2.00	---
PC	16	0.33 ± 0.005	1.072 ± 0.0012	3.25 ± 0.41	18.8 ± 1.32	51.9 ± 1.36	66.4 ± 0.96	52.9 ± 1.42	0.313 ± 0.0278
NC	16	0.32 ± 0.005	1.076 ± 0.0013	3.94 ± 0.41	18.9 ± 1.28	52.4 ± 1.40	65.3 ± 0.99	52.3 ± 1.46	0.267 ± 0.0278
NC + PV 1%	16	0.32 ± 0.005	1.073 ± 0.0013	4.00 ± 0.45	16.7 ± 1.28	51.1 ± 1.40	64.6 ± 0.96	52.3 ± 1.42	0.260 ± 0.0278
NC + DPV 1%	16	0.33 ± 0.006	1.072 ± 0.0013	2.86 ± 0.41	17.6 ± 1.28	54.1 ± 1.40	66.4 ± 0.96	54.6 ± 1.42	0.301 ± 0.0278
NC + PVP 1%	16	0.33 ± 0.005	1.074 ± 0.0012	3.49 ± 0.40	16.9 ± 1.28	54.5 ± 1.40	67.7 ± 0.99	56.0 ± 1.46	0.295 ± 0.0278
NC + PV 0.01%	16	0.32 ± 0.005	1.070 ± 0.0013	2.84 ± 0.45	17.4 ± 1.32	53.1 ± 1.45	66.8 ± 1.02	54.0 ± 1.51	0.311 ± 0.0278
NC + DPV 0.01%	16	0.32 ± 0.005	1.073 ± 0.0013	3.61 ± 0.45	18.9 ± 1.28	53.7 ± 1.36	67.0 ± 0.99	54.5 ± 1.42	0.309 ± 0.0278
NC + PVP 0.01%	16	0.32 ± 0.005	1.074 ± 0.0013	2.91 ± 0.45	18.7 ± 1.32	51.3 ± 1.40	65.5 ± 1.02	52.6 ± 1.46	0.290 ± 0.0278

Source of variation	----------*P-value*----------							
Diet	0.088	0.179	0.354	0.876	0.587	0.428	0.428	0.819

[a-c] Treatment means with no common superscript within a column differ significantly ($p \leq 0.05$). Uppercase letters indicate $P \leq 0.01$.

[1] Dietary treatment means for shell quality and bone quality parameters are averaged over 90 to 94 wk of age.

[2] Bone ash for each bone segment (distal, middle and proximal) refers to the ash content (in g) relative to the total dry bone weight (g) of that segment

[3] Two-dimensional projection of mass densities as measured by dual energy X-ray absorptiometry.

[4] SEM = standard error of the mean.

[5] PC= positive control with 3.60% Ca and 0.33% aP (with other nutrients according to the Lohmann LSL-Lite management guide); NC = negative control with 2.84% (the PC diet with a 21% reduction in Ca); NC + PV 1%, NC + DPV 1%, NC + PVP 1%, the NC diet supplemented with 1% phosvitin (PV), dephosphorylated phosvitin (DPPV) or phosvitin peptides (PVH); NC + PV 0.01%, NC + DPV 0.01%, NC + PVP 0.01%, the NC diet supplemented with 0.01% phosvitin (PV), dephosphorylated phosvitin (DPPV) or phosvitin peptides (PVH).

4.0 Chapter 4: A meta-analysis of the effects of dietary Ca on performance, eggshell quality and bone quality in laying hens

4.1 Introduction

Calcium (**Ca**) is essential for daily eggshell calcification in laying hens. Dietary Ca provides approximately 60% to 75% of the Ca needed for eggshell formation, while skeletal Ca reserves provide the remaining 25% to 40% (Driggers and Comar, 1949; Joyner et al., 1987). In total, laying hens need 2 to 3 grams of Ca per eggshell, equivalent to 10 to 15% of a hen's total body Ca (Clunies et al., 1992b; Newberry et al., 1999; de Matos, 2008).

Despite the significant role Ca plays in egg production, there have been a wide range of recommendations since the last NRC Nutrient Requirements for Poultry (9th Revised Edition), was published in 1994. In 1994, the NRC recommendation was lowered from the previous requirement of 3.74 g/hen/d (NRC, 1984) to 3.25% Ca in the diet per hen/d, which corresponds to 3.25 g/day/hen for Single Comb White Leghorn hens and 3.6 g/day/hen for Brown egg-laying hens due to differences in feed intake (FI; NRC, 1994). Some subsequent publications have agreed with the NRC (1994) recommendations (Roland et al., 1996; Bar et al., 2002; Hernández-Sánchez et al., 2006; Rodrigues et al., 2013) while others have suggested that levels between and 3.34 to 5.57 g/hen/d increase shell quality (Ahmad et al., 2003; Hernández-Sánchez et al., 2006; Lichovnikova, 2007; Valdés Narváez and Pro Martínez, 2011; An et al., 2016; Kakhki et al., 2019), productivity (Narváez-Solarte et al., 2006; Araujo et al., 2011; Pelicia et al., 2011; Vieira et al., 2011) or a combination of those parameters (Keshavarz, 2003; Rao et al., 2003; Castillo et al., 2004; Safaa et al., 2008; Rodrigues et al., 2013; Attia et al., 2020).

Although various publications exploring laying hen Ca requirements exist, several moderating factors (i.e., breed, housing, stage of production, environment, and other nutrients)

complicate the interpretation of the research results. Additionally, the intense genetic selection of laying hens for increased persistency of production, skeletal health, and shell quality over recent decades has likely also changed Ca utilization in birds (Fulton, 2012). This selection likely contributes to the variations or changes in Ca requirements reported research across various studies (Fulton, 2012; Baak, 2020). This has been commented on by many researchers (Edwards, 1983; Bolden and Jensen, 1985; Shafey et al., 1990; Hurwitz et al., 1995; Rao et al., 2003; Safaa et al., 2008; Applegate and Angel, 2014; van der Klis and Vinyeta, 2015; Bain et al., 2016).

The objective of this meta-analysis was to compile the dose-response effects of varying levels of dietary Ca on performance, shell quality and bone quality parameters from various publications while accounting for random sources of variation. To capture the impact of commercial genetic selection of laying hens over the years, research papers published between 1980 and 2021 were used. We hypothesized that genetic selection for increasing productivity by primary breeding companies in recent years has made Ca utilization more efficient thus reducing commercial laying hen Ca requirements of modern commercial laying hens expressed as a percentage of the diet.

4.2 Materials and Methods

This study was based on data obtained from the literature and did not require animal ethics approval.

4.2.1 Data Sourcing

Publications included in this meta-analysis were found using Google Scholar, Web of Science, Scopus, AGRIS, DOAJ, SciELO. Key words used while searching were the following: laying hens, dietary Ca, Ca, requirements, performance, egg production, shell quality, eggshell

quality, bone strength and bone quality. The reference lists of these publications were also used to source more potential publications.

4.2.2 Description of the Data Set

Publications from the initial search were selected based on the following criteria: 1) papers published between 1980 and 2021; 2) use of commercial (i.e. non-indigenous and non-regional) white or brown laying hens in production (first or second cycle); 3) dietary Ca levels (minimum of two levels) used as a main effect; 4) dietary Ca levels were constant and clearly defined for the experimental period during which parameters were reported; 5) the publication reported the data of at least one dependant and random variable of interest (Tables 4.1 and 4.2) or the parameter's data could be calculated from the reported data (See Appendix A). Based on these criteria, a data set containing 791 observations was compiled from 57 papers published between 1981 and 2020 and was used in the meta-analysis (Table 4.3). Summary statistics for the data used in the study are shown in Table 4.1. Publications that reported the effects of another dietary factor (i.e., Ca particle size) were included, if that factor was clearly defined for the time frame during which parameters were reported.

4.2.3 Defining Dependent, Random and Response Variables of Interest

Dietary Ca level was the independent variable of interest. The expression of dietary Ca level used in the model was selected based on the following order of preference: analyzed Ca (% or g/kg) followed by formulated or calculated Ca (% or g/kg). Random variables were factors that differed between publications and may have been expected to modulate the effect of the independent variable on a dependant variable (Table 4.2). Dependent variable categories included productivity, mineral intake, production efficiency, shell quality and bone quality parameters. Random and response variables of interest were assessed after entering the experimental details

and data from approximately half of the initial group of publications. This resulted in a detailed and comprehensive list of approximately 160 potential random and 124 potential response variables to be considered. Under-reported random and response variables were removed from the list of potential variables of interest. Additionally, random variables with very few descriptive categories across papers (e.g., cage type) were eliminated and accounted for under a broader category (e.g., housing type) to reduce redundancies. Once these adjustments were made, the remaining random and response variables were evaluated and were eliminated by consensus among the authors based on their relevance in fulfilling the objectives of the meta-analysis. After these candidate variables were determined, the experimental details and data from the remaining publications were compiled into the data set. A publication only needed to report on one of the random variables of interest (Table 4.2) to be included on the analysis.

4.2.4 Data Entry

Data from each publication were entered into a spreadsheet. Within each publication, a separate record line (i.e., row in the spreadsheet with each of the different categories as columns) was made for each treatment applied to an experimental unit (i.e., hen or group of hens) over a period-specific time. The reference software EndNote (Clarivate, Philadelphia, PA) assigned each publication with a unique identifier (Record No.), which was used as a random variable to account for systematic bias within publications. Data that represented binary states were specified as 0 or 1. Categorical data with more than 2 categories was entered using letter codes and the code UR was used if the variable was unreported. For numeric data the cell was left blank if it was unreported. Standard errors of the mean were not captured, as they would not be used in the regression model. If response variables were reported in a range (e. g., Ca intake of 3.40 to 3.72

g/hen/d) for an experimental group, then the average (i.e., 3.56 g/hen/day) was calculated for that record.

For all variables, the original units were recorded and converted to a common standard unit suitable for analysis and publication (Appendix A). Egg production was reported in percentages (i.e., hen-day or hen-housed egg production). If hen-housed egg production was reported without mortality, estimating hen-day egg production was not possible. In this case hen-housed egg production was combined into the same column as hen-day egg production as this was the best estimate. Additionally, feed conversion ratio (FCR) values reflecting feed (g or kg)/kg eggs were combined since the units both cancel, while kg feed:dozen eggs was entered separately. Specific gravity data reported in different units (i.e., g/cm^3 or g/mL of H_2O) or reported with no units were combined in the same column. Response variables measured by different machines or techniques (i.e., egg or bone breaking strength and shell thickness) were combined in the same column. Bone ash was only included if it reflected moisture-free and fat-free (defatted) bone ash. Given the lack of consensus in defining fine and large particle sizes and chronic underreporting of Ca particle sizes (mm), Ca particle size was not reported as continuous numerical data. Instead, Ca particle size was reported in discrete categories based on how particle size was described by the publication (i.e., Fine or Ground, Medium, Coarse, or Combination).

4.2.5 Statistical Analysis

A meta-analytical approach was used for analysis since the dataset was compiled from multiple publications. The different publications included in the data set were uniquely identified and data were analyzed using the mixed procedure of SAS (version 9.4. SAS Institute Inc., Cary, NC, 2016). Each dependant variable response was estimated using:

$$Y = \alpha + \beta DietaryCaLevel + \gamma_i RandomVariable_i + e,$$

where Y is the value of the dependent variable in publication; β is the regression coefficient for dietary Ca level; γ_i is the regression coefficient for the random variables included in the model and *e* is the residual error. Model level significance was evaluated using the Type 3 Sum of Squares *P*-value. Effects were reported as significant where $P \leq 0.05$, in which case the hypothesis that the fixed and random variables caused no differences in the dependant variables was rejected.

4.2.6 Random Variables Included in Each Regression Model

Random variables that were biologically relevant to the objectives of the meta-analysis were categorized as continuous (regression) or discrete (categorical) random variables (Table 4.2). Time was reported in years with the *Year* 2000 as zero to scale time closer to the present and avoid a large adjustment to the intercept of the prediction model. The final list of the random variables considered for inclusion in the model was chosen from a more extensive, initial list of potential variables. Ultimately, the final list included only those random variables that decreased the Bayesian Information Criterion (**BIC**) by one or more units when added systematically to the model. For each response variable, any random variable that did not improve the model according to this criterion was eliminated from the regression model.

4.2.7 Selection of Regression Model

The random variables that improved each response variable model were used to generate all possible combinations of random variables without repetition. This process was to determine which combination of random variables resulted in the best model (lowest BIC). If multiple combinations of random variables resulted in models with the same lowest BIC, then the most

parsimonious model (lowest BIC and least number of random variables) was chosen. The list of final moderator variables used in each regression model are summarized in Table 4.5.

4.3 Results

4.3.1 Productivity, Mineral Intake and Efficiency Parameters

Calculated Ca intake and FI both showed a positive relationship with changing levels of dietary Ca (Table 4.6). The regression model for FI showed an increase (1.54 g/hen/d) in FI per 1% unit increase in dietary Ca (P<0.001; Table 4.6) between 0.048% dietary Ca and 6.46% dietary Ca. Additionally, the regression model for Ca intake showed an increase (1.04 g/hen/d) in Ca intake per 1% unit increase in dietary Ca (P<0.001; Table 4.6) between 0.048% dietary Ca and 6.46% dietary Ca. Conversely, both FCR parameters had a negative relationship with changing levels of dietary Ca. The regression model for FCR showed a decrease of 0.022 kg feed per kg egg (P=0.009, Table 4.6) between 0.8% dietary Ca and 5.5% dietary Ca. Additionally, FCR decreased by 0.051 kg feed per dozen eggs (P=0.046; Table 4.6) per 1% unit increase in dietary Ca between 0.8% dietary Ca and 5.25% dietary Ca. For each of the FCR parameters, *Time* had a significant effect as a random variable. For FCR there was a decrease of 0.014 kg feed per egg per year (P=0.001) and increase of 0.016 kg feed per dozen eggs per year (P=0.033; Table 4.6). There was no relationship between dietary Ca level (%) and P intake, egg mass, egg weight or egg production.

4.3.2 Eggshell Quality Parameters

Between 0.8% and 6.46% dietary Ca, the regression models showed a linear absolute percentage increase of 0.24% in shell weight per 1% unit increase in dietary Ca (P<0.001; Table 4.6) and a 0.0075 mm increase in eggshell thickness per 1% unit increase in dietary Ca (P<0.001; Table 4.6). There was a 0.0015 unit increase in egg specific gravity per 1% unit increase in dietary

Ca (P<0.001; Table 4.6) between 0.8% and 5.5% dietary Ca and a 1.23 N increase in egg breaking strength per 1% unit increase in dietary Ca (P=0.016; Table 4.6) between 2.62% and 4.5% dietary Ca.

4.3.3 Bone Quality Parameters

For bone breaking strength, there was a linear increase of 12.9 N in bone-breaking strength per 1% unit increase in dietary Ca (P<0.001; Table 4.6) between 0.8% and 6.46% dietary Ca. Additionally, *Time* as a random variable showed that bone breaking strength increased by 1.97 N for each year (P<0.001). Bone ash showed a linear absolute percentage increase of 1.03% in bone ash per 1% unit increase in dietary Ca (P=0.002; Table 4.6) between 0.8% and 5.5% dietary Ca. Bone phosphorus (%) and bone Ca (%) each showed no significant relationship with dietary Ca level.

4.4 Discussion

4.4.1 Productivity

4.4.1.1 FI & Calculated Ca intake.

Feed intake and calculated Ca intake both increased with increasing levels of dietary Ca within the ranges studied. Few studies have reported an increase in FI as dietary Ca level increases (Frost and Roland, 1991; Chandramoni et al., 1998); however, the meta-analysis shows an increase of 1.53 g/hen/day of feed for every 1% unit increase in dietary Ca between 0.048% to 6.46% dietary Ca. Usually, in trials with varying levels of dietary Ca, high FI is demonstrated by hens fed the lowest dietary Ca to compensate for a Ca deficiency (Ahmad et al., 2003). This phenomenon usually manifests as a quadratic effect where the highest FI is associated with the most deficient diet and FI slowly decreases as it approaches more optimal dietary Ca levels (Scott

et al., 1971; Ahmad et al., 2003; Narváez-Solarte et al., 2006). In these studies, the low levels of dietary Ca had induced a deficiency, as indicated by a break point in egg production unlike the regression analysis in the present meta-analysis. The present analysis, however, did not show a quadratic effect and disagrees with research showing dietary Ca does not affect FI (MacIntyre et al., 1963; Harms and Waldroup, 1971; Bar et al., 2002; Pelicia et al., 2009a; Świątkiewicz and Arczewska, 2010; Araujo et al., 2011; Pelicia et al., 2011; An et al., 2016). Instead, the linear relationship between FI and dietary Ca in the present analysis suggest that the practical range of dietary Ca that should be fed to hens is included within the range reported (0.048% to 6.46%). Additionally, to see a breakpoint in the relationship more extreme levels of dietary Ca would be needed to modify the linear relationship between dietary Ca and FI observed. If the high levels of dietary Ca included in the analysis (up to 6.46% dietary Ca) were not sufficiently excessive to demonstrate a breakpoint, then hens are likely compensating via mechanisms such as decreased Ca absorption efficiency and increased Ca excretion to maintain Ca homeostasis (De Vries et al., 2010).

In choice feeding systems, laying hens can select their own diets to meet their nutritional requirements (Henuk and Dingle, 2019; Wilkinson et al., 2019). The ability for laying hens to selectively consume a Ca-rich feedstuff to meet its requirements is known as a Ca-specific appetite (Roura et al., 2013). This specific appetite is exhibited under Ca-deficient conditions and has been described in laying hens as a learned preference, rather than an unconscious homeostatic control mechanism (Hughes and Wood-Gush, 1971). This is because the bird itself must become Ca-deficient before it exhibits a change in behaviour (i.e., appetite) by learning to select for the appropriate nutrient (i.e. Ca) (Wood-Gush and Kare, 1966). In the present study, data from studies that used at least two different levels of dietary Ca were included. However, among the

publications that reported FI and Ca intake, only a small proportion of the data (3.5%) used severe deficiencies in dietary Ca levels (<1% dietary Ca), that would have induced a Ca-deprived state (Taylor and Moore, 1954; De Bernard et al., 1980; Oursler et al., 1991; Clunies et al., 1992a; Elaroussi et al., 1994; Helfrich and Ralston, 2003; Jiang et al., 2019; Jansen et al., 2020). This is understandable as severe deficiencies are generally avoided in experimental designs. Instead, it is more likely that the increase in FI per 1% unit increase in dietary Ca observed in the present study reflects birds eating to meet other nutritional needs (Classen, 2017), rather than a specific appetite for Ca. For example, when birds eat according to the energy requirements, an antagonistic relationship between dietary Ca and the energy value of a diet may occur. High dietary Ca decreases the energy value of the diet via the chelation of lipids, thus making them unavailable for absorption (Pepper et al., 1955; Edwards et al., 1960). Although unproductive, this phenomenon would drive birds to increase FI with increasing levels of dietary Ca to meet their energy requirements and may reflect the increase in FI with increasing dietary Ca levels observed in the present study. This increase in FI would also contribute to an increase in all nutrients, such as Ca in the present study.

4.4.1.2 FCR.

Since egg production (output) was not affected by dietary Ca levels, but FI (input) increased with increasing dietary Ca levels within the range studied, it was expected that FCR would increase with increasing levels of dietary Ca. Instead the results showed that both FCR parameters (kg feed:kg eggs and kg feed:dozen eggs) decreased with increasing levels of dietary Ca. These results agree with other studies showing decreased FCR with increasing levels of dietary Ca (Keshavarz, 1996; Safaa et al., 2008). Alternatively, others have reported quadratic effects between dietary Ca levels and FCR. For example, Narváez -Solarte et al. (2006) reported that FCR

decreased as dietary Ca increased from 2.6 to 3.8%, but beyond 3.8% there was no change; additionally, Pelicia et al., 2009 found that FCR decreased as dietary Ca increased from 3.0% to 3.47% but increased between 3.47% and 4.21%. While FCR did not show a quadratic effect in the present analysis, the analysis took various experiments into consideration and thus included a broader range of dietary Ca of 0.8% to 5.25% of the diet.

Time was a significant continuous random variable for FCR parameters. *Time* was included as a random variable to account for the impact of genetic selection on production parameters in laying hens over time. FCR has historically been an important selection trait for primary breeding companies (Flock, 1998) due to escalating feed cost and the need to minimize animal agriculture's environmental impact (Hume et al., 2011). The high reproductive capacity and short generation intervals associated with poultry, coupled with the moderate heritability of feed efficiency (Wolc et al., 2013; Yuan et al., 2015) explain why in the present analysis *Time* significantly decreased FCR parameters (i.e., the birds became more efficient through the years covered in the meta-analysis).

4.4.2 Eggshell Parameters

4.4.2.1 Shell Weight, Eggshell Thickness, Egg Specific Gravity, and Egg Breaking Strength.

Our results agree with the well-documented literature showing the positive effect of increasing Ca levels on shell weight (Clunies et al., 1992b; Roland et al., 1996; Sohail and Roland, 2000; Chowdhury and Smith, 2002; Narváez-Solarte et al., 2006; Safaa et al., 2008; Świątkiewicz and Arczewska, 2010) and eggshell thickness (Scott et al., 1971; Chowdhury and Smith, 2002; Safaa et al., 2008; An et al., 2016) within the range studied. Providing adequate dietary Ca is essential for shell quality as the single source of Ca for laying hens is duodenal absorption of Ca. Once absorbed Ca moves into the blood which is then removed at a rate of approximately 100 to

200 mg per h to accommodate for the high demand for Ca during shell calcification. (Etches, 1987).

Specific gravity is an indirect method of assessing shell thickness, and strength based on the flotation method (i.e., immersing eggs sequentially in saline solutions of increasing concentrations) or calculating the specific gravity using Archimedes' principle (Hamilton, 1982; Thompson and Hamilton, 1982). Consequently, given the positive relationship between dietary Ca levels and eggshell thickness, it makes sense that egg specific gravity would follow the same trend (Keshavarz and Nakajima, 1993; Keshavarz, 1996; Keshavarz, 1998b; Keshavarz, 1998a; Castillo et al., 2004). Breaking strength is a direct measure of eggshell strength, which is affected by variations in shell thickness and egg shape (De Ketelaere et al., 2002). Like shell thickness, breaking strength also showed a positive relationship with dietary Ca levels in the present analysis, which agrees with others (Watkins et al., 1977; Chowdhury and Smith, 2002; Świątkiewicz and Arczewska, 2010; Jiang et al., 2013; An et al., 2016).

4.4.3 Bone Parameters

4.4.3.1 Bone ash.

Bone ash weight as a percentage of defatted dry bone weight reflects the Ca and phosphorus status of laying hens and can be used to predict bone strength (Wilson, 1991). Bone ash, however, is limiting in that it does not reflect the different proportions of medullary, trabecular, and cortical bone present (Korver et al., 2004), the latter two of which are structural bone tissues which have the greatest influence on fracture resistance. Still, in laying hens the volume of medullary bone present has a direct, although limited, relationship with humeral breaking strength due to the large accretion of medullary bone as birds age, even with the gradual erosion of structural bone over time (Fleming et al., 1998a). In the current meta-analysis, bone ash increased with increasing levels

of dietary Ca ($P = 0.002$). While there are studies in agreement with the positive effect of dietary Ca levels on bone ash (Atteh and Leeson, 1985a; Yosefi et al., 2003), or bone ash concentration (Cheng and Coon, 1990a; Zhang and Coon, 1997) this usually involves the pre-lay period (Keshavarz, 1987) or a bone ash response to rising dietary Ca levels compared to extremely low dietary Ca levels (0.009%) (Douglas et al., 1972). Contrary to the present analysis, the literature overwhelmingly supports the idea that changing dietary levels of Ca have no effect on bone ash between 0.8% and 4.7% dietary Ca level (Atteh and Leeson, 1985b; Cheng and Coon, 1990b; Wilson, 1991; Keshavarz, 1996; An et al., 2016; Koçbeker, 2017; Pongmanee et al., 2020). Consequently, while the positive effect of dietary Ca levels on bone ash may be true in the context of the data used in this analysis (i.e., between 0.8% and 5.5% dietary Ca), it may not be an accurate representation of the biological relationship between bone ash and dietary Ca level because of the small amount of bone ash data included.

4.4.3.1 Bone Breaking Strength.

Like bone ash, bone breaking strength also increased with increasing levels of dietary Ca, which agrees with other studies (Roland et al., 1996; Narváez-Solarte et al., 2006; Koutoulis et al., 2009). This relationship makes sense considering the different sources of Ca used by the hen for eggshell calcification. Laying hens need 2 to 3 g of Ca per egg laid, the majority of which is obtained directly from dietary Ca absorption, while the rest is obtained from bone Ca reserves (Fleming, 2008; Ahmadi and Rahimi, 2011). This was first reported by Driggers and Comar (1948) by measuring the partitioning of radioactive Ca fed to hens, which ultimately found that 60 to 75% of the Ca required for eggshell calcification was obtained directly from feed rather than from bone reserves. Since hens must use skeletal Ca reserves as the remaining Ca source, the provision of adequate dietary Ca can help preserve skeletal integrity by reducing the need for bone Ca

mobilization (Farmer et al., 1986). This is important given that structural bone is not replaced while the hen is in lay, while medullary bone is replenished daily (Fleming et al., 1998b).

Increasing bone quality is an important welfare goal for many breeding companies and researchers (Preisinger, 2018; Fernyhough et al., 2020). This is especially true given that reduced bone strength has been reported in highly productive commercial hens compared to low-producing breeds (Hocking et al., 2003; Budgell and Silversides, 2004; Rodenburg et al., 2008). Additionally, the move towards cage-free housing systems has highlighted the need to maintain skeletal health in high-producing breeds, due to a higher prevalence of keel bone fractures in non-cage housing systems compared to caged systems (Freire et al., 2003; Wilkins et al., 2004; Käppeli et al., 2011; Wilkins et al., 2011; Habig and Distl, 2013). To address concerns for laying hen welfare and optimize skeletal Ca reserves for longer laying cycles, much research has been dedicated to exploring genetic factors that can be used to improve bone traits in layers (Stratmann et al., 2016; Candelotto et al., 2017; Raymond et al., 2018; Harlander-Matauschek et al., 2019; Jansen et al., 2020a). Recently, it was suggested that longer laying cycles do not adversely affect bone health if careful management of the age at first egg and well-rounded genetic selection is taken (Habig et al., 2021). Given the effects of genetic selection pressures that have occurred in laying hens, the significant effect of *Time* as a random variable on the relationship between dietary Ca levels and bone breaking strength shown in the present study makes sense. It also supports the hypothesis that genetic selection for increasing productivity by primary breeding companies in recent years has likely altered Ca utilization and thereby many metabolic processes and traits (shell quality and skeletal health).

4.5 Conclusion

Using the NRC (1994) Ca requirements as a reference, there do not seem to be negative consequences associated with over-supplementation of Ca within the Ca ranges studied as illustrated by the lack of plateaus or decreases in the response variables tested within the range studied. For some variables the lack of regression line plateaus is likely due to most studies not including severe Ca restrictions. For other variables it may be that the level of dietary Ca was not high enough to induce a response variable plateau. While the negative consequences of overfeeding Ca, such as decreases in performance (Pelicia et al., 2009), bone development (Akbari Moghaddam Kakhki et al., 2019), and phytase efficacy (Beutler, 2009), are well documented, the present study suggests that the high dietary Ca levels (above the NRC (1994) requirements) within the range studied were still within a physiologically adequate range and do not have a detrimental effect. Additionally, the lack of a significant relationship between dietary Ca and parameters such as egg production, egg mass, egg weight, phosphorus intake, bone phosphorus (%) and bone Ca (%) shows that supplementation of dietary Ca below the NRC (1994) Ca requirements (i.e., 3.25% of the diet) also does not have detrimental effects, however up to what level cannot be answered by the present analysis. Unfortunately, however, the present analysis does not allow determination of the actual Ca requirement.

4.7 Tables

Table 4.1. Summary statistics of the dependent variable parameters

	N[1]	Min	Max	Mean	SD[2]
Dietary Ca (% of diet)	791	0.048	6.46	3.58	0.95
Dependant variable					
Egg production (%)	511	4.10	98.2	81.5	13.3
Calculated calcium intake (g/hen/day) [3]	529	0.030	6.36	3.74	1.13
Phosphorus intake (g/hen/day)[4]	513	0.102	2.06	0.498	0.233
Egg mass (g)	208	15.3	66.3	51.5	6.41
Feed intake (g/hen/day)	514	62.6	144.4	106.1	14.6
Feed conversion (g feed: g eggs)	155	1.63	2.96	2.09	0.21
Feed conversion (kg feed:dozen)	55	1.27	2.51	1.63	0.27
Egg weight (g)	471	44.4	76.4	60.9	5.86
Shell weight (%)	185	7.76	12.0	9.75	0.98
Eggshell thickness (mm)	172	0.27	0.77	0.38	0.056
Egg specific gravity	311	1.0719	1.0940	1.0820	0.0050
Egg breaking strength (N)	118	22.0	50.8	38.5	7.09
Bone breaking strength (N)	51	34.3	723.0	176.1	200.9
Bone calcium (%)	38	16.5	39.3	28.3	8.48
Bone phosphorus (%)	18	9.96	21.9	17.4	4.27
Bone ash (%)	90	43.0	64.6	54.0	5.17

[1]N = number of observations.
[2]SD = standard deviation.
[3]Ca intake was calculated using the following order of preference: analyzed calcium and formulated/calculated calcium (% or g/kg)
[4]Phosphorus intake was calculated using the following order of preference: analyzed avP phosphorus, formulated/calculated avP, analyzed total P, formulated/calculated total P (% or g/kg)

Table 4.2. Biological relevance of random variables included in the regression model

	Type of variable	Biological relevance to the effect of dietary calcium on productivity, shell quality and bone quality
Record No.	Discrete	Accounts for systematic bias within publication
Time	Continuous	Genetic selection could affect hen response to dietary calcium levels (or calcium intake) and utilization. Additionally, year of publication may account unreported information from older studies (i.e., analyzed Ca in the diet).
Breed[1]	Discrete	Breed differences could affect hens' response to dietary calcium levels (or calcium intake) and utilization.
Molted	Discrete	Age-related changes and resetting of calcium sensitivity during a molt are plausible explanatory factors. Additionally, molting can increase shell quality and enhance egg production.
Calcium Particle Size	Discrete	Calcium particle size influences calcium availability as large particles are solubilized more slowly than fine particles.
Calcium Source	Discrete	Calcium source may affect the rate at which calcium is dissolved under conditions meant to mimic the gizzard. This would affect the hen's ability to use calcium, thus affecting shell and bone quality.
Heat Stress	Discrete	Heat stress can negatively affect calcium intake (via decreased feed intake). Additionally, it can negatively affect eggshell strength, weight, thickness and performance, which are response variables involving various aspects of calcium metabolism.

[1]White or brown laying hen

Table 4.3 Description of publications used in meta-analysis

	Year	Breed[1]	Initial Age	Final Age	Molted[2]	Ca Particle Size[3]	Ca Source[4]	Heat Stress[5]	Reference
1	1981	UR	29	31	N	UR	L	N	(Gilbert et al., 1981)
2	1981	W	22	63	N	F	L	N	(Hamilton and Cipera, 1981)
3	1985	W	33	43	N	UR	L	N	(Atteh and Leeson, 1985b)
4	1985	W	20	30	N	UR	L	N	(Roland et al., 1985)
5	1985	W	28	34	N	UR	L	N	(Bolden and Jensen, 1985)
6	1986	W	23	63	N	F	L	N	(Roush et al., 1986)
7	1986	W	28	32	N	UR	L	N	(Keshavarz, 1986)
8	1986	W	24	36	N	F	L	N	(Scheideler and Sell, 1986)
9	1990	UR	36	42	N	M	C	N	(Cheng and Coon, 1990a)
10	1990	UR	36	42	N	UR	L	N	(Cheng and Coon, 1990b)
11	1990	W	22	62	N	UR	L	N	(Hartel, 1990)
12	1991	UR	25	31	N	UR	L	N	(Frost and Roland, 1991)
13	1992	W	42	44	N	UR	L	N	(Clunies et al., 1992b)
14	1993	UR	20	36	N	F	L	N	(Keshavarz and Nakajima, 1993)
15	1993	B	19	23	N	UR	L	N	(Leeson et al., 1993)
16	1993	W	22	38	N	M	C	N	(Keshavarz et al., 1993)
17	1994	W	24	27	N	UR	L	N	(Roland and Bryant, 1994)
18	1994	W	22	26	N	F	L	N	(Elaroussi et al., 1994)

19	1995	W	70	101	Y	F	L	N	(Zapata and Gernat, 1995)
20	1996	W	21	32	N	UR	L	N	(Roland et al., 1996)
21	1996	W	32	44	N	UR	L	Y	(Keshavarz, 1996)
22	1998	W	58	64	N	F	L	N	(Gordon and Roland, 1998)
23	1999	W	22	35	N	UR	C	N	(Scott et al., 1999)
24	2000	B	58	59	N	C	L	N	(Smith et al., 2000)
25	2000	C	74	90	Y	UR	L	N	(Albano Jr et al., 2000)
26	2002	U	57	65	N	UR	UR	N	(Bar et al., 2002)
27	2002	W	30	34	N	UR	L	N	(Chowdhury and Smith, 2002)
28	2003	W	28	48	N	M	O	N	(Rao et al., 2003)
29	2003	W	45	65	N	UR	L	N	(Keshavarz, 2003)
30	2003	B	57	58	N	UR	L	N	(Ahmad et al., 2003)
31	2003	B	21	30	N	UR	L	N	(Lim et al., 2003)
32	2003	W	32	57	N	F	L	N	(Schreiweis et al., 2003)
33	2004	W	23	71	N	F	L	N	(Castillo et al., 2004)
34	2006	W	46	62	N	UR	L	N	(Narváez-Solarte et al., 2006)
35	2006	B	83	99	Y	F	L	N	(Pizzolante et al., 2006)
36	2006	W	79	87	Y	UR	L	N	(Hernández-Sánchez et al., 2006)
37	2007	UR	56	57	N	M	C	N	(Lichovnikova, 2007)
38	2008	B	58	73	N	M	C	N	(Safaa et al., 2008)
39	2008	B	39	55	N	UR	L	N	(Costa et al., 2008)
40	2009	B	90	108	Y	F	L	N	(Pelicia et al., 2009b)

41	2011	B	76	88	Y	F	L	N	(Cufadar et al., 2011)
42	2011	B	23	35	N	M	L	N	(Pelicia et al., 2011)
43	2011	W	25	49	N	M	L	N	(Araujo et al., 2011)
44	2011	B	40	44	N	UR	L	N	(Vieira et al., 2011)
45	2011	W	20	36	N	UR	L	N	(Valdés Narváez and Pro Martínez, 2011)
46	2012	W	42	58	N	M	L	N	(Pastore et al., 2012)
47	2013	B	16	18	N	UR	L	N	(Rodrigues et al., 2013)
48	2013	B	19	28	N	C	L	N	(Jiang et al., 2013)
49	2014	W	80	92	Y	UR	L	Y	(Nascimento et al., 2014)
50	2015	B	25	30	N	M	L	N	(Swiatkiewicz et al., 2015)
51	2016	B	70	80	N	C	L	N	(An et al., 2016)
52	2018	B	26	48	N	UR	L	N	(Świątkiewicz et al., 2018)
53	2019	W	72	81	N	M	L	N	(Akbari Moghaddam Kakhki et al., 2019)
54	2019	W	72	81	N	M	L	N	(Kakhki et al., 2019)
55	2020	W	25	37	N	UR	L	N	(Pongmanee et al., 2020)
56	2020	W	68	69	N	UR	L	N	(Bello et al., 2020)
57	2020	B	60	72	UR	F	L	Y	(Attia et al., 2020)

[a] B = Brown, C= Combination (both brown and white), UR = Unreported and W = White,
[1] B=Brown, C=Combination (both brown and white), UR=Unreported and W=White.
[2] N = No (not molted), Y = Yes (molted) and UR = Unreported
[3] F=Fine or ground, M=Mixed and UR = Unreported
[4] L= Limestone, O= Oyster shell, C= Combination (multiple sources of Ca)
[5] Publications where the birds experiences temperatures of 30°C or more

Table 4.4. Dependant and Moderator (random) variables included in each regression model

Dependant variables	**Random variables included in the final, most parsimonious model.**						
	Record No.	Time	Breed	Molted	Calcium Particle Size	Calcium Source	Heat Stress
Egg production (%)	✓						
Calculated Calcium intake (g/hen/day)[1]	✓			✓			
Calculated Phosphorus intake (g/hen/day)[2]	✓	✓					
Egg mass (g)	✓	✓					
Feed intake (g/hen/day)	✓		✓				
Feed conversion (kg feed:kg eggs)	✓	✓					
Feed conversion (kg feed:dozen eggs)		✓			✓		
Egg weight (g)	✓	✓		✓			✓
Shell weight (%)	✓						
Eggshell thickness (mm)	✓						
Egg specific gravity	✓						
Egg breaking strength (N)		✓			✓		
Bone breaking strength (N)		✓			✓	✓	
Bone calcium (%)		✓	✓	✓			
Bone phosphorus (%)		✓	✓	✓			
Bone ash (%)	✓						

[1]Ca intake was calculated using the following order of preference: analyzed calcium and formulated/calculated calcium (% or g/kg)
[2]In the following order of preference: analyzed available phosphorus, formulated/calculated available P calcium, analyzed total phosphorus, formulated/calculated total phosphorus (% or g/kg).

Table 4.5. Parameter estimates for dependant variable regression models

Model	Equation[1]	Dietary Ca Range (%)
Mineral Intake		
Calculated calcium intake (g/hen/day)	Y = 0.28 (± 0.22) + 1.04 (± 0.01)x, *P*<0.001	0.048% to 6.46%
Productivity		
Feed intake (g/hen/day)	Y = 103.6 (± 4.01) + 1.54 (± 0.41)x, *P*=0.0002	0.048% to 6.46%
Efficiency		
Feed conversion (kg feed: kg egg)	Y = 2.28 (± 0.05) - 0.022 (± 0.0081)x, *P*=0.009	0.8% to 5.5%
Feed conversion (kg feed:dozen eggs)	Y = 2.05 (± 0.14) - 0.051(± 0.024)x, *P*=0.046	0.8% to 5.25%
Shell Quality		
Shell weight (%)[1]	Y = 8.61 (± 0.20) + 0.24 (± 0.026)x, *P*<0.001	0.8% to 6.46%
Eggshell thickness (mm) [1]	Y = 0.35 (± 0.016) + 0.0075 (± 0.0016)x, *P*<0.001	0.8% to 6.46%
Egg specific gravity[1]	Y = 1.0769 (± 0.0012) + 0.0015 (± 0.00014)x, *P*<0.001	0.8% to 5.5%
Egg breaking strength (N)	Y = 18.32 (± 4.81) + 1.23 (± 0.50)x, *P*=0.016	2.62% to 4.5%
Bone Quality		
Bone breaking strength (N)	Y = 271.58 (± 193.6) + 12.92 (± 2.41)x, *P*<0.0001	0.8% to 6.46%
Bone ash (%)	Y = 50.49 (± 1.84) + 1.03 (± 0.32)x, *P*<0.0021	0.8% to 5.5%

[1] Each dependant variable response was estimated using: $Y = \alpha + \beta DietaryCaLevel + \gamma_i RandomVariable_i + e$, where Y is the value of the dependent variable in publication; β is the regression coefficient for dietary Ca level; γi is the regression coefficient for the random variables included in the model and e is the residual error.

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5.0 Chapter 5: Synthesis

5.1 General Discussion

Proper calcium (**Ca**) metabolism is essential for laying hen health and welfare and for the economic viability of the industry by allowing for proper shell quality. Chapter 3 reported the effects of dietary phosvitin (**PV**) as a potential strategy for protecting the shell and bone quality of end-of-cycle laying hens fed a Ca-reduced diet. A total of eight experimental diets were used including a positive control (**PC**), negative control (**NC**; 21% less Ca than the PC) and six NC PV-derivative diets that contained either phosvitin (**PV**; 37.4% purity), dephosphorylated phosvitin (**DPV**; 39.5% purity) or phosvitin peptides (**PVP**; 39.8% purity), each fed at a 1% or 0.01% of the diet with different amounts of each PV product were added to account for differences in purity. It was hypothesized that dietary PV would protect laying hen bone quality via increased dietary Ca digestibility while maintaining productivity and shell quality in end-of-cycle laying hens. Initially, all NC-based diets led to a drop in productivity (egg production and egg mass) in the first week of the experiment. Ultimately, both egg production and egg mass for the NC-PV diet-fed hens recovered by 94 woa and did not differ significantly compared to the PC-diet fed hens. This was because birds fed the reduced Ca diets increased feed intake (**FI**) to increase Ca intake, and increased Ca digestibility to overcome the challenge in the short term. Thus, hens have an appetite for Ca and will increase their FI to maintain Ca intake and will increase the rate of Ca absorbed from the diet to maintain Ca homeostasis. The cumulative lack of effect of the NC diet on egg production (except for NC + DPV 1% fed hens), shell quality and bone quality, suggests that the NC-diets were not deficient in Ca, at least in the short-term.

The lack of an NC-effect in Chapter 3 in the short term highlighted the robustness of modern hens to withstand dietary Ca reductions and prompted us to explore Ca requirements in laying hens. This was the foundation of Chapter 4 which involved a meta-analysis aimed to account for factors relevant to Ca utilization via the inclusion of random variables that statistically improved each response variable's regression model. Ultimately a data set containing 792 observations was compiled from 57 published papers between 1981 and 2020. Seven moderator variables (Record No., Year, Strain, Molted, Ca Particle Size, Ca Source and Heat Stress). Response parameters such as Ca intake and FI increased with dietary Ca levels within the range studied. These results suggest that the practical range of dietary Ca that should be fed to hens was within a the range tested and that even at the higher Ca ranges, hens are likely to maintain Ca homeostasis via mechanisms such as increased renal excretion. Each of the **feed conversion ratio** (FCR) parameters decreased with increasing levels of dietary Ca within the range studied. The significant effect of *Time* (i.e., year of publication) as a random variable on each of the FCR parameters reflects the impact of successful selection for feed efficiency by primary breeding companies over time. Eggshell quality response parameters such as shell weight, eggshell thickness, specific gravity, and egg breaking strength each increased with increasing levels of dietary Ca within the range studied. Bone quality response parameters such as bone breaking strength and bone ash increased with increasing dietary Ca levels within the range studied. The positive relationship between dietary Ca levels and bone ash may be due to most publications not using severe deficiencies and the lack of bone ash data included in the present analysis. Alternatively, the positive relationship between bone-breaking strength and increasing Ca levels supports existing research and suggests that increasing dietary Ca levels prevents hens from unnecessarily using Ca from the bones when the opportunity for adequate Ca intake is present.

5.2 Phosvitin as a dietary additive and the tolerance of modern laying hens to calcium reductions

Chapter 3 focused on the effect of PV as a feed additive on birds experiencing a 21% reduction in dietary Ca. At 94 woa, there was no significant dietary effect on egg production or egg mass, which suggests that the birds were performing well at the end of the experiment, however, FI was significantly increased for birds fed any of the NC diets. Ultimately, there were no treatment effects on final body weight nor any bone or shell quality trait at 94 woa. The results in Chapter 3 demonstrated that modern laying hens, even at the end of an extended laying cycle, can tolerate reductions in Ca in the short term.

Unfortunately, the lack of an NC effect in this trial did not allow us to assess if PV had a protective effect on bone health and Ca absorption in laying hens. While it may seem logical to reduce Ca even further to test the effect of PV, this also may not have worked. Dunn et al. (2021) found that there was a high positive genetic correlation between late egg production and medullary bone mineralization. Given that the birds in the present study were highly productive end-of-cycle laying hens, it is possible that an already high mineralization of medullary bone provided protection against the dietary Ca reduction. Instead, it may be more interesting to test the effect of PV at another metabolically demanding time point, such as prior to the onset of sexual maturity. Two weeks before sexual maturity, the formation of structural bone ceases and bone diameter increases in preparation for the deposition of medullary bone (Fleming et al., 1998; Whitehead, 2004). Once egg production has started, laying hens experience intense diurnal cycles of medullary bone mobilization and deposition and the gradual erosion of structural bone overtime (Whitehead, 2004). Consequently, the effect of a dietary Ca reduction prior to egg production, or at the start of egg

production may elicit a stronger metabolic effect compared to highly productive birds, with well-established Ca reserves at the end of lay. Still, given the impressive performance of end-of-lay hens tolerating a 21% reduction in dietary Ca, it was postulated that the Ca requirements of modern laying hens have likely decreased, which was the foundation of the objectives and hypothesis in Chapter 4.

5.3 Dietary calcium recommendations

The addition of large dietary Ca safety margins to feed to has been standard practice in industry over the years. This has resulted in a wide range of recommendations since the most recent publication of NRC Ca requirements in 1994 (NRC, 1994). Concurrently, the genetic potential of laying hens has changed substantially, which has resulted in birds that can produce nearly an egg a day with increased persistency of lay up to 100 woa and beyond. These changes have likely decreased the requirements of modern laying hens, including that for Ca. Initially, given the ability for end-of-cycle laying hens to tolerate such as a 21% reduction in dietary Ca in Chapter 3, it was hypothesized that hens can tolerate lower dietary Ca levels compared to current requirements and recommendations by increasing FI and efficiency of Ca absorption.

Ultimately, the research in Chapter 4 revealed that most recommendations compiled from 38 papers (3.17 to 5.57 g/hen/d), were higher than the NRC (1994) requirements of 3.25% Ca in the diet per hen/day (3.25 g/day/hen for Single Comb White Laying hens and 3.6 g/day/hen for Brown egg-laying hens). Instead, the NRC (1984) Ca requirement from nearly four decades ago (3.75 g/hen/day) was closer (but still below) the mean of the compiled Ca recommendations (4.01 g/hen/day) from the meta-analysis. Additionally, although recommendations have appeared to increase over time, there was no significant difference between Ca recommendations from different decades between 1980 and 2020. Still, given the intense genetic selection that has occurred since

1994, it is impressive for modern laying hens to have the productivity, persistency, shell quality and bone quality that they do, without a dramatic increase in Ca requirement. Still, most of the Ca recommendations from primary management guides are much higher than the recommendations from individual publications, which supports the idea that primary breeders include large safety margins in their recommendations for dietary Ca. Whether these safety margins (i.e., excess dietary Ca) are harmful to the industry was the purpose of compiling data from multiple publications to assess the dose-response effects of varying levels of dietary Ca on performance, shell quality and bone quality parameters

5.4 Effect of dietary Ca levels on productivity, shell quality and bone quality parameter

Although a myriad of research has been dedicated to the importance of Ca in laying hen nutrition, the data from multiple studies is often not combined to provide a new perspective. In Chapter 4, the data from 57 publications was combined to provide this perspective. The purpose of this was to assess the dose-response effects of varying levels of dietary Ca on performance, shell quality and bone quality parameters. This is important, since high levels of dietary Ca have adverse effects such as the formation of insoluble Ca-phytate complexes (Rama Rao et al., 2006; Selle et al., 2009; Skřivan et al., 2016), negatively affect Ca and P absorption and retention (Proszkowiec-Weglarz and Angel, 2013; Li et al., 2016), reduce the efficacy of exogenous phytase (Selle et al., 2009a), negatively affect bone mineralization (Hamdi et al., 2015), trigger bone resorption (Rama Rao et al., 2006) and negatively affect shell quality (Kebreab and Vitti, 2010; Kakhki et al., 2019) and egg production (Jiang et al., 2013). Among the significant regression models, no adverse relationships between increasing dietary Ca and the productivity, shell quality and bone quality parameters were found. Ca intake and FI increased with increasing levels of dietary Ca, while FCR (kg feed:kg eggs and kg feed:dozen eggs) decreased, each within their respective ranges. All shell

quality parameters, except egg weight which was not significantly affected, increased with increasing levels of dietary Ca. And lastly, bone breaking strength and bone ash also increased with increasing levels of dietary Ca, while bone Ca and bone P remained unaffected. The lack of an adverse effect of increasing levels of dietary Ca on these parameters may negate concerns about feeding high dietary Ca, however, there are a few caveats. Most dietary Ca levels ranged between 3 and 5% dietary Ca, leaving dietary Ca levels above that range (>5%) to be considered high dietary Ca. The range of 3 to 5% calcium is inclusive of the range of Ca recommendations from various primary breeder management guides (4.10 - 4.7%) which suggests that the latter recommendations would not be high levels of dietary Ca. Consequently, it is fair to assume that feeding excess dietary Ca in the industry is likely not an issue, Overall, however, Chapter 4 provides a better appreciation for quantifying the effects of dietary Ca level on various parameters. Additionally, it shows that the dietary reduction of Ca used in the NC diet in Chapter 3 (2.93% analyzed Ca) was likely not detrimentally low, given the responses to dietary Ca between 2 and 3% used in the regression models for egg production, egg mass, FI, feed conversion (kg feed:kg eggs), egg shell thickness, specific gravity, egg breaking strength, bone breaking strength and bone ash were comparable to the effects of dietary Ca levels that were slightly above that range (3 to 5%).

5.5 Novelty of research

There are few studies looking at the mechanism of action and the use of PV in animals, however, Chapter 3 is the first one in hens. Ultimately, the lack of an NC effect in Chapter 3 limited the ability to explore PV as an innovative supplement to proactively manage bone health in layers. Consequently, there is still potential in exploring the effect of PV on bone health and Ca absorption in layers if a more severe Ca reduction is implemented, the experimental duration is increased, or a different age range is used.

Numerous publications over the past five decades have investigated the effect of dietary Ca levels on productivity, shell quality and bone quality in laying hens, however, there is a large variability of responses to dietary Ca and a large range of Ca recommendations. Chapter 4 is the first meta-analysis involving dietary Ca and laying hens which attempted to better quantify the effect of random factors related to the effect of dietary Ca on productivity, shell quality and bone quality parameters overall. Additionally, this is the first publication that summarizes Ca recommendations from various decades in a standardized form (g/hen/day) that accounts for FI, rather than in the form of a dietary percentage. It is anticipated that the NRC Nutrient Requirements for Poultry will be revised soon, therefore, it is hoped that Chapter 4 has provided some clarity when it comes to the changing requirements of modern laying hens.

5.6 Study limitations

Various limitations were identified over the course of the research presented in this thesis. The short-term objective of Chapter 3 was to test the use of PV and PV-derived products for their ability to protect the skeletal structure of laying hens fed a Ca-reduced diet for 4 weeks. The 4-week duration was intended to serve as a pilot study to eventually test the effect of these feed additives under long-term conditions. The 21% reduction in Ca in the NC diet was intended to induce a metabolic state of Ca deficiency, to test our hypothesis, but was limited to 4 weeks in the interest of bird welfare. The lack of an NC-effect observed in the pilot study however eliminated the ability to test the hypothesis. In this context, the high genetic potential of modern laying hens was a major limitation in that it is difficult to balance between pushing these birds to their metabolic limits, without unnecessarily risking animal welfare. Regarding, phosvitin, the scale up extraction protocol yielded a low PV recovery rate, which may have confounded the results if a NC-effect

was achieved. Still, scale up extraction is necessary if commercialization of PV in the laying hen industry is pursued.

Other limitations associated with Chapter 3 included the effect of the COVID-19 pandemic on lab analysis. This section of the thesis relied heavily on production data collected during the experiment and the small amount of laboratory analysis that could be completed before university shutdowns occurred in March 2020. Digestibility data was not completed until two years later and limited analysis was completed due to restricted project funds. This also meant that other samples that were originally collected were not analyzed. This included blood samples which were collected to assess changes in osteocalcin, pyridinoline, and parathyroid hormone (PTH), during shell formation when osteoclasts are most active (15 h after oviposition) (Sugiyama and Kusuhara, 1993). Unfortunately, these samples were not analyzed due to lack of funds, staff, and time, which eliminated blood chemistry from being assess in this pilot study. The COVID-19 pandemic also did not allow for further bird work after March 2020 for this thesis due to research and funding restrictions. The unknown timelines associated with the pandemic prompted exploration of alternative ways to finish this thesis. The lack of NC effect observed in Chapter 3 prompted the decision to explore Ca requirements and recommendations in laying hens, which was catalyst for Chapter 4. This meta-analysis allowed for an opportunity to conduct research safely during a pandemic, without relying on changing restrictions, timelines, and funds.

The meta-analysis itself had its own unique limitations. The purpose of Chapter 4 was to perform a meta-analysis by combining the data from multiple studies to provide a new perspective and potential knowledge regarding the effect of dietary Ca in laying hens. The two major potential limitations associated with this type of analysis are publication bias and search bias. Publication bias is extremely difficult to avoid as researchers often only publish favorable results. Unfavourable

or unremarkable results are typically not attractive to publish, especially from the perspective of funders who often want to develop products for commercialization. Still, the results of such studies would add valuable insight into the metabolic response and mechanism of poultry. In the present thesis, Chapter 3 is a prime example of an experiment that did not provide remarkable results regarding a feed additive but did lead to further questions about hen nutrition and biology. Search bias is another limitation affected by the key words selected and the search engines used to look for publications. In Chapter 4, a consistent set of key words was used as a search strategy for the meta-analysis. This consistency allowed for repeatability of the Materials and Methods used but limited the types of publications included. For example, key words focused on publications using varying levels of Ca (Ca, dietary Ca, requirements) limited the inclusion of publications focusing on other nutrients (i.e., phosphorus, protein), feed additives (i.e. prebiotics and organic acids), enzymes (i.e., phytase) and specific metabolic states (i.e. Ca depletion, low Ca diet) which also used varying levels of Ca.

Selection bias was another limitation that was considered in Chapter 4 in the present thesis. To avoid the selection of unsuitable publications for inclusion a strict set of criteria in selecting publications was developed. This was intended to reduce dissimilarities among papers, which can affect the final conclusions drawn. While these criteria provided guidance in the selection of publications, it did not help with the unanticipated challenge accounting for the underreporting of moderator variables (i.e., housing, management, details on experimental diets or designs). Ultimately, the moderator variables included and considered in the analysis were not only selected based on how they related to Ca metabolism and utilization but were also limited by what was available in the publications chosen based on the selection criteria.

5.7 Direction of Future Research

There are many opportunities for future studies based on the current research. As previously mentioned, Chapter 3 demonstrated the resilience of end-of-cycle modern layers to reduced dietary Ca, which ultimately did not allow us to assess the effect of PV. Given that a 21% reduction in dietary Ca was considered severe, this prompted us to question if the Ca requirements for modern layers has changed and if modern laying hens have increased efficiency of Ca utilization. Due to the COVID-19 pandemic, using bird work to explore these questions was not possible, however, testing these questions with early, mid and end of lay production hens, would contribute a better understanding of the metabolic capabilities of the modern laying hen.

Additionally, given the lack of an NC-effect in Chapter 3 there is still an opportunity to assess the effect of PV in laying hens. PV elicits an effect on Ca availability and bone incorporation in rodent models (Jiang and Mine, 2000; Jiang and Mine, 2001; Choi et al., 2005), which are attractive characteristics for laying hens as the industry moves towards non-cage housing systems and longer laying cycles. If future research can address what levels of dietary Ca in modern laying hens induce a dietary deficiency, with proper measures and endpoints for preventing unnecessary pain and suffering, this would be a useful future direction that would help with testing feed additives in laying hens.

Chapter 4 also provided opportunities for future studies. Although the analysis in this chapter was based on Ca, it has provided a method for exploring the effects of other nutrients or additives among various publications. Once such nutrient that was discussed during the development of Chapter 4 was P. Given that Ca requirements are highly linked to P requirements, collecting information on P from each of the publications was a priority. Collecting P related information however revealed challenges that were beyond the scope of the present meta-analysis

but would be a potential future direction. For example, available P was a potential random variable that we attempted to include in the analysis. Among the publications, P level was reported one of six ways: formulated/calculated total P (% or g/kg), formulated/calculated available P (% or g/kg), analyzed total P (%) and analyzed available P (%). To include this in the meta-analysis, we needed to select the most suitable way of expressing the amount of P available for utilization by the bird which was limited to avP (available phosphorus) because it is assumed to be in a form useable by the animal. Soon it became apparent that 262/792 lines of data (33%) did not report any form of available P and would therefore be eliminated from the analysis if available P was added as a random variable. It was also discussed that 90/262 (34%) of the publications that did not state any form of available P, had stated some form of total P (formulated, calculated, or analyzed). Initially it was suggested that an estimate of available P could be calculated by entering the diet specifications of that publication into a feed formulation program. Ultimately, however, this still would have involved assumptions because some publications with dietary Ca levels with multiple levels of phosphorus (e.g., 3.0% Ca with 0.250%, 0.300%, 0.350% and 0.400% available P), still only reported Ca as a main effect which meant that the available P level was not clearly defined. These same issues also contributed to the exclusion of the Ca:P ratio as a moderator variable from the meta-analysis. Ultimately it was decided that accounting for available P was too complicated and beyond the scope of the original intent of Chapter 3, however, it would be a great opportunity for a secondary meta-analysis with moderator factors specific to P availability and utilization.

5.8 Overall Implications

Findings in Chapter 3 have revealed the need to test feed additives such as PV in laying hens under a more severe dietary Ca reduction, or at a different point in the laying phase. Without this, the effects of PV on Ca bioavailability, absorption and bone incorporation can not be properly

addressed. The secondary implications from this thesis chapter were that modern laying hens, even at end of lay, are tolerant to moderate dietary reductions in Ca, which may reflect the results of intense genetic selection which has resulted in an increasingly robust laying hen. Ultimately, these findings suggested that Ca requirements in modern laying hens have likely changed due to increased Ca utilization or alternative adaptations to support egg production, shell quality and bone quality (i.e., skeletal Ca mobilization).

The meta-analysis allowed for quantification of productivity, shell, and bone parameter dose responses to dietary Ca over various decades. Using the NRC (1994) Ca requirements as a reference, there does not seem to be negative consequences associated with over-supplementation of Ca within the Ca ranges studied. This is illustrated by the lack of plateaus or decreases in the response variables tested within the range studied. This also suggests that the Ca recommendations from primary breeding management guides are likely not detrimentally high and that modern layers can tolerate lower levels. Ultimately, the implication from these findings is that if producers are dealing with issues related to Ca utilization but are feeding Ca according to the primary breeder recommendations, they should not focus on the level of dietary Ca as the issue. Instead, they should reflect on their overall management and consider other nutritional strategies, such as Ca particle size, Ca feeding strategy and the source of Ca.

5.9 Conclusions

This thesis investigated PV as a feed additive and Ca requirements and recommendations of laying hens. While the trial in Chapter 3 did not provide further insight into the use of PV as a feed additive, it did highlight the need to revaluate how to induce a Ca deficiency in laying hens and consider the robustness of the modern laying hens to nutritional challenges. In the case of Ca nutrition, modern layers may be able to adapt to reduced dietary Ca via an increase in FI and Ca

digestibility to overcome the challenge in the short term, which suggests that hens have an appetite for Ca. Overall, these results suggest that primary breeder feeding guidelines have large safety margins for producers and that laying hens can also tolerate substantial dietary reduction in Ca in the short term.

In exploring Ca recommendations, many researchers agree that discrepancies in Ca recommendations exist because many internal and external factors (strain, environmental factors, and other nutrients) need to be considered. Attempting to account for some of these factors via the meta-analysis in Chapter 4 helped summarize and quantify the effect of dietary Ca levels on performance, shell quality and bone quality parameters, which ultimately showed that within the range reported increasing levels do not have a detrimental effect on performance, shell quality and bone quality parameters. The results of the response variable regression models were supported overwhelmingly by existing literature and have also suggested that the Ca requirements for optimal performance of modern laying hens have likely increased.

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Appendices

Appendix A

Conversion Calculations for parameters used in the meta-analysis data set

Age from Days to Weeks

$\frac{day(s)}{} * \frac{1\ week}{7\ days}$ = Age (weeks)

Calcium or Phosphorus in g/kg to %

$\frac{g}{kg} * \frac{1\ kg}{1000\ g} * 100\%$ = Dietary calcium or phosphorus (%)

Calcium/Phosphorus Intake

$\frac{Dietary\ Ca\ (\%)}{} * \frac{1}{100\%} * Feed\ intake\ (\frac{\frac{g}{hen}}{day})$ = Calcium or phosphorus intake (g/hen/day)

Phosphorus in mg to g

$\frac{Phosphorus\ (mg)}{} * \frac{1\ g}{1000\ mg}$ = g

Shell weight (%)

$\frac{Shell\ weight\ (g)}{} * \frac{1}{Egg\ weight\ (g)} * 100\%$ = Shell weight (%)

Eggshell Thickness (µm to mm)

$Eggshell\ Thickness\ (\mu m) * \frac{1\ mm}{1000\ \mu m}$ = Eggshell Thickness (mm)

Egg or Bone Breaking Strength (kgF to N)

$\frac{kgF}{} * \frac{1\ N}{9.80665\ kgF}$ = Eggshell Breaking Strength (N)

Egg Breaking Strength (gF to N)

$\frac{Eggshell\ Breaking\ Strength\ (gF)}{} * \frac{1\ kgF}{1000\ gF} * \frac{1\ N}{9.80665\ kgF}$ = Eggshell Breaking Strength (N)

Bone Calcium or Phosphorus (mg/g → %)

$\frac{mg}{g} * \frac{1\ g}{1000\ mg} * 100\%$ = Bone Calcium (%)

Bone Calcium or Phosphorus (g/kg to %)

$\frac{g}{kg} * \frac{1\ kg)}{1000\ g} * 100\%$ = Bone Ca or P (%)

Bone Ash (g/kg to %)

$\frac{(g)}{kg} * (\frac{1}{1000g}) * 100$ = Bone Calcium (%)

Calcium recommendations (% to g/hen/day)

$\frac{Dietary\ Calcium\ Recommendation\ (\%)}{} * \frac{1}{100\%} * Feed\ Intake\ (\frac{\frac{g}{hen}}{day})$= Calcium Recommendation (g/hen/day)

Appendix B

Figure 1 Calcium intake (g/hen/day)

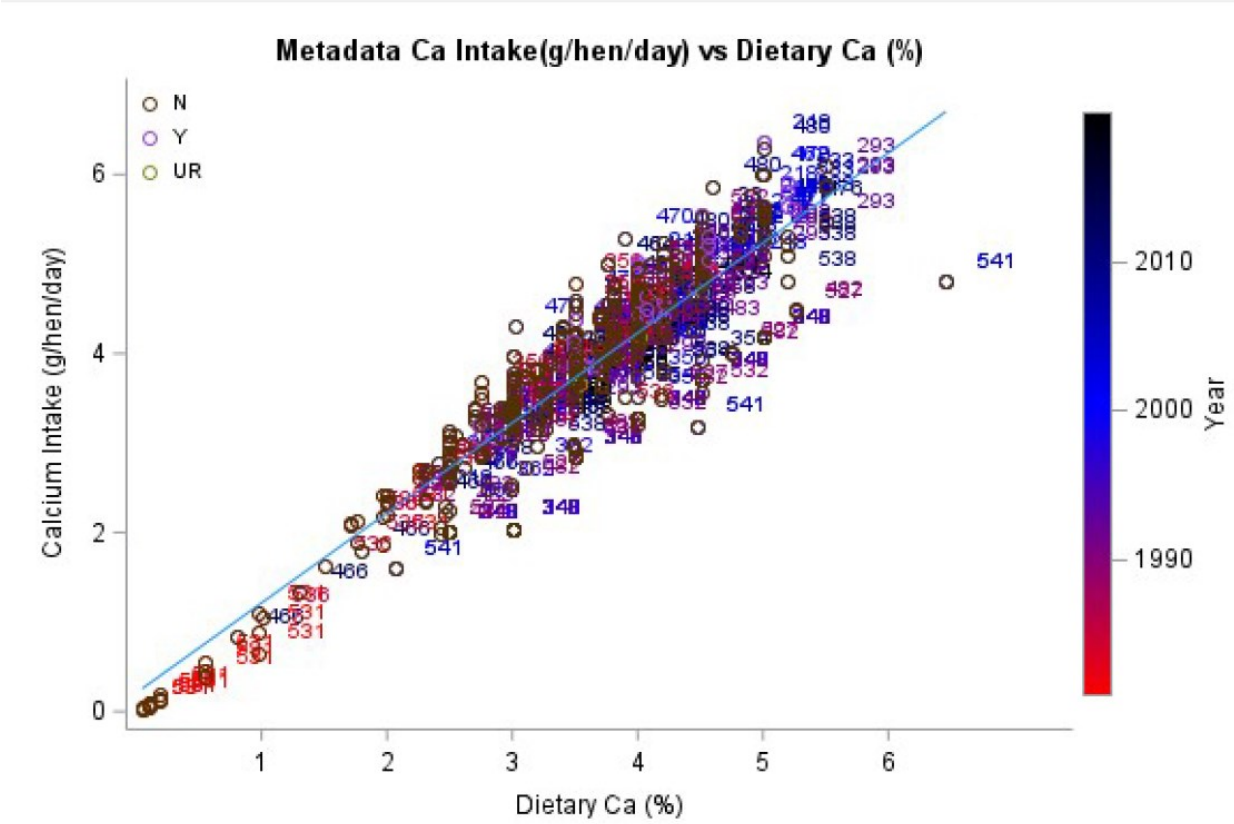

Figure 1. Impact of dietary calcium (%) on calcium intake (g/hen/day) in laying hens (Y = 0.28 (+ 0.22) + 1.04 (+ 0.01)x, *P*<0.001)

Figure 2 Feed intake (g/hen/day)

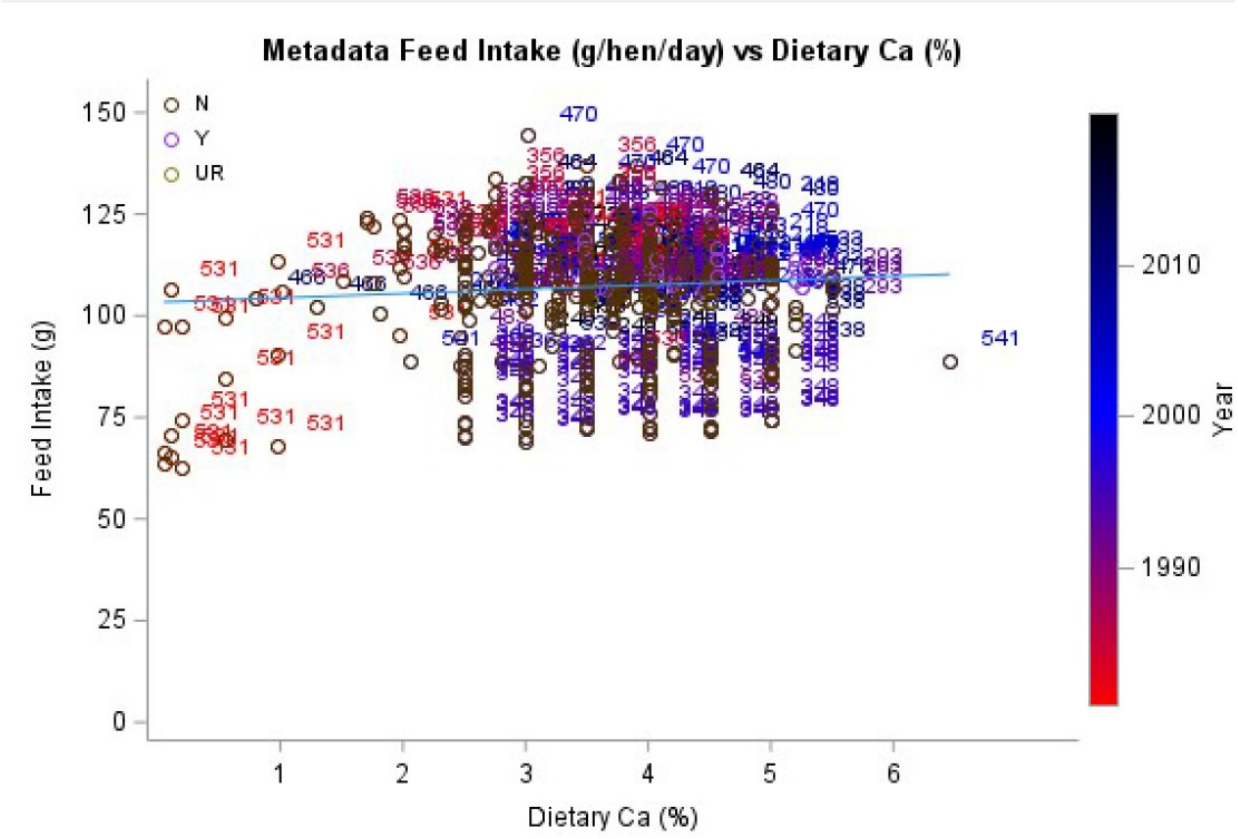

Figure 2. Impact of dietary calcium (%) on feed intake (g/hen/day) in laying hens (Y = 103.6 (+ 4.01) + 1.54 (+ 0.41)x, *P*=0.0002)

Figure 3 Feed conversion (kg feed: kg eggs)

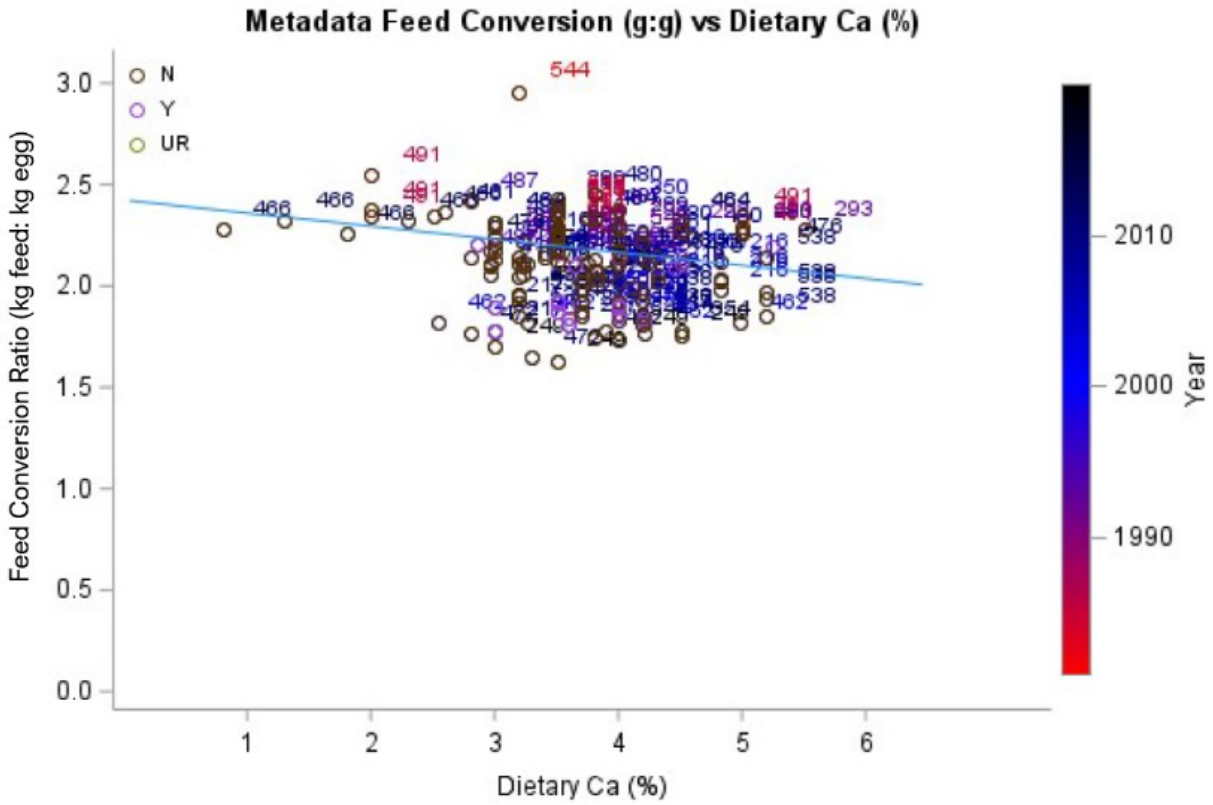

Figure 3. Impact of dietary calcium (%) on feed conversion (kg feed:kg eggs) in laying hens (Y = 2.28 (+ 0.05) - 0.022 (+ 0.0081)x, P=0.009)

Figure 4 Feed conversion (kg feed:dozen)

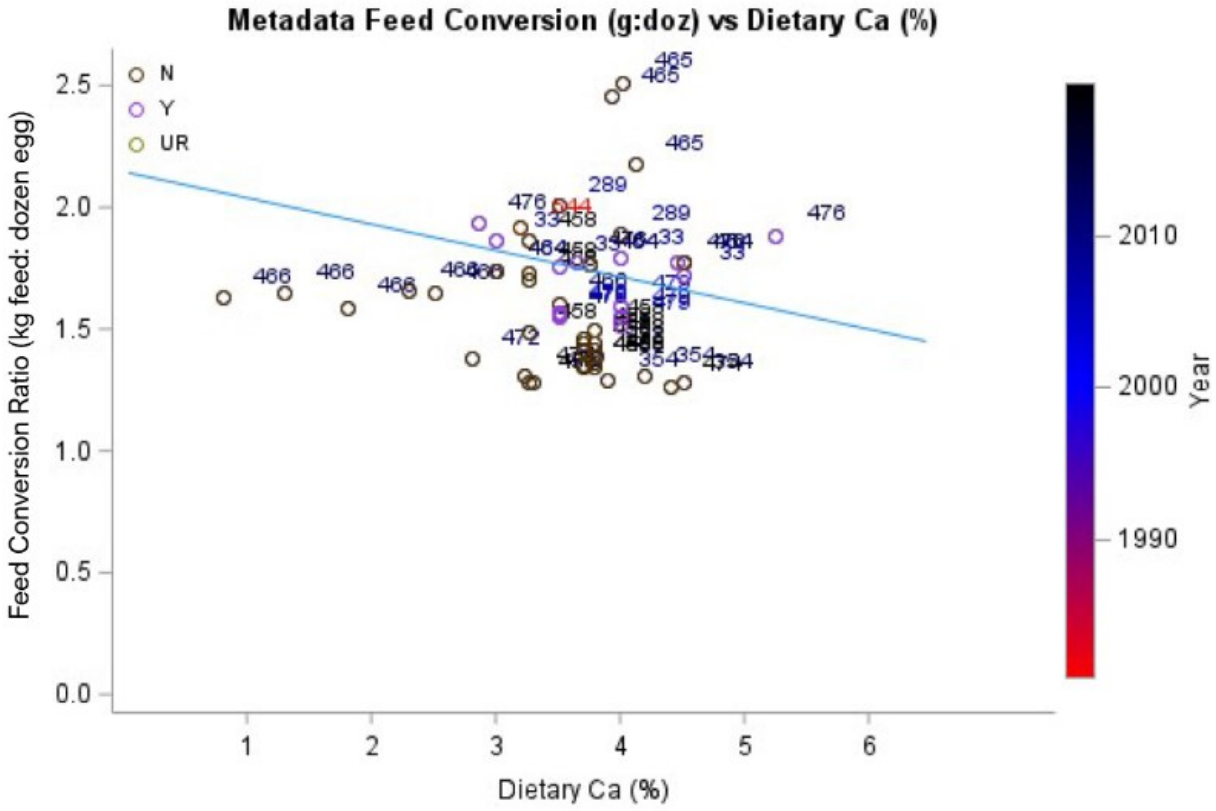

Figure 4. Impact of dietary calcium (%) on feed conversion (kg feed:kg dozen) in laying hens (Y = 2.05 (+ 0.14) - 0.051(+ 0.024)x, *P*=0.046)

Figure 5 Shell weight (%)

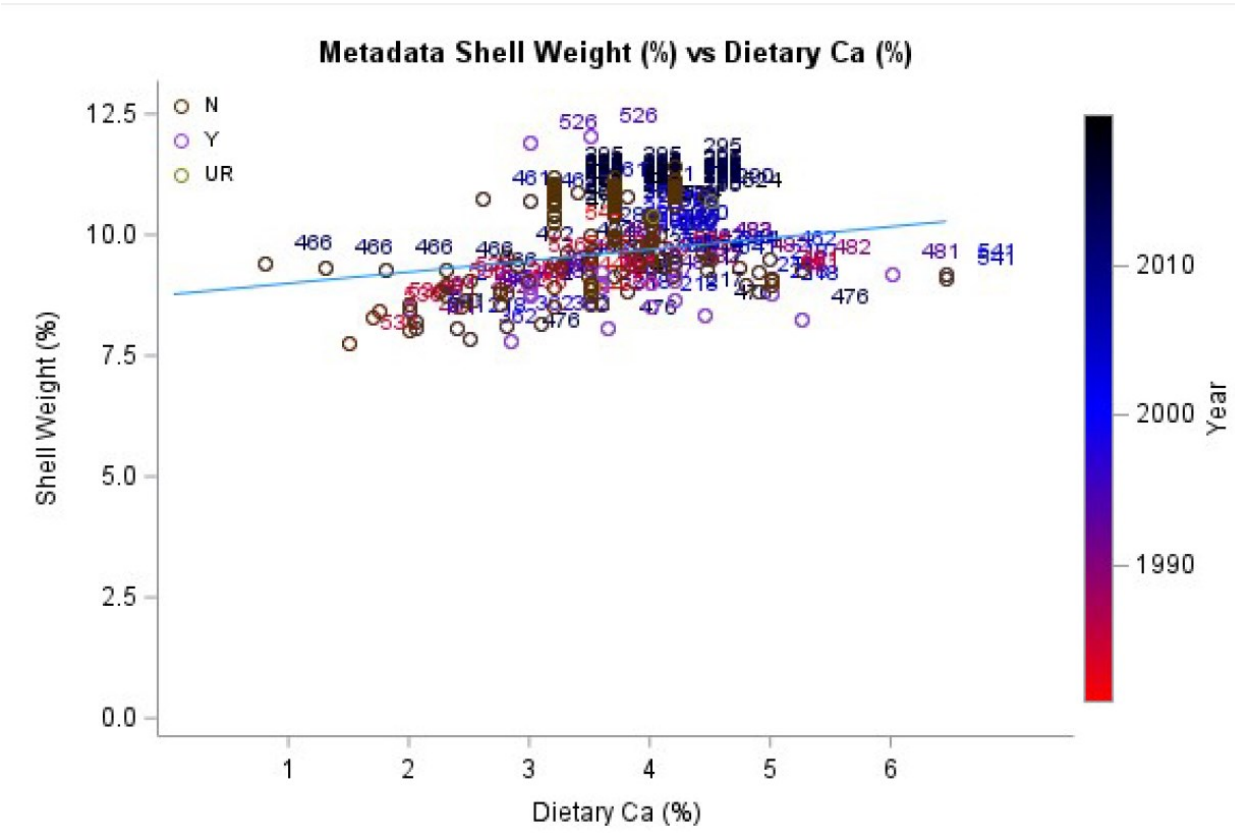

Figure 5. Impact of dietary calcium (%) on shell weight (%) in laying hens (Y = 8.61 (+ 0.20) + 0.24 (+ 0.026)x, P<0.001)

Figure 6 Eggshell thickness (mm)

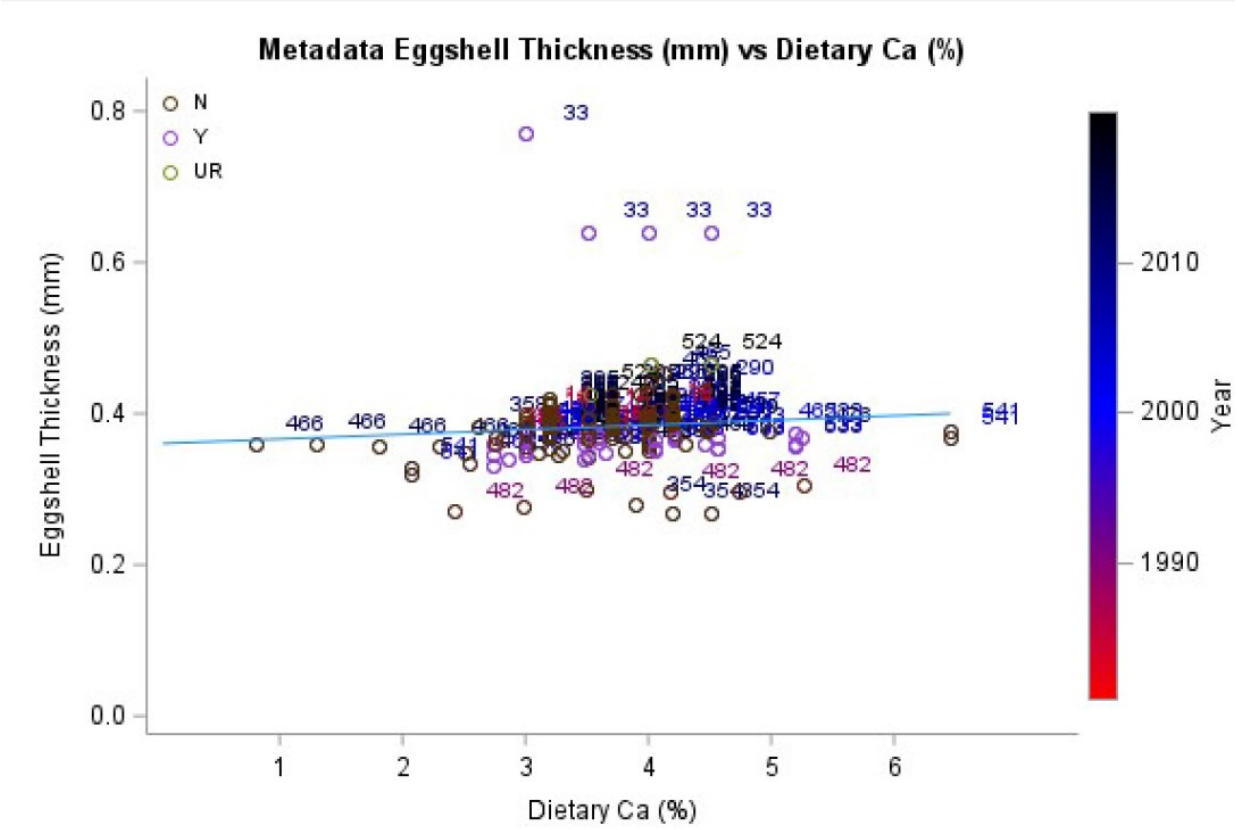

Figure 6. Impact of dietary calcium (%) on eggshell thickness (mm) in laying hens (Y = 0.35 (+ 0.016) + 0.0075 (+ 0.0016)x, *P*<0.001)

Figure 7 Egg specific gravity

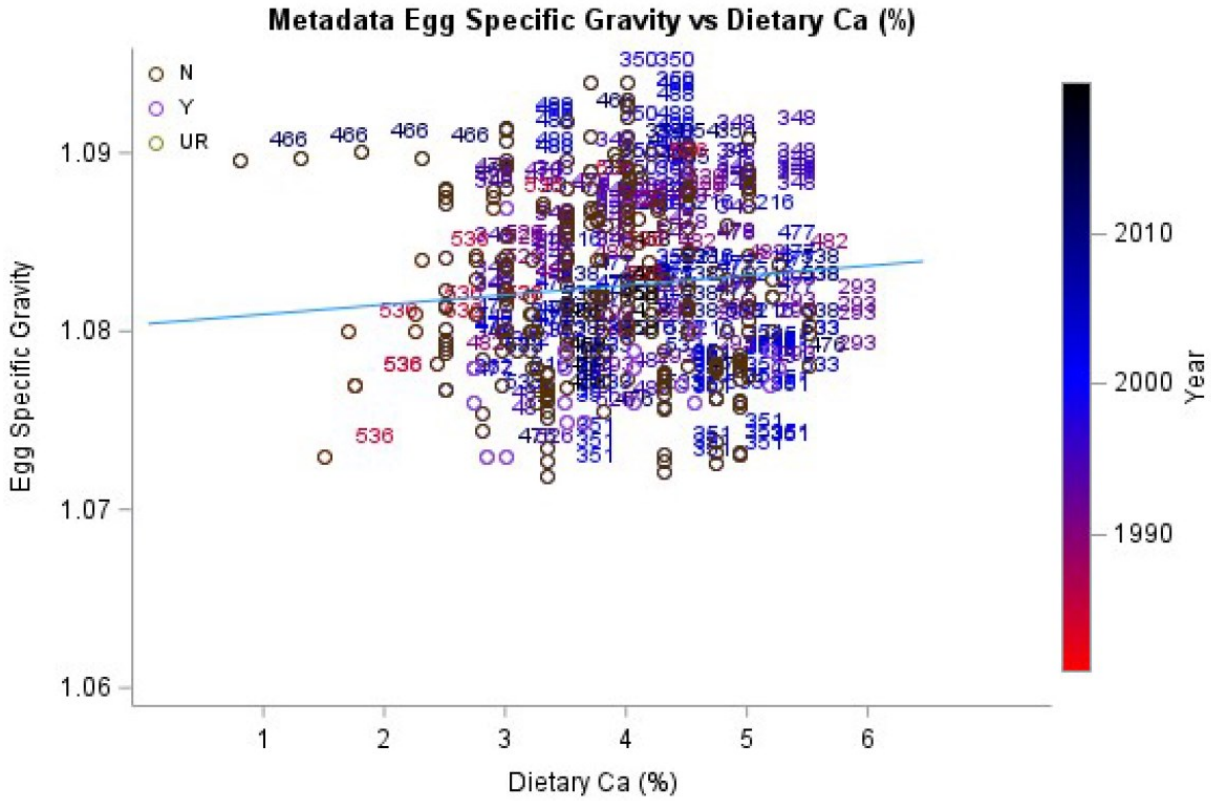

Figure 7. Impact of dietary calcium (%) on egg specific gravity in laying hens (Y = 1.0769 (+ 0.0012) + 0.0015 (+ 0.00014)x, *P*<0.001)

Figure 8 Egg breaking strength (N)

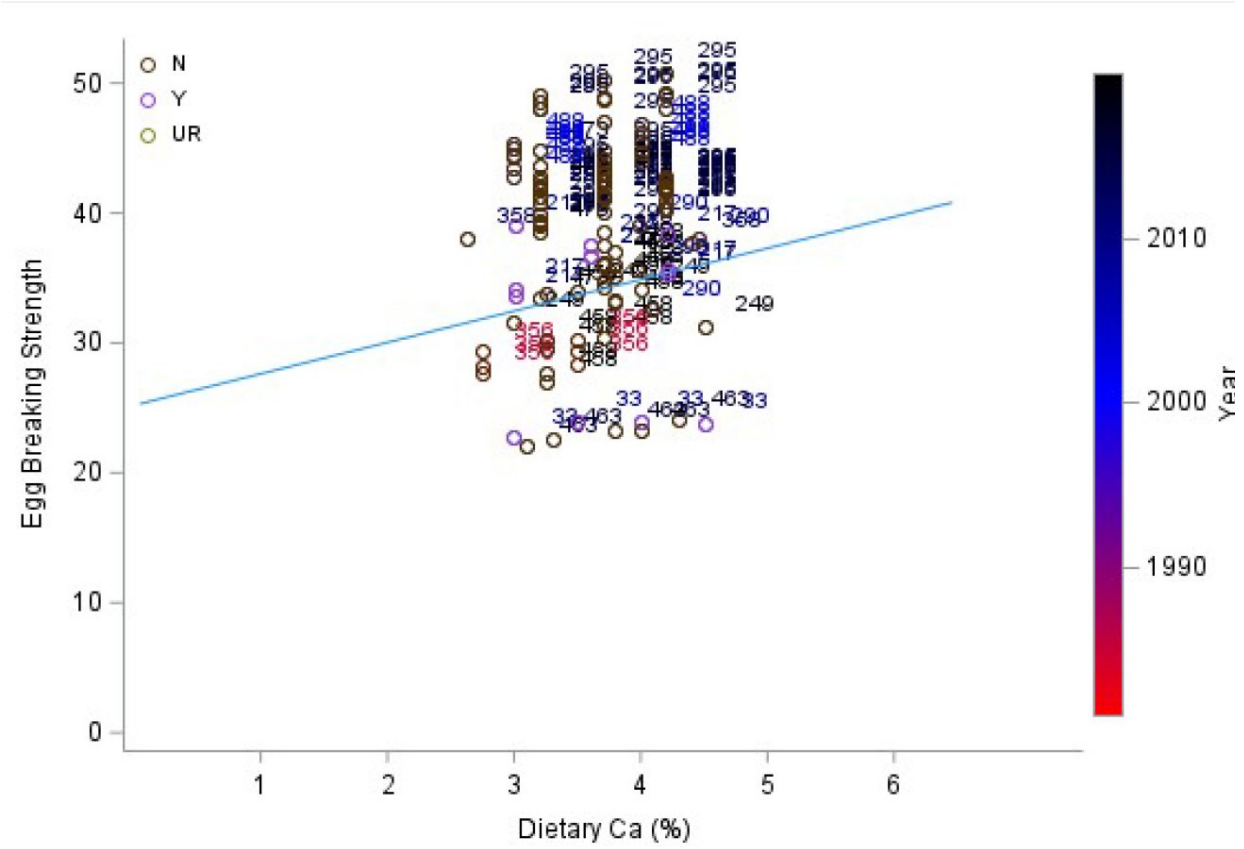

Figure 8. Impact of dietary calcium (%) on egg breaking strength (N) in laying hens (Y = 18.32 (+ 4.81) + 1.23 (+ 0.50)x, *P*=0.016)

Figure 9 Bone breaking strength (N)

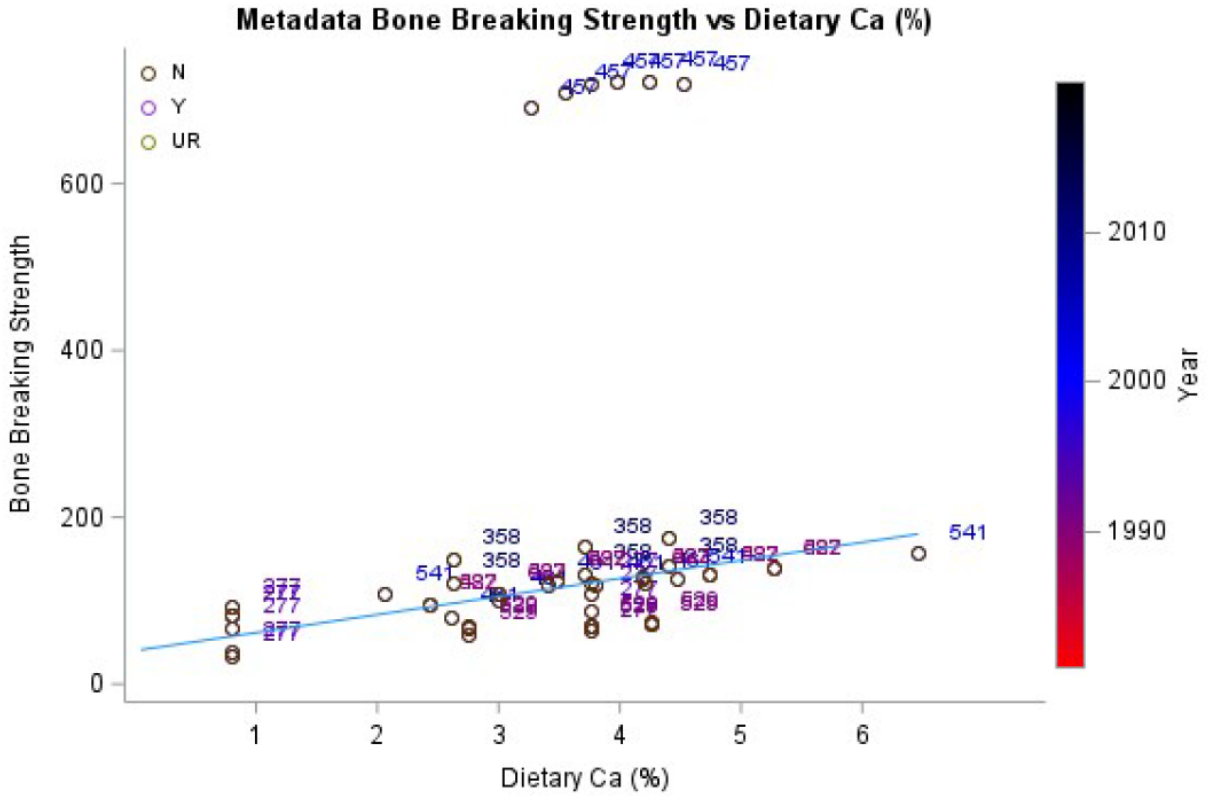

Figure 9. Impact of dietary calcium (%) on bone breaking strength (N) in laying hens (Y = 271.58 (+ 193.6) + 12.92 (+ 2.41)x, *P*<0.0001)

Figure 10 Bone ash (%)

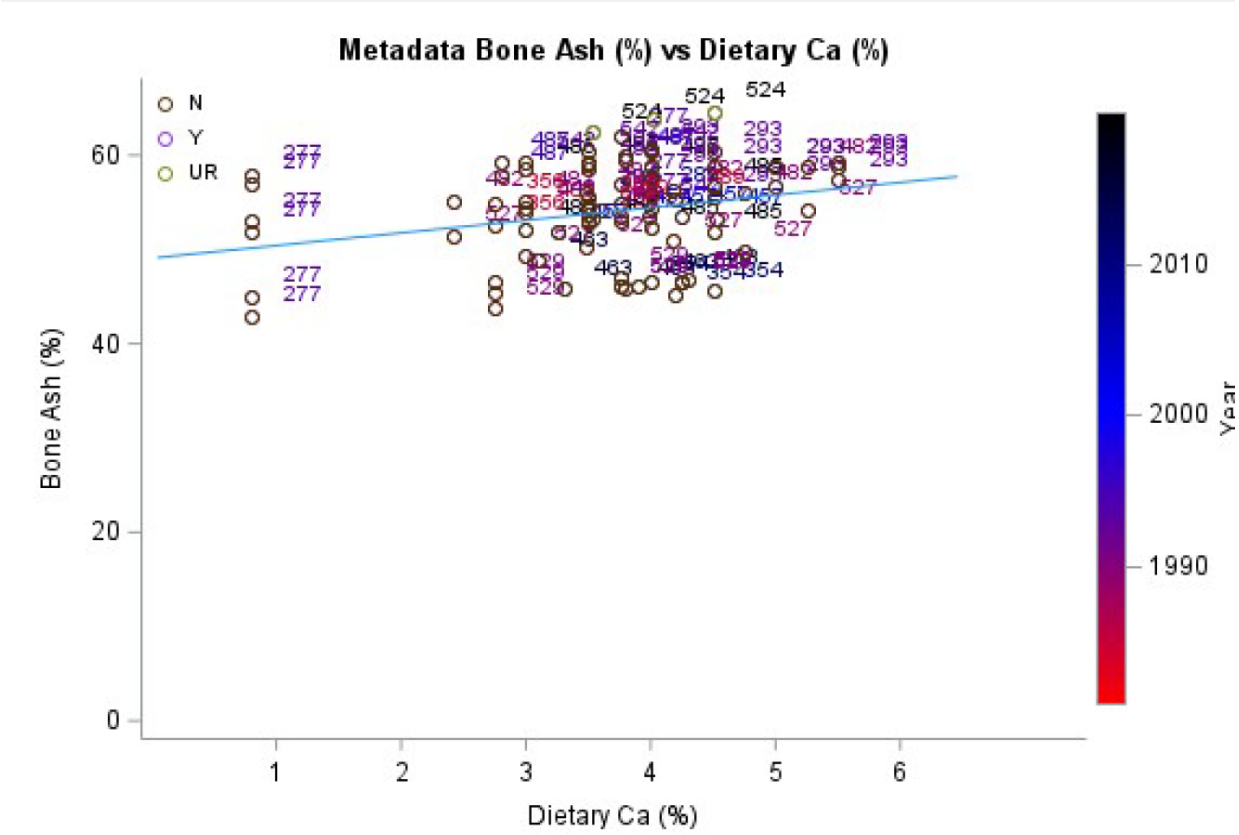

Figure 10. Impact of dietary calcium (%) on bone ash (%) in laying hens (Y = 50.49 (+ 1.84) + 1.03 (+ 0.32)x, *P*<0.0021)

Figure 11 Calcium recommendations

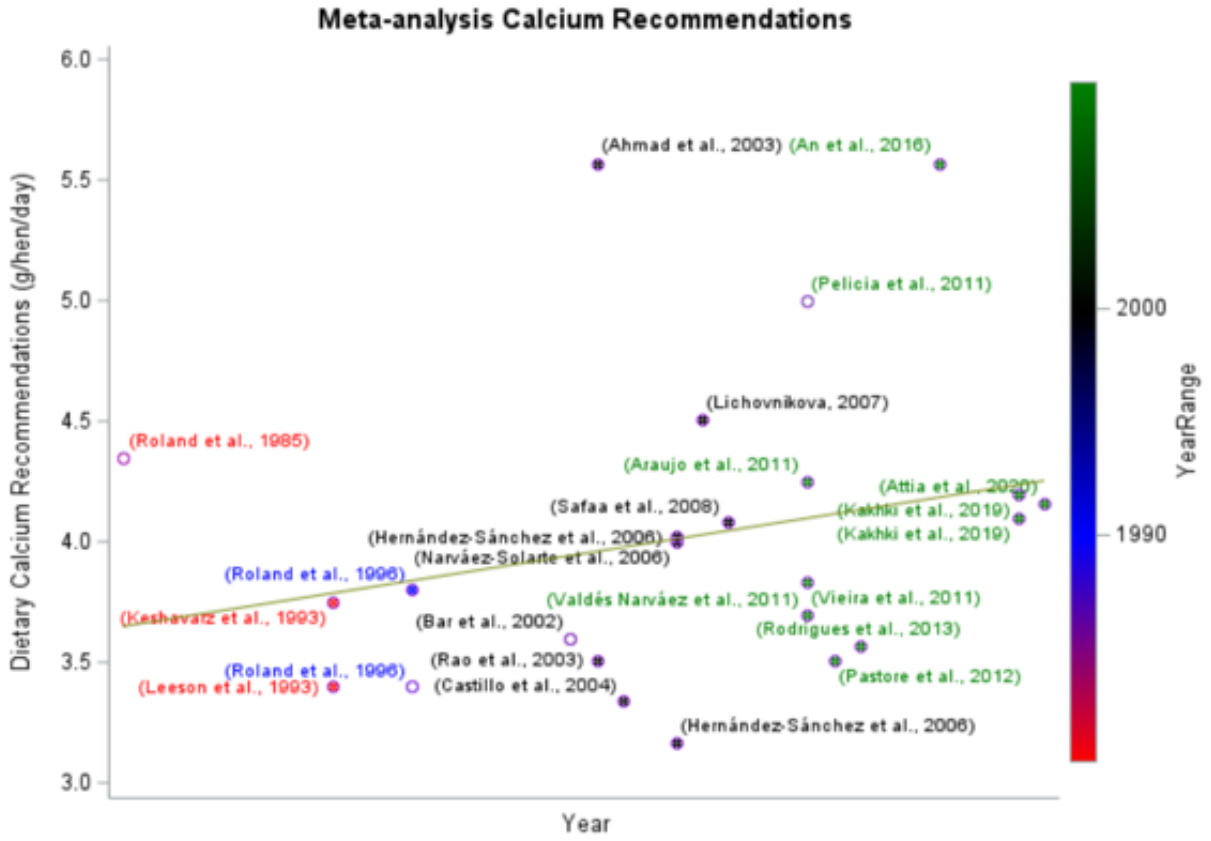

Figure 11. Calcium recommendations (g/hen/d) from various publications between 1980 and 2020.