7-2015

Novel Biomarkers for Calcium and Phosphorus Metabolism in Breeder Hens and Broilers

Andrew Dick Magnuson
University of Arkansas, Fayetteville

Novel Biomarkers for Calcium and Phosphorus Metabolism in Breeder hens and Broilers

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Poultry Science

by

Andrew Magnuson
Cornell University
Bachelor of Science in Animal Science, 2013

July 2015
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

_____
Dr. Craig Coon
Thesis Director

_____
Dr. Charles Maxwell
Committee Member

_____
Dr. Narayan Rath
Committee Member

Abstract

Broiler breeder hens are subject to the dual expectation to not only maintain a high production of eggs for an extended time period, but to produce eggs which can support the life of chicks which will be used for either meat production or as parent stock. Egg fertility and hatchability are heavily influenced by the thickness of the egg shell, the mineral calcium carbonate shell of the egg necessary for protecting the embryo growing inside. Many factors affect egg shell quality including age of the hen, diet, environmental conditions, genetic strain, stress, disease, and nutrition. Laying hens will mobilize calcium from the medullary bone to synthesize the calcium carbonate of the egg shell and use the calcium they absorb from their diet to replenish this medullary bone. Phosphorus is necessary for many cellular functions in animals and it also affects the availability of calcium making the ratio between the two minerals crucial not only egg production but also bone and whole body health. Previous studies have indicated a phosphorus retention threshold in breeder hens and broilers, the point when the amount of available phosphorus in the diet begins to be released into the excreta instead of being utilized in the body. In order to gain further understanding of why this phosphorus retention exists many elements involved in the whole body homeostasis of calcium and phosphorus in laying breeders have been investigated. The first study involved 6 dietary treatments of diets which consisted of 6 graded levels of non phytate phosphorus (NPP) ranging from 0.15% to 0.40% NPP with increments of 0.05 to determine the amount of phosphorus necessary for optimal egg production and to find out FGF23's involvement in the retention threshold of phosphorus. Results showed that between 0.20% and 0.25% NPP that phosphorus retention dropped from 33% to 26% while FGF23 levels increased from 0.15% to 0.20% NPP and remained at the same concentration regardless of further increases in NPP in the diet. In the second study the relationship between

bone health and egg shell quality was studied through the use of biomarkers for Osteoblast and Osteoclast activity: Tartrate acid resistant phosphatase (TRAP), and Bone alkaline phosphatase (BAP) respectively in both breeder hens and their progeny. Breeder hens were selected upon egg shell quality through Dual Energy X-ray Absorptiometry (DEXA) and split into two groups with either good shell quality of specific gravity >1.80 or poor egg shell quality with specific gravity <1.80 and their eggs were placed and grown until 2 weeks of age. Egg shell quality was shown to correlate negatively with TRAP and positively with BAP. Progeny of the poor egg shell quality hens had lower levels of BAP and high levels of TRAP compared with the progeny of the good egg shell hens.

Key words: bone homeostasis variables, shell quality, phosphorus metabolism, biomarker

Table of Contents

Introduction

Introduction

In egg laying animals calcium and phosphorus are the most important macro minerals necessary for egg shell development. Formation of calcium carbonate in the shell gland of laying hens requires calcium from both the diet and or medullary bone to meet the amount needed for egg shell synthesis. Proper amounts of calcium and phosphorus and the ratio between the two are necessary for diets for laying hens due to how important the minerals are for not only egg shell synthesis, and bone turnover, but also for maintaining calcium and phosphorus homeostasis throughout the body and circulating blood supply. While approximately 99% of calcium is stored in the body in the form of $Ca_{10}[PO_4]_6[OH]_2$ hydroxyapatite in the bones and teeth, for mammals, it is also used in the circulatory system, extracellular fluid, muscle, and other soft tissue for regulating vascular constriction, vasodilation, nerve transmission, hormonal secretion, muscle function, and intracellular signaling (Ross et al., 2011). Only 85% of phosphorus in the body is found in the form of hydroxyapatite due to its essential use in the creation of the following compounds: nucleic acids for DNA and RNA, ATP, creatine phosphate, cell membrane phospholipids, and many different enzymes and hormones. Phosphorus is also used for cell growth and maintenance, muscle contractions, kidney function, regulation of heart rate, transmission of nerve impulses, maintaining body blood pH, and it is used sometimes as a co-factor for other minerals and vitamins including vitamin D, iodine, magnesium, and zinc (USDA, 2005). Proper balance of calcium and phosphorus in the body is essential as circulating levels of the two minerals are under tight circulation through many hormones and when one is in excess it will be promptly be removed via the kidney, or have its absorption through the S.I. blocked lowering digestibility.

Sources of phosphorus and calcium used in poultry diets are quiet costly due to the high amounts needed for egg shell production, and bone growth due to the monogastric G.I. system of avians with no post-gastric fermentation unlike other livestock. Limestone is the most common source of calcium in poultry feed obtained commonly from oyster shells and it is almost 100% available depending on the particle size which will change the transit time through the G.I. tract. Dicalcium phosphate and monocalcium phosphate are synthetic sources phosphorus and calcium created through the reaction of calcium carbonate and phosphoric acid and they are the most used source of phosphorus supplementation in poultry diets. Other sources of phosphorus may come plant matter in the diet in the form of either phosphate (PO_4^{3-}) or pyrophosphate ($P_2O_7^{4-}$). Both of these forms of phosphorus are consider organic sources of P, which is mostly compromised of phytate. The inorganic forms of phosphorus are known as non-phytate phosphorus (NPP). The purpose of NPP or phytic acid is to serve as a storage molecule for both phosphorus and inositol in plants, however it is mostly unavailable in poultry with up to only 1/3 of the phosphorus comprised of NPP being digestible. Excess phosphorus in the diet leads to phosphorus accumulation in poultry litter which can have detrimental effects for the environment (Pote et al., 1997). Phosphorus deficiencies can lead to rickets in growing chicks, and osteoporosis, osteomalacia, and thinner egg shells in laying hens (Long et al., 1984; Nieves, 2005; Roberts, 2004). The proper amount of calcium and phosphorus and ratio between the two in the diet is necessary to prevent wastage or deficiency as the amount of either one in the blood changes how the body views the other.

Studies have shown that the current NPP requirements for breeder hens and broilers overestimate the amount of phosphorus that is truly needed (Leske and Coon, 1999; Manangi and Coon, 2008; Ekmay and Coon, 2012). Work by Coon et al. has shown that current industry

requirement of 0.40% NPP may be almost twice that which is necessary for proper maintenance in breeder hens and growth in broiler hens. Throughout the studies by Coon et al. there is a threshold where phosphorus excretion increases dramatically and plasma inorganic phosphorus plateaus, approximately 0.23% NPP. Depending on the amount of calcium in the diet the threshold where phosphorus excretion begins to rapidly increase and blood phosphorus plateaus changes. This value of 0.23% NPP is much closer to the NRC requirement (1994) of 0.25%NPP and 3.25% calcium for laying breeder hens. Currently however there is no physiological explanation for why this phosphorus threshold exists although it may be tied into several hormones responsible for regulating calcium and phosphorus metabolism including one or more of the following: Parathyroid hormone (PTH), Calcitonin, Phosphate regulating endopeptidase homolog, x linked (PHEX), Matrix extracellular phosphoprotein (MEPE), Dentin matrix acid phosphoprotein (DMP1), Receptor activator of nuclear factor kappa-B ligand (RANKL), and Fibroblast growth factor 23 (FGF23). Egg shell synthesis from laying hens doesn't solely depend on the available calcium and phosphorus from the diet but also from the reserves in the bone.

Bone turnover is a major contributor to the calcium necessary for egg shell synthesis in the egg shell gland. Osteoclasts are specialized cells in bone which are responsible for the resorption of calcium and phosphorus from the hydroxyapatite mineral complex in the bone. Osteoclasts dissolve the protein-mineral complex through the collagenase cathepsin K, and tartrate resistant acid phosphatase (TRAP)(Väänänen et al., 2000). Osteoblasts conversely are cells in the bone used for building the mineral complex through the enzyme activity of bone alkaline phosphatase (BAP) which will create a high concentration of phosphorus through cleaving phosphate groups off nearby molecules. Once a high concentration gradient of both calcium through active transport and phosphorus through BAP activity are achieved then

hydroxyapatite can be synthesized at the site of bone construction through the following reaction: $6\ HPO_4^{2-} + 2\ H_2O + 10\ Ca^{2+} \rightleftharpoons Ca_{10}(PO_4)_6(OH)_2 + 8\ H^+$ (Neuman et al., 1958). Blood concentration of plasma and calcium isn't solely dependent upon bone mobilization and what the birds eat, however, as calcium and phosphorus absorbed through the S.I. are under regulation from the body.

Calcium enters the blood system through the G.I. tract via calcitonin, the active form of vitamin D and cytosolic calcium binding protein (calbindinD(9k)). There are two mechanisms under which calcium is absorbed through the S.I.: transcellular active transport of which a majority occurs in the duodenum and upper jejunum; and paracellular passive transport that occurs through the whole intestine. The transcellular transport consists of three steps: entry across the brush border, regulated by the calcium selective ion channel (CaT1), intracellular diffusion, controlled by calbindinD(9k), and lately extrusion, mediated by Ca-ATPase. The transcellular absorption of calcium will change depending on the bodies need for calcium whereas the paracellular absorption is constant (Bronner, 2003). Both CaT1 and calbindinD(9k) are regulated by calcitriol and thus will lower in function when the synthesis of the active form of Vitamin D is inhibited (Zhuang et al., 2002). Phosphorus is absorbed through the type IIb sodium phosphate cotransporter (Na-Pi-IIb cotransporter) which is located in the brush border membrane of the epithelium in the duodenum and jejunum (Angel, 2007). The rate of phosphorus absorption through the S.I. is controlled through its transporters as shown when broilers were challenged with a low phosphorus total the gene expression and total protein of the NaPi-IIb increase, and lower in response to high concentrations of phosphate (Fang et al., 2012). Controlling the amount of phosphorus and calcium entering the blood supply isn't the only way

the body regulates their levels as excretion of the two macro minerals through the kidneys is the fastest way for the body to lower their concentrations (Wideman, 1983).

Plasma phosphorus is found in one of three different states including ionized, complexed, and protein bound (Fuchs, 1980). Only the protein bound form which composes 25% of the plasma phosphate is un-filterable by the glomerulus of the kidney while ionized and complexed, that is bound to Ca2+, Mg2+, and Na+, can be filtered. The type IIa sodium phosphate cotransporter (Na-Pi IIa), the complement to the S.I Na-Pi IIb, is the main transporter for the reabsorption of phosphorus in the kidney and is located in the apical membranes of the epithelial cells of the renal proximal tubules (Biber et al., 1996). The type I sodium phosphate cotransporter has also been located in the cortex of the kidney but it is not phosphorus specific and seems to be used for net ion balance (Broer et al., 1998). Roughly 97% of calcium that is filtered through the glomerulus is recovered due to how tightly regulated its concentration in the blood is. Unlike phosphorus transport, which occurs principally in the proximal tubule, calcium reabsorption occurs throughout the kidney including the proximal tubule, thick ascending limb, distal convoluted tubule, and the connecting tubule (Arjen et al., 2006). Parathyroid hormone is the principal hormone for regulating the excretion of phosphorus through the kidney in response to calcium levels, its function however is to increase ratio of calcium to phosphorus either by increasing calcium or decreasing phosphorus. Another hormone fibroblast growth factor 23 is entirely dedicated to being phosphaturic, a factor which increasing the concentration of phosphorus in the urine, which acts principally on the kidney by increasing phosphorus excretion by downregulating the Na-Pi type IIb transporter and decreasing the conversion of cholecalciferol to calcitrol (Liu et al., 2007). Laying hens face another complication for calcium

and phosphorus homeostasis as the shell gland will remove calcium from the blood changing the way the S.I., kidney, and bone will behave.

Regulation of phosphorus and calcium is critical for laying hens as the shell gland will remove calcium from the blood to synthesis the calcium carbonate while leaving behind the phosphorus to be excreted. Approximately 95% of dry egg shell is calcium carbonate, 0.3% phosphorus, and 0.3% magnesium and other trace minerals (Roberston, 2001). Genetics and the amount of calcium available to the hen during synthesis will determine egg shell quality. Other factors which affect egg shell thickness include: time spent in the shell gland, time of day when the shell is being synthesized, age of the hen, health of the bird, environmental conditions, and source of calcium in the diet (particle size). It takes roughly 25 hours to lay an egg beginning from an egg cell being released from the ovary to the final product coming out of the cloaca with 20 hours of that being time spent creating the egg shell in the shell gland (Joyner et al., 1987) During egg shell formation the ratio of calcium to phosphorus necessary for synthesis of calcium carbonate is 20:1, while the ratio of calcium to phosphorus liberated from the bone is 2.5:1 (Whitehead, 2004). Boorman (2001) reported that excess dietary phosphorus causes less calcium deposition in egg shells, perhaps due to less skeletal mobilization due to higher circulating plasma P levels which prevents calcium from being liberated through osteoclast bone resorption. Medullary bone is used as a calcium reservoir for egg shell synthesis while the structural cortical bone isn't mobilized (Bar, Hurwitz, 1984).

In summary the first objective of this study is to gain further understanding of the complex mechanisms by phosphorus retention threshold in breeder hens and broilers between the relationship of the kidney, S.I., and bone with FGF23 as the focus due to its phosphaturic nature. The second objective is to find out the relationship between bone turnover and egg shell quality

through the biomarkers of bone resorption and: building tartrate resistant acid phosphatase and bone alkaline phosphatase for osteoclast and osteoblast activity respectively.

Chapter 1

Literature Review

Phosphorus & Calcium Modulation

Phosphorus is necessary for many cellular activities used to maintain homeostasis in animals including the synthesis of nucleic acids, bioactive signaling proteins, phosphorylating enzymes, and hydroxyapatite (Berndt et al., 2005). Calcium is used in many essential cellular functions as well including mediating nerve transmission, muscle function, intracellular signaling, hormone secretion, vascular contraction, and vasodilation. Both minerals are regulated through their concentrations in the blood and the surrounding tissue. Parathyroid hormone is the chief hormone for maintaining adequate levels of calcium in the blood as it works with the bone, kidney, and indirectly the small intestine. The individual concentrations of calcium and phosphorus are important but the ratio between the two minerals also affects how available each of them is to the body due to them complexing in the blood. When the ratio of calcium to phosphate increases more calcium ions are free in the circulation. Free calcium in the blood is principally used for bone synthesis in growing broilers, and for egg shell synthesis in mature laying breeder. Phosphorus is necessary for hydroxyapatite synthesis; however, it is not as important for calcium carbonate synthesis in laying hens and will often be removed to make calcium more available. Modern nutritionists used a phosphorus requirement in the diets of poultry is that much higher than necessary to be safe due to how important phosphorus is for growing broilers and laying hens (Waldroup, 1999). Work by Coon et al. has shown that when broilers and breeders are fed diets consisting of NPP levels of 0.23 or lower that phosphorus in the excreta, and blood begin to decline signifying that there is a phosphorus retention threshold. Understanding this phosphorus threshold is important not only for maximizing the phosphorus we use in poultry diets but also to improve bone and egg shell quality. Fibroblast growth factor 23 (FGF23) is a hormone entirely devoted to being phosphaturic which will act to increase

phosphorus in the urine and prevent more phosphorus from entering the blood. Given that FGF23 is dedicated to regulating phosphorus independent of calcium it is very likely that it is involved in this phosphorus retention threshold

Hormone & Factor Regulation

While phosphorus and calcium are necessary for many cellular functions in the body, the accumulation of the two minerals in the tissue can lead to soft tissue calcification, excess buildup of calcium salts in soft tissue, which can lead to cellular death and organ failure (Bertazzo, 2013). The main hormone for the regulation of calcium in the blood is parathyroid hormone (PTH), an 84 amino acid long hormone produced by chief cells in the parathyroid gland. The main function of PTH is to bring calcium blood levels within a certain range depending on the state of the body which can vary depending on age, bone development, production (milk, egg shell synthesis), disease, time of day, and many other factors. PTH acts upon many different portions of the body including the bone where it will liberate calcium from the hydroxyapatite through indirect stimulation of osteoclasts to cause bone resorption. Receptors for PTH in the bone are on osteoblasts which then cause RANKL, a promoter of osteoclast formation, to be released which inhibits the expression of Osteoprotegerin, a suppressor of osteoclast activity. Kidney function is also altered in response to PTH as Na-Pi IIb cotransporter are downregulated in response to PTH which will decrease the amount of phosphorus reabsorbed and lead to increased excretion. The conversion of the inactive form of vitamin D, cholecalciferol, to the active form calcitriol in the kidney is upregulated by PTH. Vitamin D causes more calcium to be absorbed through the small intestine through increased expression of the calcium transporting protein calbindin.

Calcitonin is the antagonist hormone of PTH as its main function is to reduce blood calcium levels. It is a 32 amino acid long hormone produced by the parafollicular cells in the thyroid. Calcitonin will inhibit osteoclast function in the bones and increase osteoblast activity, bone building and hydroxyapatite synthesis. Net absorption of calcium through the small intestine is lowered in response to calcitonin. Reabsorption of calcium in the kidney is lowered due to calcitonin increasing the amount of calcium in the urine. The reabsorption of phosphorus is also lowered in response to calcitonin, one function that is the same for PTH (Boron, 2004).

Receptor activator of nuclear factor kappa-B ligand (RANKL) is a tumor necrosis factor that is involved in both the immune system and for regulation of bone turnover and remodeling. RANKL is found is many tissues and organs including skeletal muscle, liver, colon, adrenal gland, thymus, pancreas, epithelial cells, prostate, mammary gland, and osteoclasts. While RANKL isn't directly involved in the balance of calcium and phosphorus it part of the regulation for bone resorption and bone building which are impacted by the hormones PTH and Calcitonin. Osteoclasts are the principle cells for bone resorption as they dissolve the hydroxyapatite and collagen through the enzymes TRAP and cathepsin K which liberates calcium and phosphorus into the blood (Wada et al., 2006). RANKL is synthesized in response to the bodies needed to resorb bone and it will bind to its receptor RANK on osteoblasts. Once osteoblasts are stimulated by RANKL they will differentiate into osteoclasts. Osteoprotegerin (OPG) is a cytokine receptor that acts as a decoy receptor for RANKL. The purpose of OPG is to reduce the amount of osteoclasts that are formed, once more in response to the current conditions of the body. The female sex hormone estrogen has been found to increase the amount of OPG, effectively increasing bone mineral density and preventing osteoporosis (Bateman et al., 2002). Given that

RANKL is necessary for the differentiation of osteoclasts it is effectively a middle man between mineral concentration and bone mobilization.

Phosphate-regulating neutral endopeptidase, X-linked (PHEX) is a newly discovered enzyme involved in bone mineralization and renal phosphate reabsorption. PHEX is thought to oppose the effects of phosphaturic hormones such as FGF23, and factors which oppose mineralization such as Osteopontin. Downregulation of PHEX has been shown to induce osteomalacia, hypophosphatemia, and rickets (Barros et al., 2013). PHEX is different from most of factors regulating phosphorus homeostasis in that its presence will increase the reabsorption of phosphorus from the kidney.

Sclerostin is a glycoprotein secreted by osteocytes, mature osteoblasts, and chondrocytes, cartilage cells, that down regulate the synthesis of bone formation. The proposed pathway for how this occurs is that Sclerostin inhibits the Wnt signaling pathway, a cascade of proteins that cause a net signal transduction necessary for cell proliferation, cell migration, and cell fate specification. Without the Wnt signaling pathway new bone cannot be formed due to the inability for the cells to coordinate and grow together to form new tissue. The Wnt signaling pathway has also been shown to regulate calcium inside cells (Nusse et al., 1992). Ultimately the differentiation of osteoblasts is inhibited by sclerostin produced by osteocytes and chondrocytes as part of an internal regulation of the bone use in bone remodeling and turnover.

Osteopontin is protein that regulates the mineralization throughout the whole body and is primarily used to prevent soft tissue calcification. Osteopontin has an overall negative charge due to containing many negatively charged amino acids including aspartate, glutamic acid, and serine. The negative charge allows for Osteopontin to bind to calcium available at crystal

surfaces in different bio-minerals including hydroxyapatite. Many different cells throughout the body synthesis Osteopontin including fibroblasts, osteoblasts, osteocytes, odontoblasts, chondrocytes, dendritic cells, macrophages, smooth muscle, myoblasts, endothelial cells, brain, kidney, placenta, and the uterine wall. Strangely Osteopontin is synthesized by all 3 cell types involved in bone formation and synthesis, osteoblasts, osteoclasts, and osteocytes. Osteoclasts produce Osteopontin in order to begin bone resorption as it is the first anchor for breaking down hydroxyapatite. Osteopontin is also found in the urine where its main function is to inhibit kidney stone formation (Addison, 2008). Osteopontin is antagonized by the bone mineralization stimulating hormone PHEX which will rapidly degrade Osteopontin upon contact in order to stop bone resorption.

Osteocalcin is a hormone produced by osteoblasts which links tissue development with bone growth. The target site of Osteocalcin's function is the beta cells of the pancreas to produce insulin, and adipose cells to create adiponectin, a hormone which increases sensitivity to insulin (Lee et al., 2007). Osteocalcin has also been found to increase testosterone production in males through acting upon leydig cells in the testis (Karsenty, et al., 2014). While the exact mechanism behind Osteocalcin and bone growth is unknown it has been found to be positively correlated with bone mineral density and seems to be produced by Osteoblasts during periods of bone mineralization.

Matrix extracellular phosphoglycoprotein (MEPE) is a glycoprotein produced by both osteoblasts and osteocytes which upregulates the synthesis of FGF23 and inhibits bone mineralization. MEPE has been shown to inhibit PHEX, the principal enzyme involved in promoting bone mineralization. Abnormally high levels of MEPE have been associated with

osteoporosis and osteomalacia further supporting its suppression of bone mineralization (Yamada, et al., 2004).

Dentin matrix acidic phosphoprotein 1 (DMP1) is a protein produced by osteoblasts that promotes bone mineralization and formation. The effects of DMP1 mirror the opposite of what MEPE does by both down regulating FGF23 and stimulating the release of the enzyme PHEX. Mutations in the genes coding for DMP1 have been shown to cause hypophosphatemia which can ultimately lead to rickets and osteomalacia indicating how important DMP1 is for bone formation (Hirst, 1997).

Fibroblast growth factor (FGF23) is a 251 amino acid long hormone that is synthesized by osteocytes in the bone in response to excess phosphorus accumulation. Among all of the other proteins secreted in the bone FGF23 is unique in that it acts not only locally by indirectly suppressing bone mineralization through antagonizing PHEX, but also by acting on serval organs including the kidney, parathyroid gland, pituitary gland, and choroid plexus. The receptor for FGF23, Klotho, has been found predominately in the kidney and PTH where FGF23 has the most profound effects: promoting the net secretion of phosphorus into the urine and increasing the synthesis of PTH (Liu et al., 2007). Another impact FGF23 has on the kidney is its inhibition of the conversion of cholecalciferol to calcitriol, indirectly reducing the amount of calcium and phosphorus absorbed through the small intestine. Factors which affect the expression of FGF23 include circulating phosphorus, calcitriol, PHEX, DMP1, MEPE, and phosphorus buildup in tissue. Overall the purpose of FGF23 is to reduce phosphorus independent of calcium or the calcium to phosphorus ratio making it the only hormone that influences the kidney Na-Pi type IIb cotransporter that isn't affected by calcium.

Bone building and resorption

Growing breeders and broilers undergo two stages of bone development: Endochondral ossification which is response for longitudinal growth in long bones, and intramembranous ossification necessary for long bone widening. These two processes are how cortical bone is remodeled in hens which are moderated through osteoclast activity through the activity of many cofactors including PHEX and Osteopontin. Beginning at sexual maturity in female breeders increased estrogen production causes the formation of medullary bone, a specialized type of bone found only in lizards and birds. Medullary bone acts as a reservoir of calcium to be mobilized when needed for egg shell synthesis or for maintain calcium homeostasis throughout the body. A majority of medullary bone is located in the long flat bones such as the tibia and femur. Throughout a laying period hens can lose a lot of bone due to being at a net calcium deficiency while maintaining egg production, however, bone can be rebuilt and brought back to normally mineral density once out of production. While hens may also draw heavily upon their mineral reserves in their bone they are still able to maintain bone strength due to preserving their collagen crosslinks. Hydroxyapatite the mineral portion of the bone isn't the only part which gives it strength however, as the collagen crosslinks which bind the mineral together as shown to be just as important for bone strength (Wrath, 2000).

The two cell types responsible for bone resorption and are Osteoclasts, specified cells responsible for bone resorption through enzyme activity of TRAP and cathepsin K, and Osteoblasts which are the antagonists of Osteoclasts as their function is to build bone and form

hydroxyapatite and collagen. The hormones and cofactors which upregulate the mineralization and formation of new bone include: DMP1, PHEX, and Calcitonin. The hormones and cofactors which downregulate the mineralization and formation of new bone include: MEPE, Osteopontin, Osteocalcin, Sclerostin, PTH, RANKL, Vitamin D, and FGF23. During the laying period the egg shell gland in hens will rapidly deplete the calcium from the blood to synthesis calcium carbonate. When calcium levels in the blood reach a certain threshold the factors and hormones mentioned above will begin to mobilize hydroxyapatite from the bone to liberate more calcium, along with reabsorbing more calcium through the kidney and absorbing more calcium from the small intestine. Depending on the time of day under which the egg shell synthesis occurs will the body of the hen depend more on the calcium in the bone or in the diet. During the normal day-night sequence egg shell synthesis will occur during the night when the G.I. tract is empty causing more bone to be mobilized to meet the requirements of the shell gland. Once calcium homeostasis is restored after the egg shell is synthesized the medullary bone can be rebuilt utilizing calcium from the diet through osteoblast activity via bone alkaline phosphatase.

Kidney Regulation

The principal hormones which regulate phosphorus and calcium levels, PTH, Calcitonin, and FGF23, act not solely on the bone or S.I. but on the kidney to increase or decrease blood mineral levels through net secretion or retention. Phosphorus and calcium are both separated from the blood when filtered through Bowman's capsule and the reabsorption of each mineral depends upon specific transporters within the kidney. Approximately 97% of calcium is absorbed under normal conditions signifying how important retaining calcium is to body homeostasis. A majority of calcium reabsorption occurs in the proximal tubule and thick ascending limb of Henle's loop through passive diffusion. The remainder of the reabsorption

occurs in the distal convoluted tubule, and connecting tubule via transcellular reabsorption. Phosphorus reabsorption in the kidney fluctuates much more than calcium is it is the preferred mineral to remove in order to increase the calcium to phosphate ratio. The sodium phosphatase type IIa cotransporter is the principal transporter of phosphorus in the kidney and it is present in the proximal tubule. The Na-Pi type IIa transporter is pH sensitive and basic conditions will greatly increase its rate of transportation. Another transporter is phosphorus in the kidney, the type 1 Na-Pi cotransporter is found only in the cortex and is used for net ion balance, minimizing its influence on phosphorus balance (Werner et al., 1991). Phosphorus reabsorption is increased in response to PHEX, the bone mineralization promoting hormone synthesized by osteoblasts and osteocytes during bone growth and remodeling. Phosphorus reabsorption is decreased in response to PTH, Calcitonin, and FGF23 in order to either achieve a net high amount of calcium in the blood or to prevent bone mineralization.

Small Intestine absorption and control

While adult laying hens have reservoirs of calcium and phosphorus stored in the hydroxyapatite of their medullary bone they are still reliant upon mineral intake through their diet to replenish this bone. Growing broilers are completely reliant upon the minerals in their diet making the uptake of calcium and phosphorus through the G.I. essential for maintaining mineral homeostasis. Calcium uptake through the S.I. is regulated through the formation of calcitriol, the active form of vitamin D. Hormones and factors which affect the conversion of cholecalciferol to calcitriol include PTH, Calcitonin, and FGF23. In the presence of calcitriol calbindin is able to bind free calcium in the lumen of the small intestine and transporter it across the brush border membrane. The majority of calcium absorption occurs in the duodenum and upper jejunum. When there is a higher concentration of calcium in the diet then the body will preferably absorb

it through paracellular passive diffusion involving the transportation of calbindin bound calcium through the brush border membrane. Under conditions of lower calcium the body will use transcellular calcium transport to maximize its absorption (Bronner, 2003). Phosphorus is reabsorbed in the duodenum and jejunum via the type IIb sodium phosphate transporter expressed in the brush border membrane. The phosphate absorption in the duodenum is less sensitive to changes in phosphorus in the lumen while expression of the type IIb cotransporters in the jejunum will change depending on the phosphate concentrations (Fang, 2012).

Phosphorus Retention Threshold

Laying broiler breeder hens must not only fulfill the phosphorus and calcium requirements in their body for cellular homeostasis but also deposit a portion of that calcium into the eggs they synthesis every day. Calcium liberated from hydroxyapatite will lead to phosphorus loss and will change the bird's phosphorus requirement. Total phosphorus requirements for laying breeders thus are complex and are effect not only by the amount of phosphorus required to synthesis egg, but also what they lose due to bone mobilization and digestibility of the diet they consume (Ekmay, 2010). The NRC daily NPP requirement for broiler breeders is 250mg/100g of feed per hen, or 0.25% of the total diet. Currently the industry requirement is 0.40% NPP in the diet which well exceeds that is needed for breeder production, however, due to how essential phosphorus is for bone and cellular health in breeders nutritionists use this high value as a safety margin. Studies have shown that increasing NPP in the diet above 0.4% has no benefit on production while studies which have lowered the available phosphorus below this requirement show that there is little change in egg production, weight, fertility, hatchability, or hatching weight until below the NRC requirement of 0.25% NPP (Leske and Coon, 1999; Manangi and Coon, 2008; Ekmay and Coon, 2012). Chandramoni et al. (1998)

found that breeders feed 0.32% available P did not have improvements in egg production, shell weight, SWUSA, and egg content when the NPP was increased. Keshavarz (2000) reported that there was no difference in egg production when layer hens were fed low levels of NPP, 0.15%-0.25%. Feeding high levels of NPP (1.0%) has affected the bone ash and strength of progeny compared to 0.2% NPP (Triyuwanta and Nys, 1992), however, bone ash and strength for the same chicks at week 2 and 7 weren't different. Plumstead et al., (2007) found decreased fertility in breeders fed NPP below 0.37% but a total increase in chicks per hen. Coon (2007) reported that increasing NPP above 0.23% in broilers marked a break point where phosphorus in the excreta would increase linearly with increasing NPP in the diet.

Chapter 1

Effects of dietary NPP intake on production performance, phosphorus retention, and FGF23 expression in broiler breeders.

Introduction

Phosphorus is an essential mineral for basic cellular function including the synthesis ATP and nucleic acids, and is also necessary for hydroxyapatite bone formation. Breeder broiler hens during egg production have high demands for calcium and phosphorus in their diet with the current requirement estimated at 0.40% NPP to fulfill their metabolic needs. Breeder hens rely upon medullary bone to supply calcium for the shell gland to make calcium carbonate synthesis and a lot of phosphorus is wasted during this process due to very little phosphorus being utilized in egg shell synthesis. Previous studies have shown that both breeder hens and broiler chicks have a phosphorus retention threshold at approximately 0.23% NPP where the amount of phosphorus in the urine remains constant and then suddenly increases rapidly with increasing NPP in the diet. The mechanism behind this phosphorus threshold is unknown as performance parameters such as body weight gain and feed conversion ratio continue to increase along with NPP from 0.23% to 0.40% despite the overall decrease in phosphorus retention. New proteins and hormones involved in phosphorus and calcium metabolism have been discovered over recent years, one of them being fibroblast growth factor 23 (FGF23). FGF23 is secreted by osteocytes in the bones in response to high levels of phosphorus in the blood and high concentrations of calcitriol and is the only known phosphaturic factor which acts independently of calcium. Recent work involving colostomy of breeder hens and broilers has shown that phosphorus excreted during the change in retention ends up in the urine signifying that the kidney is responsible for the phosphorus removal which points towards FGF23, as a phosphaturic factor, regulating this retention threshold. The purpose of this study is to further investigate this phosphorus retention threshold in breeder hens at 0.23% NPP to physiologically find out why this occurs and the possible connection with FGF23 concentration in the blood.

Materials and Methods

All procedures regarding the use of live animals in this study were carried out in accordance with the Animal Use Protocol 13002, which was approved by the University of Arkansas Institutional Animal Care and Use Committee.

Animals and Handling

A flock of 850 Cobb 500 hens was delivered to the production house at the age of 20 weeks. Each hen was assigned an identification number and individually caged (47 cm high, 30.5 cm wide, 47cm deep) with separate feeders and water nipples. Hens were offered standard Cobb feed daily using the amounts recommended on the Cobb Breeder Management Guide (Cobb-Vantress, 2005). Daily allotted feed intake was restricted and was increased every 8 percent increase in egg production beginning from 5% production to peak. The farm where the birds were kept was environmentally controlled with regards to temperature (22 °C) and humidity. Lighting schedule began with 12 hours per day at week 21 of age and increased one hour for the next two weeks until reaching its apex at 14 hours per day. Light duration was further increased to 15 and 16 hours at 20%, and 50% egg production respectively. As soon as the flock reached the farm their egg production was recorded daily, and egg weights recorded two days a week. Eggs which were soft shelled, cracked, dirty, or double yolk were recorded as such.

Balance Study

Beginning at week 32 a total of 144 breeder hens of average body weight were switched from the standard Cobb feed to 6 experimental diets, 24 hens per diet, containing 0.15% NPP through .40% NPP increasing at 0.05% increments. All egg weights were recorded daily starting at the beginning of the balance study as well to account for phosphorus in the eggs. At week 35 a

total collection of excreta was conducted for 5 days during which all excreta was collected. Excreta and egg samples were freeze dried and sent to the University of Arkansas Central Analytical Laboratory for mineral Ca, and P analysis. At the end of the fourth week of the balance study blood from drawn from the hens from the wing vein with regards to oviposition and stored in heparinized blood collecting tubes. Hens were then euthanized through CO2 gas asphyxiation and right tibias removed from measurement of breaking strength and bone ash concentration. Blood samples were immediately centrifuged using the methodology described by Tuck (2009) for plasma separation, and then stored at -20 °C until they were analyzed for plasma inorganic phosphorus, or fibroblast growth factor 23.

Plasma chicken specific fibroblast growth factor 23 was analyzed using a quantitative competitive immunoassay test kit (Neobiolab, USA). Plasma inorganic phosphorus was measured using a colorimetric assay involving the formation of phosphomolybdate complex from phosphorus and ammonium molybdate to create a molybdenum blue color complex (Pointe Scientific, USA). Tibias were analyzed for bone-breaking force by the sheer force measurement method described by Wilson (1991), utilizing an Instron Universal Testing Machine (Model 1123, Instron Corp., Canton, MA). Following the sheer test, the tibias were defatted in a container of 180 alcohol for 24 hours followed by refluxing in petroleum ether in a Soxhlet apparatus for 48 h. The defatted tibia samples were oven-dried at 110°C for 24 hours and ashed in ceramic crucibles for 24 h at 600°C. Ash content was determined as dry, fat-free tibia and expressed as grams of ash/bone and as a percentage of the defatted tibia weight.

Statistical Analysis

A completely randomized design was used to analyze data through the use of the ANOVA procedure of SAS (version 9, SAS Institute, Cary, NC). When necessary trend

contrasts were used to determine line shape. Data was also analyzed for linear and quadratic regression to determine curvilinear responses to dietary NPP. All statements of significance are based on testing at $P \leq 0.05$.

Results

No statistical differences were found between egg production and increasing NPP in the diet (0.135) (Table 2). Egg production as a percentage of all the hens in the experiment versus each individual treatment fed a graded level of NPP was found to not be significantly different across treatments (P=0.512). There was no effect of NPP in the diet on mortality (P=0.499). Change in body weight of the hens from the beginning of the study and at the end was not effected by NPP in the diet (P=0.499), however hens fed 0.40 NPP, the highest level of NPP, had the highest body weight average. There were no treatment effects on egg weight, however breeders fed 0.40% NPP had highest the egg weight average. Total excretion of phosphorus increased with total phosphorus in the diet ($P < 0.0001$) including an inflection point at 0.25% NPP where phosphorus excretion increased much more rapidly (Table 3). Total phosphorus retention decreased at 0.25% NPP in the diet and continued to decrease as total NPP in the diet increased. Total phosphorus excretion was highest above 0.30% NPP which coincide with the different in total phosphorus retention. Total phosphorus retention did not show a significant linear response to increase NPP in the diet. Diet had no effect on tibia bone ash percentage (P= 0.563), however hens fed the diet comprising of the highest level of NPP, 0.40%, had the highest percentage of bone ash.

There was a significant diet effect plasma inorganic phosphorus as the concentration of phosphorus in the blood increased with NPP in the diet (P= 0.06)(Table 4). Plasma inorganic phosphorus did not have a significant linear response with NPP in the diet as it showed the

greatest increase between 0.15NPP and 0.25NPP; afterwards it plateaued at approximately 10mg/dl. Plasma FGF23 did not show a significant linear response to increase in NPP in the diet. There was a significant relationship between dietary NPP and plasma fibroblast growth factor 23 ($P < 0.001$). Plasma FGF23 increased between 0.15NPP and 0.20NPP afterwards it remained around the same concentration regardless of increasing NPP.

Discussion

Phosphorus and Calcium Homeostasis for Laying Hens

The currently accepted phosphorus requirement for broiler breeders doesn't fully account for the phosphorus deposited into the egg. For an average sized egg the concentration of P is approximately 116 mg, most of which is concentrated in the yolk and some in the egg shell and albumen (Romanoff et al., 1949). Due to lack of information the current requirement for NPP is close to 600mg/day or higher despite over half of it not being utilized. The mobilization of hydroxyapatite through osteoclast activity not only liberates calcium, but also phosphorus in the circulation which will cause a loss of phosphorus into the urine. As bone is mobilized for calcium the phosphorus requirement of breeders will increase due to this phosphorus excretion. Higher levels of total available phosphorus in the diet have been correlated with thinner egg shells (Boorman et al., 2001), perhaps due to the negative feedback of excess phosphorus in the plasma on bone resorption. The relationship between calcium and phosphorus is tightly regulated through many hormones and controlled through interactions between the kidney for excretion, and S.I. for absorption, and bone for replenishment of plasma mineral levels. High levels of phosphorus in the blood inhibiting bone resorption to release calcium for calcium carbonate synthesis is consistent with the findings of Plumstead et al. (2007) which showed an increase in total egg production when phosphorus intake was lowered. Bone health of laying hens may

actually dependent upon bone turnover through resorbing bone to mobilize calcium, and then rebuilding the medullary bone from calcium absorbed from the S.I. When bone resorption is low so to is bone formation (Van de Velde et al., 1984). Bones aren't comprised solely of the mineral complex hydroxyapatite but also water, and collagen protein. The protein crosslinks between the mineral portions of the bone contribute to the bones overall strength (Rath et al., 1999). In the current study hens were fed diets containing a constant concentration of 3.25% calcium and graded levels of NPP to isolate interaction between phosphorus levels in the diet and production parameters including egg production, egg weight, excreta phosphorus, and bone tibia ash. By maintaining a constant concentration of calcium and changing available phosphorus across diets the ratio of Ca:iP in the diet can be seen as well.

The current study shows that dietary non phytate phosphorus levels ranging from 0.15% to 0.40% in the diet had no impact on egg production for hens. This is consistent with that was seen in by several other investigators (Triyuwanta et al., 1992; Keshavarz, 2000; Boling et al,, 2000; Ekmay et al., 2010) that was were no difference in egg production for breeder hens, or laying hens fed 0.15% to 0.25% NPP. Ekmay et al. (2010) reported that lowering dietary NPP concentrations to 288mg/day would not affect egg production even at peak production when the hens have the greatest demand for calcium carbonate synthesis. While there was no difference when lowering NPP down to 0.20% of the diet or the equivalent of 288mg/day, when NPP was decreased even further to 0.15%, 216mg/day, was egg production significantly affected and mortality increased (Ekmay, 2009). Breeders fed diets with very low concentrations of available phosphorus (0.09%) with phytase were still able to maintain the same egg production as breeders fed diets containing high NPP (Plumstead et al., 2008). Phytase cleaves phosphate off of the inositol structure of phytic acid making the phosphate available for the hens to absorb through

the S.I. making phytase activity effectively increase the net NPP. It seems that broiler breeders have different phosphorus requirements not only depending on the amount of calcium in the diet, but also depending on their age and state of production. Under normal management as hen's approach and peak production they will be given more feed and longer hours of light in order to further stimulate egg production. Given what is known about the 25 hour cycle of egg synthesis most of the egg shell is made during the night when the G.I. tract and gizzard are empty, causing hens to rely upon medullary bone reserves to provide calcium necessary for the shell gland. Medullary bone is replenished during the day through osteoblast activity by drawing upon calcium absorbed from the S.I. from the diet. During peak production hens have less time to restore this medullary bone due to the constant egg production whereas as hens become older they will lay eggs in clutches and have a day in-between laying eggs to give the bone time to be rebuilt. While egg production was unaffected by dietary NPP in this study another aspect of this study to consider is that the birds were only offered these diets of graded NPP for a period of 28 days. Layer hens and broiler breeders are very resilient and will continue to lay eggs despite being at a nutrient deficiency for longer periods of time. Work by Ekmay et al. (2010) has shown the effect of feeding hens low NPP diets for long time periods of approximately 40 weeks with similar results as to what was seen in this study which may indicate that the hens can adapt for lower available phosphorus diets.

Total egg production from each treatment versus egg production for the entire flock of birds involved in the study was not significantly different for any individual treatment. This further supports that there was no impact on egg production from available phosphorus in the diet ranging from 0.15 to 0.40%. The principal calcium regulating hormone parathyroid hormone will act on the bone, kidney, and indirectly through the S.I. to raise calcium blood concentration.

While mobilizing hydroxyapatite from the bone will increase calcium and phosphorus, PTH will act to lower phosphorus in the blood by decreasing resorption of phosphorus in the kidney from the Na-Pi type IIb cotransporter and preventing phosphorus from being absorbed through the S.I. via the Na-Pi type IIa cotransporter. Calcium circulating through the blood will complex with phosphorus ions making it unavailable unless the concentration of phosphorus is decreased which is the reason why the body will excrete phosphorus when calcium is low. Hens fed the graded levels of NPP for the current study had the same concentration of calcium, 3.25% of the diet. Given that egg production was unaffected by the different levels of NPP means that the hens were able to complete the synthesis of calcium carbonate in the shell gland through the mobilization of their bones, and that they were able to rebuild their medullary bone at the same reason with sufficient calcium from the diet. During the day when osteoblasts rebuild the hydroxyapatite the excess phosphorus from the diets containing more than 0.25% NPP, or 288mg/day was removed via the kidney and excreted into the urine. Abnormally high concentrations of phosphorus can be easily removed through the kidney but chronic excess phosphorus in the blood can lead to calcium deposition in the soft tissue such as the adipose tissue, muscle tissue, and the organs of the body. While calcification occurs due to calcium build up, excess phosphorus in the blood can be responsible for this as higher calcium retention is caused by complexed calcium build up since it doesn't behave as free ionized calcium in the blood. As the complex of calcium and phosphorus increases eventually calcification will begin to occur in the arteries and tissue regardless of proper kidney function.

Mortality was not significantly affected by the graded levels of NPP in the diets. Ekmay et al. (2012) reported a sharp increase in mortality when feeding below 0.20% NPP, 288mg/day during a 40 week study. When he reached 0.15 NPP, 216mg/day at at peak production, egg

production remained constant but mortality was significantly affected. The limitation of the current study as stated earlier is the short length of 28 days. If the study were to continue for longer than the effect of low phosphorus on mortality would be made more apparent as breeders can make up for mineral deficiencies in the diet through relying upon medullary bone reserves.

There was no significant change in body weights of the hens from the beginning of the study versus their body weight at the end due to NPP in the diet. Historically body weight is indicative of the net homeostasis of an animal. If the animal is energy or protein deficient they will mobilize body reserves in order to compensate for what they aren't receiving from their diet. Mineral balance isn't as simple as that however, as changes in body composition due to mineral deficiency or excess may that be spotted through body weight alone. In a hypothetical situation where the hens were completely deficient or either calcium or phosphorus they would mobilize medullary bone to maintain egg production for a limited time before going out of production due to not being able to maintain mineral homeostasis. Less severe deficiencies would most likely be associated with decreased egg production for the same reason of mineral homeostasis. Calcium or phosphorus excess would be characterized by soft tissue calcification which can lead to joint problems and cardiovascular disease. If the breeders were under duress their feed consumption would decrease which would be attributed by a drop in egg production, or going out of production entirely, and a decrease in body weight. While body weight alone may not be indicative of the hens meeting the entire requirement for mineral balance from their diet it also rules out that nothing drastic has occurred.

Average egg weight was not significantly affected by the graded levels of NPP in the diet. Eggs produced by breeder hens are used to host life to ultimately create offspring to be grown to become parent stock themselves or to be used for meat production. Fertility and

hatchability of eggs are positively correlated with egg weight and egg shell thickness. Breeders while create smaller eggs often before stopping production due to being limited on the resources necessary to create them. The three components of an egg: the yolk, albumen, and egg shell are all synthesized from the hen and need amino acids, lipids, and calcium to create. The albumen of the egg is comprised almost entirely of protein and can be readily synthesized as long as the hen has enough protein in the diet with the proper amino acid profile. The yolk being made mostly of lipid and some protein is reliant upon the energy homeostasis of the hen to simply have enough calories at its disposal to be able to complete lipogenesis. Egg shell on the other hand requires the two essential minerals calcium and phosphorus, and ratio between the two in order to create the calcium carbonate. Eggs with lower weights tend to have lower specific gravities due to thinner egg shells. The more calcium that is available to the shell gland during egg shell synthesis the thicker the egg shell will ultimately be. While not a direct measurement of egg shell thickness, total egg weight can be used to approximate if the hens were meeting their requirements necessary for egg production. Due to egg weights not being significantly different due to NPP in the diet we can conclude that the hens were able to receive the nutrients from their diet necessary to normal eggs capable of fulfilling the nutritional requirements of the progeny.

Phosphorus Threshold & Impact of FGF23 as a Phosphaturic Regulator

Total excreta phosphorus was significantly affected by the NPP in the diet. Hens fed diets containing 0.15, and 0.20% NPP were not significantly different in their total phosphorus excretion, but were significant compared to the hens fed 0.25, 0.30, 0.35, and 0.40% NPP. Not much research has been conducted on hens fed low levels of NPP but previous work by Coon et al. has shown that there is an inflection point at approximately 0.23% NPP for both broilers and breeders where the amount of phosphorus in the excreta will rapidly increase (Leske and Coon,

1999; Manangi and Coon, 2008; Ekmay and Coon, 2012). Phosphorus will normally be excreted in response to parathyroid hormones attempt to maintain the proper Ca:P ratio in the blood as the excess complexing of calcium can lead to soft tissue calcification. It seems that birds reach the threshold where the body will begin to remove phosphorus from the system that it deems too much when available phosphorus in the diet reaches 0.23%. Work by Coon et al. has also shown that the phosphorus excretion threshold will also change depending on the amount of calcium in the diet which is consistent with the mechanism for PTH. Another hormone, Fibroblast growth factor 23, is released from the bone in response to high concentrations of phosphorus making it an entirely phosphaturic hormone which will reduce the reabsorption of phosphorus in the kidney through the Na-Pi type IIa cotransporter. The mechanism behind this phosphorus excretion threshold is unknown but it is most likely due the body trying to prevent tissue calcification.

Total phosphorus retention was significantly affected by NPP in the diet. Hens fed the diets containing 0.15, and 0.20% NPP had retentions that were not significantly different from each other, as seen with the total excreta phosphorus, but were significantly different from the hens fed 0.25, 0.30, 0.35, and 0.40% NPP. The phosphorus retention mirrors what was seen with the phosphorus excretion as the retention begins to rapidly decline past the 0.20% NPP diet declining from 34% retention to 22%. Even though the total available phosphorus in the diets offered to the hens was increasing, the rate of excretion was increasing at the same rate causing the retention to lower. Non-phytate phosphorus is 100% digestible and can be entirely absorbed into the body through the Na-Pi type IIb cotransporters in the brush border membrane of the duodenum and jejunum. The phosphate entering the blood must be exceeding the threshold of phosphorus that the body can tolerate and is being excreted in response, most likely due to a

combination of PTH, and FGF23. Both hormones act on the kidney by reducing the amount of phosphorus reabsorbed while PTH will stimulate osteoclast bone resorption and FGF23 will inhibit the synthesis of calcitriol to prevent more phosphorus from entering via the S.I., effectively lowering the digestibility of phosphorus.

Tibia bone ash was not significantly different for the hens fed diets containing different levels of NPP. While there was no significant difference between treatments the ash percentage from the hens fed the 0.40% NPP diet was the greatest. Work by Ekmay et al. (2010) found that feeding 0.15% NPP caused significant decrease in bone ash percentage, but no difference at 0.20% NPP and higher. The limitation of this current study once more is the 4 week duration which is not sufficient time to see the chronic effects of feeding below the required mineral concentration for laying breeder homeostasis, whereas the work by Ekmay et al. was done over a 40 week period. Everyday laying breeders will mobilize calcium from the hydroxyapatite of the medullary bone through osteoclast activity involving the enzymes TRAP and collagenase cathepsin K to break down the mineral and protein portions of the bone respectively. Once the egg shell is synthesized and the calcium demand from the egg shell gland is gone can the bones begin to rebuild utilizing calcium from the diet through osteoblast activity via bone alkaline phosphatase to create new hydroxyapatite and collagen crosslinks. If the body is in a mineral deficient then the medullary bone will eventually become depleted and the bone ash percentage will decrease as the hydroxyapatite is removed, whereas is there is a mineral surplus then the opposite situation will occur with medullary bone increasing and ash percentage going up. The body has a limit on the amount of bone synthesis which can occur due to perhaps prevention of soft tissue calcification as the body will even remove calcium when it is in excess through calcitonin activity to lower calcitriol and calcium reabsorption.

Plasma inorganic phosphorus was significantly affected by dietary NPP in the diet. Hens fed diets consisting of 0.15, and 0.20% NPP were not significantly different from each other, but they were significantly different from the hens fed 0.25, 0.3, 0.35, and 0.4% NPP. Plasma inorganic phosphorus has an inflection point at 0.25% where the phosphorus levels plateaued and ceased to decline, whereas at a 0.15 and 0.20% NPP the plasma phosphorus was steadily increasing until the plateau. The plasma phosphorus plateau inflection point somewhere between 0.20 and 0.25% seen here mirrors the phosphorus excretion threshold which indicates that once the NPP percentage in the diet reaches a certain level that plasma phosphorus will reach its maximum and the body will begin to remove phosphorus via the kidney. As mentioned earlier the body will remove phosphorus in order to prevent excess complexing of calcium and ultimately soft tissue calcification. As seen by the phosphorus plateauing in the plasma the body must have reached the point where it cannot allow any more phosphorus in the plasma and starts to throw it out of the system and into the urine through the kidney, and by reducing the amount of phosphorus absorbed through the S.I. most likely through the activity of PTH and FGF23.

Plasma fibroblast growth factor 23 was significantly affected by the NPP in the diet. Hens offered the diet containing 0.15% NPP had a significantly lower concentration of FGF23 compared to the hens offered 0.20, 0.25, 0.30, 0.35, and 0.40% NPP. The amount of FGF23 in the blood at 0.15% NPP is almost half of what is seen at 0.20% NPP and it plateaus in a similar pattern with what is seen with plasma inorganic phosphorus. The inflection point for FGF23 is in-between 0.15 and 0.20% NPP where the amount of plasma inorganic phosphorus in the blood reaches a point where FGF23 is secreted from the bone in order to reduce the phosphate in the body. Once can assume that at lower levels of NPP that FGF23 would decrease in the blood as well due to it being synthesized in response to high concentrations of circulating plasma

phosphate. What is interesting is that the plateau relationship of FGF23 is very similar to plasma inorganic phosphorus, except that it is shifted to over to the left by perhaps 0.05 NPP indicating that the phosphate in the blood causes FGF23 secretion to peak, followed by maximal phosphorus excretion at the next graded level of NPP. Once more the reason for preventing phosphorus build up in the blood is to limit the amount of complexed calcium there is in the system to avoid tissue calcification. There may also be a saturation of phosphorus and calcium in the cells of the muscle, adipose, and organs throughout the body which is causing a feedback loop to signal the removal of phosphorus. While FGF23 has a similar pattern compared to the excreta phosphorus retention, and plasma inorganic phosphorus inflection points, it doesn't match up with the 0.23% NPP threshold, as its threshold appears to be between 0.15 and 0.20% NPP. When FGF23 reaches maximum production from the bone there may be other hormones which are produced as part of the regulation of calcium and phosphorus that may explain the disparity in the thresholds.

In conclusion there was no difference in egg production, egg weight, mortality, tibia bone ash, or body weight for hens offered diets comprised of 0.15 through 0.40% at the 6 graded levels differing at 0.05% NPP. Total excretion of phosphorus was significantly affected as it began to rapidly increase past 0.20% NPP. Plasma inorganic phosphorus was also significantly affected by NPP with the same inflection point as the phosphorus excretion between 0.20 and 0.25% NPP. Plasma inorganic phosphorus was significantly affected by NPP with a plateau pattern similar to the plasma inorganic phosphorus and an inflection point between 0.15 and 0.20% NPP. The optimum level of phosphorus for egg production of breeder hens is somewhere above 0.20% NPP in the sweet spot where the body doesn't throw out NPP but has enough to make the eggs and preserve bone integrity and cellular maintenance.

Table 1: Composition (%) of experimental diets and nutrient contents

	Treatment[1]					
Ingredient	1	2	3	4	5	6
Corn	67.30	67.08	66.86	66.64	66.42	66.20
Soybean meal	20.12	20.15	20.19	20.23	20.27	20.31
Poultry Fat	2.62	2.69	2.76	2.83	2.91	2.98
Limestone	8.26	8.09	7.93	7.76	7.60	7.43
Dicalcium Phosphate	0.27	0.55	0.82	1.09	1.37	1.64
Salt	0.33	0.33	0.33	0.34	0.34	0.34
Sodium Bicarbonate	0.20	0.20	0.20	0.20	0.20	0.20
Methionine	0.29	0.29	0.29	0.29	0.29	0.29
L-Lysine HCl	0.19	0.18	0.18	0.18	0.18	0.18
Choline	0.11	0.11	0.11	0.11	0.11	0.11
Mineral Premix[1]	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin Premix[2]	0.10	0.10	0.10	0.10	0.10	0.10
Ethoxyquin	0.02	0.02	0.02	0.02	0.02	0.02
Nutrient						
CP (%) (calculated)	15.5	15.5	15.5	15.5	15.5	15.5
Crude fat (%)	5.1716	5.2371	5.3025	5.3680	5.4335	5.4989
Ca (%) (calculated)	3.25	3.25	3.25	3.25	3.25	3.25
Total P (%) (calculated)	0.3803	0.4306	0.4809	0.5313	0.5816	0.6319
Total P (%) (analyzed)	0.43	0.45	0.48	0.56	0.63	0.66
NPP (%) (analyzed)	0.15	0.21	0.26	0.32	0.35	0.41

[1]Diet 1 = 0.15% NPP; Diet 2 = 0.20% NPP; Diet 3 = 0.25% NPP; Diet 4 = 0.30%; Diet 5 = 0.35% NPP; Diet 6 = 0.40% NPP

[2]Provided per kg of diet: Zn, 150.6mg; Mn. 180mg; Fg 20.16mg; Cu, 2.04mg; Se. 03mg.

[3]Provided per kg of diet: Vitamin A, 13200 IU; Vitamin E, 66IU; Vitamin D3, 3950ICU; Niacin. 74.25 mg; D-Panthothenic acid, 33mg; Riboflavin, 19.8 mg; Pyridoxine, 5000mg; Thiamine, 3.3mg; Menadione, 3.3mg; Folic acid, 3.3 mg; Bitoin, 0.33mg; Vitamin B12, 0.0297.

Table 2: Production performance parameters from hens fed diets containing 6 different levels of nonphytate phosphorus from 32 to 36 weeks of age[1]

	Egg Production	Hen House Egg Production[2]	Mortality	Change in body weight	Egg weight
	(Egg/hen)	(% of total production)	(% of total)	(%)	(g)
Diet[3]					
1	15.4	0.169	0	107.65	64.3
2	15.9	0.174	0	108.53	65.2
3	14.6	0.160	0	109.08	64.7
4	14.7	0.161	4.16	103.24	65.0
5	15.3	0.167	0	106.40	64.8
6	15.2	0.167	0	109.22	66.2
SEM	0.43	0.04	2.3	1.6	0.7
P Value	0.135	0.512	0.216	0.499	0.13

[1]Values are presented as means ± SEM for the 4 week production period.

[2]Defined as total eggs per hen, corrected for mortality.

[3]Diet 1 = 0.15% NPP; Diet 2 = 0.20% NPP; Diet 3 = 0.25% NPP; Diet 4 = 0.30%; Diet 5 = 0.35% NPP; Diet 6 = 0.40% NPP

Table 3: Phosphorus retention and tibia ash in 33 week old broiler breeder hens fed graded levels of dietary NPP from 32-36 weeks of age.

	Total Feed P (mg)	Total Excreta P (mg)	Total P Retention (%)[1]	Tibia Bone Ash (%)
Diet[2]				
1	576.2	380.8^{A}	34.05	56.04
2	603	401.0^{A}	33.49	54.22
3	643.2	475.5^{AB}	26.07	56.72
4	750.4	579.3^{B}	22.80	56.91
5	844.2	629.3^{B}	25.46	56.42
6	884.4	664.3^{B}	24.88	56.82
SEM	NA	52.2	6.8	3.72
P Value	NA	0.0001	0.03	0.563

[1]Retention defined as (intake-excretion)/intake x 100.

[2]Diet 1 = 0.15% NPP; Diet 2 = 0.20% NPP; Diet 3 = 0.25% NPP; Diet 4 = 0.30%; Diet 5 = 0.35% NPP; Diet 6 = 0.40% NPP

[A-B]Means within a column not sharing a letter are significantly different ($P<0.05$)

Table 4: Plasma Inorganic Phosphorus and Fibroblast Growth Factor concentrations in 36 week old broiler breeder hens fed graded levels of NPP.

	Plasma Inorganic Phosphorus (mg/dl)	Plasma Fibroblast Growth Factor 23 (pg/ml)
Diet[1]		
1	7.56[A]	129.8[A]
2	8.82[AB]	201.2[B]
3	10.32[B]	205.3[B]
4	9.87 [B]	205.1[B]
5	10.64[B]	206.9[B]
6	10.23[B]	220.2[B]
SEM	2.25	87.2
P Value	0.06	0.001

[1]Diet 1 = 0.15% NPP; Diet 2 = 0.20% NPP; Diet 3 = 0.25% NPP; Diet 4 = 0.30%; Diet 5 = 0.35% NPP; Diet 6 = 0.40% NPP

[A-B]Means within a column not sharing a letter are significantly different ($P<0.05$)

Chapter 2

Literature Review

Mechanisms Regulating Egg Shell Synthesis

Broiler breeder hens unlike layer hens produce eggs which are used to grow chicks which will quintessentially be grown for meat production or be used as parent stock for future generations of chick production. Many factors can affect egg quality and production including age of the hen, disease, genetic strain, diet, environmental conditions, and stress (Solomon, 1990). Given that breeder hens have been selected over the past decades for egg production they now face the metabolic cost of producing an egg once a day for several days in a row during clutching. Eggs can be broken down into three components: yolk, albumen and egg shell. The yolk is the most available portion for hens to create due to lipogenesis not requiring much contribution from the hen's body reserves other than energy. Albumen is mostly protein which will require essential amino acids which can force hens to metabolize muscle in case of a deficiency in the diet. Egg shell is approximately 95% calcium carbonate $CaCO_3$ and 5% calcium phosphate including magnesium carbonate and select insoluble proteins. Egg shell thickness has a huge impact on fertility and hatchability of chicks making thicker shells essential for supporting life within (Mcdaniel et al., 1978). Due to how essential both calcium and phosphorus are for body homeostasis they are not as available to be used for shell synthesis compared to energy for yolk and amino acids for albumen. Breeders rely on two sources to supply the calcium and phosphorus necessary for egg shell synthesis: the diet that they consume and their medullary bone.

Bone resorption & building

Specialized cells with the bone known as osteoblasts and osteoclasts will both build and resorb bone respectively in order to maintain bone health and supply calcium to the shell gland.

Osteoclasts are formed through the differentiation of osteoblasts under the presence of RANKL, and macrophage colony stimulating factor (M-CSF). The formation of osteoclasts is tightly regulated as they ultimately determine the rate of bone resorption and thus their differentiation is tied to many hormones are factors including MEPE, DMP1, Sclerostin, PHEX, PTH, Osteoprotegrin, and RANKL (Schoppet et al., 2002). Factors which are designated to increase free calcium and phosphorus in the blood will upregulate the formation of osteoclasts while those which oppose resorption will act to inhibit its differentiation. Osteoclasts are multinucleated cells which have the sole purpose of breaking down both hydroxyapatite and the collagen crosslinks which give bone strength (Vaananen et la., 2000). Structurally they are large cells with an abundance of mitochondria, lysosomes, ribosomes, and golgi complexes (Dacke et al., 1993). Osteoclasts are located in a group together on the surface of the bone which are referred to as resorption bays, or Howships's lacunae (Basle et al., 1988). The active site where osteoclastic activity occurs is characterized by a ruffled border that invades the surface of the bone tissue where hydroxyapatite and collagen are being degraded through enzymatic activity. Osteopontin is a substrate protein for Osteoclast activity as it will bind to calcium on the surface of bio minerals including hydroxyapatite (Teitbelbaum, 2000). Tartrate resistant acid phosphatase (TRAP) and Cathepsin K are the enzymes produced by osteoclasts which serve to degrade hydroxyapatite and collagen respectively (Schlesinger et al., 1997). Osteoclasts also release hydrogen ions through carbonic anhydrase which forms bicarbonate and protons from water and carbon dioxide in order to decrease the immediate pH which increases the activity of TRAP and Cathepsin K.

While bone resorption is important for maintaining calcium levels in the blood for the shell gland, rebuilding bone is just as important in order to prevent osteoporosis and

osteomalacia. Osteoblasts are created through differentiation of mesenchymal stem cells found in the bone marrow and on the surface of bones. Mesenchymal also differentiation into chondrocytes, cells necessary for the creation of cartilage and collagen in the bone which support the mineral complex (Blair et al., 2008). Bone synthesis begins with chondrocytes following vascularization and building cartilage within the designated area the body has determined to become bone. Osteoblasts form from the mesenchymal stem cells and begin to replace the cartilage laid down with hydroxyapatite. Many factors affect the rate of bone growth including bone morphogenetic proteins (BMPs) which determine where bone differentiation occurs and where to leave spaces for joints in between bones (Lee et al., 2013). Transforming growth factor beta, another factor necessary for osteoblast function, mediates the differentiation of cartilage prior to osteoblast activity. Fibroblast growth factors control many different aspects of bone cell growth and differentiation during embryonic growth and some are used in the adult body for mineral regulation such as FGF23. Osteoblasts build bone through the enzymatic activity of bone alkaline phosphatase (BAP) which synthesizes hydroxyapatite from free calcium under basic conditions. Many factors and hormones regulate osteoblast bone building including Calcitonin, PTH, Sclerostin, DMP1, MEPE, Osteopontin, and PHEX. Calcitonin stimulates osteoblast mineralization in order to remove calcium from the blood. Parathyroid will upregulate bone resorption and osteoclast activity and down regulate osteoblast bone building in order to increase plasma calcium. Sclerostin is made by osteoblasts during bone mineralization as part of negative feedback on themselves. Phosphate regulating endopeptidase homolog, x linked is an enzyme that hasn't been fully explored but is known to be linked to bone mineralization as its absence causes osteomalacia and rickets (Barrow et al., 2013). Dentin matric acidic phosphoprotein 1 stimulates the release of PHEX which promotes bone mineralization indirectly (Thurner et al.,

2010). Matrix extracellular phosphoglycoprotein inhibits PHEX by binding to it and making it unavailable, preventing bone mineralization. Osteoblasts themselves release Osteocalcin, a hormone which promotes muscle, fat, and testosterone production, during bone mineralization.

Alkaline phosphatases are a group of hydrolase enzymes which remove phosphate from other molecules including proteins, nucleotides, and alkaloids. Different isoforms of alkaline phosphatase are synthesized in the intestine, liver, bone, kidney, and the placenta of pregnant woman. Levels of total alkaline phosphatase have been used extensively in both human and animal research to diagnosis many different conditions including billary obstruction, osteomalacia, osteoblastic bone tumors, leukemia, lymphoma, Paget's disease, sarcoidosis, hyperthyroidism, hyperparathyroidism, pregnancy, hypophosphatasia, achdonroplasia, aplastic anemia, and Wilson's disease (Kress et al, 1999; Garnero et al., 1999; Raisz et al., 2000). Bone alkaline phosphatase in particular is made by osteoblasts in developing bone to promote hydroxyapatite synthesis through the removal of pyrophosphate (Harada et al., 1986). Initially hydroxyapatite crystals are synthesized on the surface membrane of osteoblasts and chondrocytes which are then deposited between collagen fibrils in the order bone (Blair et al., 2007). Pyrophosphate, $P_2O_7^{4-}$, is a potent inhibitor of hydroxyapatite synthesis while inorganic phosphorus, PO^{4-} promotes hydroxyapatite synthesis as it is directly utilized in its synthesis along with calcium (Orimo, 2010). Bone alkaline phosphatase concentrations in the blood are indicative of bone building and can be used to evaluate the bone homeostasis of animals.

Tartrate resistant acid phosphatase (TRAP) is a glycosylated metalloprotein enzyme which catalyzes phosphate ester hydrolysis and is characterized by having high activity in acidic conditions and by being resistant to tartrate inhibition. Many cells synthesize TRAP for different cellular functions including macrophages, osteoclasts, and dendritic cells (Luchin et al., 2000).

Osteoclasts use TRAP to digest hydroxyapatite during bone resorption by secreting it to work in conjunction with Osteopontin. Osteopontin will bind to calcium on the surface of the hydroxyapatite crystalline calcium lattice and make it available for enzymatic cleavage by TRAP to remove the phosphate bones which hold the mineral complex together (Ek-Rylander et al., 1994). Migration of osteoclast cells throughout the bone is dependent upon TRAP activity to increase the size of resorption sites to facilitate cellular movement. TRAP activity from osteoclasts has also been shown to directly inhibit bone sialoprotein, a protein responsible for the growth of the initial hydroxyapatite crystals necessary for bone mineralization (Hayman, 2008). High concentrations of TRAP are correlated with osteoporosis, ostemalacia, hypophosphaturia, Gaucher's disease, leukemia, and other metabolic bone diseases (Angel et a., 2000).

During animal growth cortical bone is elongated and widened through the osteoblast and chondrocyte function. Vascularization and angiogenesis regulated by growth factors such as vascular endothelial growth factor (VEGF) is necessary for supplying the bone cells with nutrients for growth which ultimately dictates how much bone can be formed (Tischer et al., 1991;. Part of bone growth is remodeling, the process through which osteoclasts break down existing hydroxyapatite and collagen in order for osteoblasts to build new bone in its place. Remodeling is necessary not only for bone turnover to replace old collagen protein and renew it, but also for the reshaping of bones during elongation (Raggatt et al, 2010). Rigorous exercise can cause micro fractures in the bone which are minute breaks in-between the mineral portions of the bone. While these fractures may seem detrimental they are the stimulus for new bone growth which leads to stronger bones with a high mineral density (Brighton et al., 1997). Studies have shown the profound effects of exercise on bone growth, angiogenesis, and tissue development in broiler chicks (Acar et al., 1995;Wideman et al., 1995). Tibial dyschondroplasia seen in growing

broiler chicks is characterized by the incomplete ossification of bone cartilage at the epiphysis and diaphysis of the tibia. Given that growing broiler chicks barely move more than necessary than for eating and drinking past day 10 of age, the lack exercise and thus stimulus for bone growth and vascularization is apparent from the rate of ascites and osteomalacia. Cortical bone isn't the only type of bone in chickens as a specialized known as medullary bone acts as a reservoir for calcium exists in laying hens.

At sexual maturity female birds and reptiles develop medullary bone which is used for supplying calcium to the shell gland during egg shell synthesis (Blom et al., 1941). Medullary bone is formed primarily in the leg bones including the tibia and the femur. Estrogen stimulates the osteoblast cells to create medullary bone while inhibiting osteoclast resorption. During clutches which consist of laying eggs for several days in a row hens will draw heavily upon the medullary bones which can osteoporosis and a weakening of the bone with an increased chance of fracture. In between laying eggs hens will rebuild their medullary bone utilizing calcium from the blood which comes from the G.I. tract and the diet.

Small Intestine & Kidney Regulation

Calcium levels in the blood are under tight regulation through parathyroid hormone interactions between the bone, kidney, and small intestine to make sure that enough calcium stays within the system for cellular process, but also to prevent too much from accumulating which can lead to soft tissue calcification. Calcium introduced into the body of breeders through the diet must pass through the gizzard to be broken down into small particles and then traverse the small intestine. Particle size of calcium effects how long the calcium will stay in the G.I. tract and ultimately increase its absorption as particle size increases (Zhang et la., 1997). Free calcium

is absorbed primarily in the duodenum and jejunum through transportation via the calcium binding protein calbindin. When calcium levels in the blood are low PTH will increase the conversion of the inactive form of vitamin D, cholecalciferol, to the active form calcitriol. Vitamin D increases the absorption of calcium and phosphorus in intestines by upregulating calbindin (Deluca et al., 1971). During blood filtration in the kidney through Bowman's capsule free calcium is removed from the blood and must be reabsorbed. Approximately 97% of calcium is reabsorbed under normal conditions as the body has two mechanisms for the recovery of calcium: passive transportation in the proximal tubule, thick ascending limbs of Henle's loop, and the distal tubules. When the body wants to increase calcium reabsorption in response to PTH there are active transporters for calcium which further increase the amount reabsorbed (Luo et al., 2003).

Egg Synthesis & Shell Gland Function

The time it takes from when the yolk is released from the ovary to the point where the fully developed egg leaves the cloaca is approximately 25 hours. Avian species only retain one ovary and fallopian tube due to evolutionary design to be light weight. The fallopian tube serves a different purpose in egg laying species as the yolk travels through the tube it will be surrounded by albumen and ultimately egg shell to form a complete egg. Egg yolks begin as minute follicles in the ovary which will undergo hypertrophy in response to rising levels of estrogen, follicle stimulating hormone (FSH), and luteinizing hormone (LH). Ovules release in birds are much larger compared to mammals due to the cells being saturated with lipids and cholesterol. Egg yolk composition depends upon which fatty acids are available to the hen during the time of lipogenesis which can cause diet and stress to affect yolk synthesis and ultimately egg fertility and hatchability. Once released from the ovary they will travel through the infundibulum

and down through the magnum which is the site where inner and outer shell membranes are added to the yolk, including some water and mineral salts. Next the developing egg will travel to the Isthmus where albumen will surround the yolk which gives eggs the protein layer necessary for growing embryos. Lastly the incomplete egg will arrive in the shell gland where calcium carbonate will enclose the egg and form around the shell membranes on the outside of the albumen. Once the egg is complete it will travel to the cloaca and will be laid once the hen feels safe enough to do so.

The egg shell gland will begin the egg shell calcification process in response to gonadal hormones progesterone, estrogen, FSH, LH, and prostaglandin synthesized during the eggs develop. The given point of time where the egg is during egg synthesis, known as oviposition, is controlled through photostimulation during day and night cycles which influence the fluctuation of these gonadal hormones. Release of LH is dependent upon melatonin release from the pineal gland, the part of the brain which responds to light and gives animals a sense of time (Sharp, 1992). Modern poultry production practices utilize hours of day light in the brooding program to stimulate hens to begin egg production and it is due to the indirect control of LH from light that this is possible. Rate of egg shell synthesis and the amount of time a developing egg spend within the egg shell gland is dependent upon these gonadal hormones. Control of calcium which is sent to the shell gland has been shown to be independent of these gonadal hormones however, as studies have shown that the shell gland itself can change the calcium homeostasis of the hen (Nys et al., 1986;Nys et al., 1984; Eastin, 1994). Egg shell synthesis occurs primarily during the night for laying hens which coincides when the G.I. tract is empty for birds fed restricted diets which is practiced in America for broiler breeders. Laying hens are often fed ad libitum and due to their unrestricted access to feed they will have calcium in the G.I. during the time for egg shell

synthesis. The egg shell gland has been shown to increase the concentration of calbindin in the S.I. in response to increased calcium demand. Broiler breeder hens rely entirely upon calcium from the hydroxyapatite of the medullary bone during the night due to not having G.I. calcium to absorb. Blood calcium decreases by approximately 18% during egg shell gland calcium carbonate synthesis and this is constant throughout the entire process indicating that the hens are able to replenish calcium at the same rate as the shell gland can use it (Winget et al., 1958). The method of how the egg shell gland removes calcium from the blood is unknown as only organically bound calcium has been shown to be removed from the total blood supply during egg shell synthesis while this organic matter is not found in the egg suggesting either direct removal of bound calcium, or removal of ionic calcium which is very rapidly disassociated from the bound form (Burmester, 1941). If plasma calcium levels were to drop below 80% the normal concentration then the shell gland will absorb less calcium leading to thinner egg shells (Bradfield, 1951). Calcium carbonate $CaCO_3$ synthesis in the egg shell gland is driven by the reaction between carbonate ions and calcium which attach to the forming calcite lattice of the egg shell. Carbonic acid in the shell gland is continuously dehydrated to carbon gas due to carbonic anhydrase which creates the carbonate ion concentration necessary for shell formation (Taylor, 1970). Overall the factors which effect egg shell thickness include calcium availability, photoperiod, health, bone density, and the inherent genetics of the hen.

Chapter 2

The relationship between production parameters, egg shell quality, bone remodeling, TRAP expression, and BAP expression in laying broiler breeder hens and their progeny.

Introduction

Egg shell thickness of eggs laid by breeder broiler hens can change not only the fertility and hatchability of eggs but also the bone quality of the progeny which hatch from them. For these reasons selecting for hens which lay thicker egg shells is essential to improving broiler development, and for selecting for future generations of chicks which will potentially become breeders themselves. Enzymes tartrate resistant acid phosphatase (TRAP) and bone alkaline phosphatase (BAP) correlate with the activity of the bone degrading and bone building cells osteoclasts and osteoblasts respectively. Previous studies have shown there is a link between bone health and mineral concentration and the ability of hens to lay thicker egg shells. Due to hens relying mostly upon medullary bone to supply calcium for calcium carbonate synthesis in the egg shell the ability of hens to mobilize bone and rebuild it is important for their continued performance of laying eggs and making good egg shells. The purpose of this study is to find out of the enzymes TRAP and BAP are correlated with good or poor egg shell quality and to see if their concentrations with in the progeny of these hens has some relationship.

Materials and Methods

All procedures regarding the use of live animals in this study were carried out in accordance with the Animal Use Protocol 13002, which was approved by the University of Arkansas Institutional Animal Care and Use Committee.

Animals and Handling

A flock of 850 Cobb 500 hens was delivered to a production house at the age of 20 weeks. Each hen was assigned an identification number and individually caged (47 cm high, 30.5 cm wide, 47cm deep) with separate feeders and water nipples. Hens were offered standard Cobb

feed daily using the amounts recommended on the Cobb Breeder Management Guide (Cobb-Vantress, 2005). Daily allotted feed intake was restricted and was increased every 8 percent increase in egg production beginning from 5% production to peak. The farm where the birds were kept was environmentally controlled with regards to temperature (22 °C) and humidity. Lighting schedule began with 12 hours per day at week 21 of age and increased one hour for the next two weeks until reaching its apex at 14 hours per day. Light duration was further increased to 15 and 16 hours at 20%, and 50% egg production respectively.

Breeder Selection & Sampling

As soon as the flock reached the farm their egg production was recorded daily, and egg weights recorded two days a week. Eggs which were soft shelled, cracked, dirty, or double yolk were recorded as such. Beginning at 30 weeks of age eggs which were weighed during the two days every week were also subjected to being measured through dual energy x-ray absorptiometry (DEXA) (GE ®Lunar Prodigy) for 16 weeks to determine egg shell mineral concentration and thickness. Regression equations developed for determining egg shell parameters from the DEXA scanning (England, 2012) were used to select 20 hens which laid worst egg shell quality eggs (Specific gravity <1.80) and 20 hens which produced best egg shell eggs (Specific gravity >1.80). Specific gravity of each egg were measured through immersion in salt solutions ranging in specific gravities ranging from 1.60 to 1.095 with a concentration gradient of 0.005 between solutions. After hens were selected for poor or good egg shell quality they were subjected to whole body scanning on the DEXA to select for good or poor bone quality. Equations for body composition for the DEXA Salas et al. (2012) were used for determining whole body composition of the hens.

At the end of the trial blood from drawn from the hens selected for good or poor egg shell, or bone quality from the wing vein with regards to oviposition and stored in heparinized blood collecting tubes. Hens were then euthanized through CO2 gas asphyxiation and right tibias removed from measurement of breaking strength and bone ash concentration. Blood samples were immediately centrifuged using the methodology described by Tuck (2009) for plasma separation, and then stored at -20 °C until they were analyzed for tartrate resistant acid phosphatase (TRAP) and chicken specific bone alkaline phosphatase (BAP).

Plasma chicken specific bone alkaline phosphatase was analyzed using a quantitative competitive immunoassay test kit (Neobiolab, USA). Plasma tartrate resistant acid phosphatase was analyzed using a micro-titer plate spectrophotometer absorptiometry technique described by (Lau et al., (1987). Tibias were analyzed for bone-breaking force by the sheer force measurement method described by Wilson (1991), utilizing an Instron Universal Testing Machine (Model 1123, Instron Corp., Canton, MA). Following the sheer test, the tibias were defatted in a container of 180 alcohol for 24 hours followed by refluxing in petroleum ether in a Soxhlet apparatus for 48 h. The defatted tibia samples were oven-dried at 110°C for 24 hours and ashed in ceramic crucibles for 24 h at 600°C. Ash content was determined as dry, fat-free tibia and expressed as grams of ash/bone and as a percentage of the defatted tibia weight.

Progeny Generation & Sampling

Hens which were selected for poor or good egg shell quality were artificially inseminated twice a week starting at week 46 through week 50. Semen used was collected from broiler breeders on the farm through abdominal massage method (Burrows, 1937). Collected semen was pooled and sperm cell concentration was measured using an IM Micro-reader using an optical

density of 381 nm (King et al., 2000). Lake solution was used in order to dilute semen to a concentration of 2x10^6 cells/50 ul for a consistent concentration of sperm using for insemination. Following the A.I. eggs were collected for a 6 day duration from the selected hens and stored in an egg cooler until they were placed for incubation and hatching. Live hatched chicks from either the good or poor egg shell quality hens were given individual wing bands and raised in separate floor pens. Chick mortality was measured daily along with water nipple adjustments. Chicks were offered standard commercial starter diet for 14 days and then had blood drawn from the external jugular vein. Chicks were then euthanized through CO2 asphyxiation and right tibias removed for breaking strength and bone ash measuring. Chicks were scanned prior to tibia removal for whole body composition using the DEXA through the equations develop by England (2012). Plasma from the chicks was also subjected to analysis for both TRAP and chick specific BAP using the same analysis that was done for the parent stock.

Statistical Analysis

A completely randomized design was used to analyze data through the use of the ANOVA procedure of SAS (version 9, SAS Institute, Cary, NC). When necessary trend contrasts were used to determine line shape. Data was also analyzed for linear and quadratic regression to determine curvilinear responses to dietary NPP. All statements of significance are based on testing at $P \leq 0.05$.

Results

No statistical differences were found between egg production and egg shell thickness (P=0.15)(Table 1). Total egg production of all the birds involved in the study versus the percentage from each selected group for egg shell quality was not significantly different

(P=0.35). Mortality was correlated with the egg shell thickness hens lay (P= 0.031) with approximately 3 birds dying out of the 20 that were selected for poor egg shell quality and 1 which died from the good shell group. Egg weight was not significantly different between good and poor egg shell groups (P =0.33). The ratio of egg shell weight to total egg weight was significantly different between good and poor egg shell groups with good eggs having a high ratio than poor (P<0.0001)(Table 2). Shell weight per unit surface area was significantly different between good and poor egg shell groups with more dense eggs having a higher SWUSA (P<0.0001). Shell calcium percentage of the total egg was significantly different between good and poor egg shell groups with good eggs having a higher concentration than poor (P=0.022). Specific gravity was significantly different good and poor egg shell groups with good eggs having a higher density than poor eggs (P<0.0001). Egg shell thickness was significantly different between good and poor egg shell groups with good eggs having a larger thickness than poor eggs (P<0.0001). Bone alkaline phosphatase was significantly higher in good egg shell hens than poor egg shell hens (P=0.023) (Table 3). Tartrate resistant acid phosphatase activity was higher in poor egg shell hens than good egg shell hens (P=0.0022). Tibia ash percentage was not significantly different in the bones of good and poor egg shell hens (P=0.46)(Table 4). Breaking strength was significantly higher in good egg shell hens compared to poor egg shell hens (P= 0.04). Bone alkaline phosphatase was significantly higher in the blood of the progeny born from good shell hens compared to the progeny of the poor egg shell hens (P= 0.0017)(Table 5). Tartrate resistant acid phosphatase activity was significantly higher in the progeny born from poor egg shell hens compared to the progeny of the good shell hens (P= 0.0022). Body weight gain was not significantly different between the progeny of either good shell hens or poor shell hens (P= 0.562). Tibia ash was significantly higher in the progeny of the good egg shell hens

compared to the progeny of the poor shell hens (P= 0.025). Breaking strength was significantly higher from the progeny of the good egg shell hens compared to the progeny of the poor shell hens (P= 0.003).

Discussion

Nutritionists attempt to meet the metabolic requirements of hens through accounting for the energy necessary for maintenance, amino acids for protein turnover, and minerals and vitamins necessary for bone and cell health. Laying hens have additional requirements unlike other production animals as they avian and create eggs instead of undergoing pregnancy. During gestation mammals will devote bodily reserves and nutrients from the diet to sustain the growing embryo(s), but the process has a definite end with a refractory time period in between the animal becoming pregnant again. Lactating animals requires energy, protein, and calcium to synthesize which puts strain on animals which are used for dairy production due to unnaturally prolonged lactation. Avian species conversely produce eggs on a daily basis for a series of days in a row known as a clutch. While clutching occurred naturally prior to human intervention modern broiler breeder hens and laying hens produce eggs at a much higher quantity and frequency than ever before (Wiemeyer et al., 1993). Selection for high egg production and egg shell quality has changed the behavior of the birds to be more aggressive when eating and the regulation of the hormones involved in egg synthesis. Breeder hens in particular are hard to select for the best characteristics of egg production due to their progeny being utilized for something completely different, meat production and growth. Current feeding regiments require that breeder hens have their feed restricted in order to prevent too much growth due to their high appetite from also having broiler tendencies. Laying hens in most North American practices have ad libitum access to feed allowing for a constant supply of nutrients from the G.I. tract to support egg synthesis.

The three principal components of an egg, yolk, albumen, and egg shell are partially created utilizing reserves from the hens body along with nutrients which come directly from the diet and G.I. tract. Compositionally, the average hen is 80% lean tissue, 16% fat mass, and roughly 4% mineral mass while the eggs they create are 74% water, 11.8% lipid, 12.8% protein, and 1.4% ash which include minerals. Egg yolk is approximately 48% water, 17% protein, 33% fat and 1% ash, egg albumen is 88% water, and 11% protein, and egg shell is 98% calcium carbonate and 2% trace minerals and protein. Egg synthesis requires 25 hours from release of the yolk from the ovary until completion, of which approximately 21 hours is spent building the calcium carbonate shell of the egg in the shell gland. Egg shell is synthesized through the incorporation of carbonate ions, concentrate through the enzymatic activity of carbonic anhydrase, and its reaction with free calcium ions absorbed from the blood. Maintaining proper calcium level in the blood is important not only egg shell synthesis but for the bone and cellular health of the hen.

While there are many different methods to evaluate the health of birds during egg production the simplest way and most straightforward method is to record their egg reproduction in response to experimental conditions to evaluate how it is impacted. Given that egg production was not significantly different ($P = 0.15$) between the good (58.3 eggs/hen) and poor (60.3 eggs/hen) egg shell quality birds one can state that the rate of formation of eggs and the ability for hens to consistently create thicker or thinner eggs shell is independent. Studies have shown that by increasing the photoperiod of birds in experimental housing, by having more ours of light than darkness, that the egg shells became thicker. Age, health, genetic strain, and nutritional content of the diet are also factors which determine the egg shell quality as older birds are shown to have lower calcium retention preventing them from utilizing calcium from their diet and bone to create calcium carbonate as efficiently as younger birds. Calcium from the diet is not only

used to rebuild medullary bone, the hen's bodily reserve of calcium of egg synthesis, but also is directly incorporated into the shell if present in the G.I. tract. There is variation between hens within a flock of the same genetic strain as some birds will be more prone to produce thicker or thinner egg shells which may be coupled with their bone health. The total production of eggs versus each individual grouping for the good (49.2% of total production) or poor (50.8% of total production) egg shell selected hens was not significantly different (P= 0.35) that egg production remains consistent between the two groups at age 30 to 46 weeks, but this may not be true as the birds become older. Mortality as significantly different (P =0.031) between the two groups with 3 times as many birds dying from the poor shell group, 7.5% of hens within that group, compared to the good shell hens with a mortality of 2.5% of the group. Nothing decreases the production of a hen more than death which makes this a very important parameter to measure when considering flock management. While some hens are prone to stop eating and subsequently falling out of production, some will continue to lay eggs despite being at a metabolic deficiency either due to not eating all of their feed or perhaps due to a genetic disposition which causes lower retention of nutrients. Laying hens are most vulnerable to sudden changes in the concentration of calcium in their blood to the cyclic nature of laying eggs and how much of a demand these is for the mineral from the shell gland. Severe calcium deficiency leads to tetany which is the inability to uncontract the muscles throughout the body which can quickly lead to death without intervention. Retention of calcium is exceedingly important for laying hens as it determines efficiently they can make egg shells, build bone, and maintain cellular homeostasis and whole body health under conditions which stress their reserves of calcium. Total egg weight was not significantly different between the good, 64.8g, and poor, 63.0g, egg shell groups (P = 0.33) which is not too surprising considering that the egg shell constitutes approximately 10% of

the whole egg by weight which would require a very drastic change in shell quantity in order for it to solely impact total egg weight.

Individual eggs were scanned through dual energy X-ray absorptiometry which allowed for in depth analysis, when paired with regression equations for each parameter, of each component of the egg without having to take it apart and manually measure everything. The ratio of the weight of the egg shell to the weight of the total egg was significantly different between the good, 0.093 ratio shell:egg, and poor, 0.076 ratio shell:egg, egg shell groups ($P<0.0001$) which in conjunction with the fact that the total egg weights were not significantly different signifies that not only did the eggs from the good shell groups have more shells, but also slightly less albumen and yolk. Hens have an internal limit on the size of the average egg due to very large eggs being unable to physically exit the cloaca which can cause the egg to become indefinitely trapped in the shell gland until the body resorbs it. Gonadal hormones regulate the rhythm of how long developing eggs spend in each portion of the reproductive tract of the hen through muscular peristalsis, and while this is unlikely to change within the same strain of bird there may be some differences in order the total time spent developing which can change the size of eggs. Shell Weight per Unit Surface Area was significantly different between good, 80.65 mg/m^2, and poor, 65.25 mg/m^2, egg shell selected hens ($P<0.0001$) with a higher value for the good shell hens that is consistent with the other parameters. Having a high shell weight per surface area essentially means that the eggs were overall more dense from the good shell hens compared with the poor. The egg shell is 98% calcium carbonate which essentially makes it entirely mineral based and thus much denser compared to the yolk and albumen which are made up of a large percentage of water. Egg shell calcium was significantly higher in the good, 6.08%, egg shell hen selected group compared with the poor, 4.83%, shell group ($P= 0.022$) which is

once more consistent with higher concentrations of shell that is made up of calcium carbonate. Chick embryonic chorioallantoic membrane (CAM) utilizes the calcium carbonate egg shell to supply calcium for bone development in the growing chicken embryo. While not much is needed in the chick compared to the total amount present in the shell, it has been shown that the presence of more shell calcium has increased the calcium transport of CAM which leads to more bone formation. Specific gravity was significantly different between the good, 2.28, and poor, 1.80, groups ($P<0.0001$) selected for egg shell quality with the good having a high specific gravity which is once more consistent with all of the other egg shell parameters. Specific gravity is essentially another method of equating substance density utilizing water as a reference, but this has been used as the standard for measuring egg density since the beginning of poultry science. The density value from SWUSA derived from the DEXA is just as accurate as specific gravity and faster to utilize (Onyango et al., 2003). Shell thickness was significantly different between the good, 1.086 mm, and poor, 1.07 mm, egg shell groups ($P<0.0001$) with the good shell hens having eggs with a higher shell thickness which complements the results from every other parameter of egg shell quality. Shell thickness is essential for protecting the growing embryo within the egg to prevent physical trauma from the outside, and to prevent microbes from entering. A huge cost in the breeder broiler and laying hen industry is attributed to broken eggs due to thin shells making it important not only for growing chicks but also from a production standpoint to have thicker shells.

Bone alkaline phosphatase (BAP) was significantly higher ($P= 0.023$) in the good egg shell hens with a concentration of 326.49 pg/ml versus the poor shell concentration of 253.19 pg/ml. Bone alkaline phosphatase is a correlated with the bone building activity of osteoblast cells in the bone as the enzyme is necessary for hydroxyapatite synthesis. The principal function

of BAP is to convert the inhibitor of bone mineralization, pyrophosphate, into a necessary element used in hydroxyapatite synthesis, inorganic phosphate. High concentrations of BAP in the blood correlate with increase bone synthesis which indicates that the hens which produce thicker egg shells are also rebuilding their bones at a higher rate than the hens which create thinner shells. Given that hens can mobilize up to 10% of their total bone over the span of a day signifies how well adapted laying hens are at breaking down their hydroxyapatite to be mobilized for the shell glands calcium carbonate synthesis. The ability of hens to breakdown and rebuild their bone is shown to diminish with age but it is also highly affected by the genetics as certain hens within a flock are pre-disposed to create thicker or thinner shells which relies on their ability to turnover their bone very rapidly. One possible explanation for the relationship between BAP, bone turnover, and egg shell thickness would be that hens which are well adapted to breaking down and rebuilding their medullary bone have a higher calcium retention and are able to more efficiently utilize the calcium and phosphorus released from the bone and which enters the blood from the G.I. tract. Higher BAP may also signify higher concentrations of osteoclasts within the bone which allows them to more rapidly respond to ambient calcium increases from the diet to prevent Calcitonin from signaling the excretion of calcium. Phosphorus regulation is also affected by osteoblast activity as the resorption of bone causes free phosphorus to enter the blood alongside calcium which causes the phosphorus to be excreted through the kidney due to it not being utilized in egg shell synthesis. Hens which have higher calcium retention as more efficient at keeping their bones strong and perhaps for the same reason are better adapted to creating thicker egg shells.

Tartrate resistant acid phosphatase (TRAP) activity was significantly higher (P= 0.0022) in the poor egg shell hen group, 4985.2 (U), compared to the good egg shell hen group. Tartrate

resistant acid phosphatase activity correlates with osteoclast cell formation and bone resorption as it is the principal enzyme used in the degradation of hydroxyapatite (Lindunger et al., 1990). Laying hens constantly resorb their medullary bone to supply calcium for egg shell synthesis making TRAP very important in the daily cycle of egg formation and bone resorption. Upregulation of TRAP is associated with many bone metabolic disorders including osteoporosis, osteoclastoma, and leukemia indicating that increased osteoclast activity or number and bone resorption may be indicative of bone health problems (Ek-Rylander et al., 2002). One possible explanation as to why hens which lay thinner egg shells have higher concentrations of TRAP is that they have lower calcium retention. Given that the poor egg shell hens cannot utilize calcium resorbed from the bone as efficiently in the shell gland compared to the thicker egg shell hens, they end up needing more calcium in order to accomplish the calcium carbonate synthesis. In order to compensate for the lower efficiency in using calcium the poor egg shells birds resorb more bone to compensate which ultimately leads to more bone being resorbed. Another theory is that the poor egg shell hens have bones which are more resilient to mobilization which causes osteoclast activity to increase in order to bring calcium blood levels back to normal when the shell gland pulls calcium out of the blood. While the shell gland can synthesis calcium carbonate readily as long as calcium is present the poor shell hens may have trouble replenishing their blood calcium which causes this increase TRAP activity.

Tibia ash percentage was not significantly different (P= 0.46) between the good, 0.58%, and poor, 0.57%, egg shell groups. Mineral concentration in the bone is directly correlated with ash percentage as the hydroxyapatite calcium and phosphorus crystalline lattice is inorganic and makes up 70% of the total bone. The collagen cross fibers which link the mineral portions of the bone together, however, are organic and constitute approximately 20% of the total bone.

Collagen not only strengthens bones but it gives them some flexibility so they bend slightly instead of breaking when under pressure (Tanck et al., 2006). The breaking strength was significantly different ($P = 0.04$) between the good, 7.26 kg/mm, and poor, 5.34kg/mm, egg shell groups. Despite having tibia ash percentages which are almost the same the good shell hens had bones which were stronger which signifies that the amount of collagen crosslinks must have been higher, or that the hydroxyapatite in the bone of the poor shell hens was incompletely calcified. While medullary bone is conventionally utilized for bone resorption and calcium mobilization in the breeder hens there is also another type of bone which can be mobilizes and which is responsive to high levels of TRAP: cortical bone. High concentrations of TRAP in the poor shell hens may have caused them to inadvertently mobilize the hydroxyapatite and calcium from their cortical bone as well as their medullary bone which may explain why their breaking strength is lower despite having ash percentages that are almost the same. If the poor shell hens mobilize cortical bone their bones will become weaker much more quickly due to cortical bone being used to support the animals frame and bear load, while medullary bone isn't structural but simply acts as a reservoir for calcium to be utilized in the shell gland. Another factor to consider is that the blood taken from these hens was with regard to time of oviposition, within 30 minutes of them laying an egg. TRAP levels will increase in the blood during the period of egg shell synthesis while BAP will increase during the period of bone rebuilding and mineralization. The levels of TRAP and BAP constantly fluctuate throughout the day during the cycle of egg synthesis and while their expression may appear abnormal at a given point the hens don't maintain the same enzyme concentration constantly which will cause enzyme concentrations to reflect how many osteoblasts or osteoclasts are within the bone.

Concentration of BAP in the blood was significantly different (P= 0.0017) between the progeny of the good, 372.3 pg/ml, and poor, 312.4 pg/ml, egg shell hen groups. Two week old broiler chicks are rapidly synthesizing both fat and muscle tissue as they've been selected to do for decades. One major complication associated with rapid weight gain in broiler chicks is that their total body mass far exceeds what their organs, bones, respiratory, and circulatory systems are capable of supporting causing them to suffer from a myriad of metabolic disorders (Robinson et al., 1991). One problem chicks are susceptible to is tibial dyichonplasia, the incomplete ossification of the epiphyseal plates of the tibia which causes the growth plates on their tibias to be soft and potentially break. Chicks with stronger bones have been correlated with higher growth rates and feed conversion due to their bone growth being able to keep pace with their muscle and fat. Breeder broiler chicks in particular will become laying hens themselves when they reach adulthood and need to have the machinery in order to produce calcium carbonate from their bones. Given that the hens which produced thicker egg shells have higher concentrations of BAP compared with the hens which produce thinner egg shells, and the progeny of the thicker shell hens have higher concentrations of BAP than the progeny of the poor shell hens indicate that the higher concentration of the enzyme is heritable and may be passed along. While the synthesis of BAP may not necessarily be upregulated in the progeny but the actual osteoblast cell count, the cells in the bone which produce BAP during bone mineralization. The chicks from the thicker egg shells were also exposed to more calcium during their embryonic development due to increase calcium transport from CAM which may also explain why they have more osteoblast activity compared to the progeny of the thinner shelled eggs.

Activity of TRAP was significantly higher (P = 0.022) in the progeny of the poor, 23590.73 U, egg shell producing hens compared with the progeny of the good, 18012.86 U, egg

shell producing hens. TRAP isn't solely used in the resorption of hydroxyapatite from bones but also used in bone remodeling, the process through which bone is reshaped and elongated during growth (Minkin, 1982). Osteoclast activity has also been associated with bone stress (Darden 1996). Micro fractures in the bone stimulate osteoclasts to resorb the damage bone and for osteoblasts to lay down new bone in its place. The tremendous strain rapidly growing broilers place upon their bones may explain why TRAP activity is so high in the progeny compared with their parents, at almost a 9 fold difference in activity. TRAP levels in the breeders were also most likely lowered due to them being taken during the day at the time of oviposition when the body is demineralizing the medullary bone by utilizing calcium from the diet. While TRAP activity indicates more bone repair in growing broilers this also tells us that the chicks from the thinner shell producing hens were more prone to having bone fractures than the progeny of the thicker shell producing hens.

Body weight gain was not significantly different ($P = 0.562$) between the progeny of the good egg shell producing hens, 285.6 g, and the progeny of the poor egg shell producing hens, 285.4g. The overall growth of the chicks was not impacted at two weeks of age due to the difference in egg shell density that they were born from which suggests that they were both gaining muscle and fat tissue at their genetic potential and that bone strength was not impacting their growth up to this point. Tibia ash percentage was significantly different ($P = 0.025$) between the progeny of the good egg shell producing hens, 0.459%, and the poor egg shell producing hens, 0.422%. Higher tibia ash percentage in the progeny of the good shell producing hens is consistent with the BAP-osteoblast activity being higher in these chicks than the progeny of the poor egg shell procuring hens, and vice versa for TRAP-osteoclast activity. Breaking strength was significantly ($p= 0.03$) different between the progeny of the good egg shell

producing hens 1.61 kg/mm, and the progeny of the poor egg shell producing hens, 1.47 kg/mm. Increased breaking strength of the progeny of the good egg shell hens is consistent with their higher concentrations of BAP and lower TRAP which indicate they have strong hydroxyapatite and collagen crosslinks within their bones. The progeny of the poor egg shell hens seem to be vulnerable to micro fractures and must repair their bone at a higher rate than the progeny of the good egg shell hens which is the reason why their TRAP activity is so high and which ultimately may be due to lower BAP activity causing less bone to be made in these chicks.

Hen genetics quintessentially determine how fit the animal will be for egg production as the upregulation of specific hormones, enzymes, transporter, and other factors can make the animal more or less efficient at utilizing calcium, the principal nutrient for egg shell synthesis. Decades of selection for egg production and shell quality have improved these traits, however, there is a limitation on how much scientists can select for from simple production parameters and more in-depth analysis is needed. Selecting hens for egg shell thickness not only benefits from the increased the fertility and hatchability of the eggs but also from increased calcium retention in the progeny which will help them with bone growth and egg development once fully grown. Progeny from the hens selected for thicker egg shells benefited from having higher concentrations of BAP, perhaps due to having more osteoblasts in their bones. The good shell quality laying hens had higher concentrations of BAP in their blood relative to the poor egg shell producing hens which indicate a relationship between bone turnover and shell gland calcium carbonate synthesis. Data in this study suggests that either osteoblast formation or BAP activity, and osteoclast formation or TRAP activity can be inherited and that hens which have higher calcium retention can pass those traits to their progeny.

Table 1: Production performance parameters from hens selected for egg shell quality from 30 to 46 weeks of age.[1]

	Egg Production (Egg/hen)	Hen House Egg Production[2] (% of total production)	Mortality (% of total)	Egg weight (g)
Egg Shell Quality[3]				
Good	58.3	49.2	5.0	64.8
Poor	60.3	50.8	15.0	63.0
SEM	2.0	NA	5.0	2.5
P Value	0.15	NA	0.031	0.33

[1]Values are presented as means ± SEM for the 16 week production period.

[2]Defined as total eggs per hen, corrected for mortality.

[3]Egg Shell Quality: Hens were defined as having good egg shell quality, having an average egg specific gravity great than 1.08, or having poor egg shell quality, having an average egg specific gravity less than 1.08.

Table 2: Egg shell quality parameters from hens selected for egg shell quality from 30 to 46 weeks of age.[1]

	Shell:Egg Weight	SWUSA[2] (mg/m^2)	Shell Calcium (%)	Specific Gravity	Shell Thickness (mm)
Egg Shell Quality[2]					
Good	0.093	80.56	6.08	2.28	1.086
Poor	0.076	65.25	4.83	1.80	1.07
SEM	0.032	21.4	1.52	0.54	0.025
P Value	0.0001	0.0001	0.022	0.0001	0.0001

[1]Values are presented as means ± SEM for the 16 week production period.

[2]Egg Shell Quality: Hens were defined as having good egg shell quality, having an average egg specific gravity great than 1.08, or having poor egg shell quality, having an average egg specific gravity less than 1.08.

Table 3: Blood parameters from hens selected for egg shell quality from 30 to 46 weeks of age.[1]

	Bone Alkaline Phosphatase (pg/ml)	Tartrate Resistant Acid Phosphatase (U)[2]
Egg Shell Quality[3]		
Good	326.49	2203.4
Poor	253.19	4985.2
SEM	108.2	2842.1
P Value	0.023	0.0022

[1]Values are presented as means ± SEM for the 16 week production period.

[2]Activity of TRAP (U) was defined by the procedure by Lau et al. as the amount of NPP cleaved under 1 hour of incubation at 37C.

[3]Egg Shell Quality: Hens were defined as having good egg shell quality, having an average egg specific gravity great than 1.08, or having poor egg shell quality, having an average egg specific gravity less than 1.08.

Table 4: Bone parameters from hens selected for egg shell quality from 30 to 46 weeks of age.[1]

	Tibia Ash (%)	Breaking Strength (Kg/mm)
Egg Shell Quality[2]		
Good	58.0	7.26
Poor	57.2	5.34
SEM	5.1	2.72
P Value	0.46	0.04

[1]Values are presented as means ± SEM for the 16 week production period.

[2]Egg Shell Quality: Hens were defined as having good egg shell quality, having an average egg specific gravity great than 1.08, or having poor egg shell quality, having an average egg specific gravity less than 1.08.

Table 5: Blood parameters from progeny of hens selected for egg shell quality at 2 weeks of age.

	Bone Alkaline Phosphatase (pg/ml)	Tartrate Resistant Acid Phosphatase (U)[1]
Egg Shell Quality[2]		
Good	372.3	18012.86
Poor	312.4	23590.73
SEM	130.4	6156.6
P Value	0.0017	0.0022

[1]Activity of TRAP (U) was defined by the procedure by Lau et al. as the amount of NPP cleaved under 1 hour of incubation at 37C.

[2]Egg Shell Quality: Progeny were created from hens which were defined as having good egg shell quality, having an average egg specific gravity great than 1.08, or having poor egg shell quality, having an average egg specific gravity less than 1.08.

Table 6: Growth & Bone parameters from progeny hens selected for egg shell quality at 2 weeks of age.[1]

	Body Weight Change (g)	Tibia Ash (%)	Breaking Strength $(Kg/mm)^2$
Egg Shell Quality[2]			
Good	285.6	45.9	1.61
Poor	285.4	42.2	1.47
SEM	15.2	6.9	0.54
P Value	0.562	0.025	0.03

[1]Values are presented as means ± SEM for the 16 week production period.

[2]Egg Shell Quality: Hens were defined as having good egg shell quality, having an average egg specific gravity great than 1.08, or having poor egg shell quality, having an average egg specific gravity less than 1.08.

References

Acar, N., F. G. Sizemore, G. R. Leach, R. F. Wideman, R. L. Owen, and G. F. Barbato. "Growth of Broiler Chickens in Response to Feed Restriction Regimens to Reduce Ascites." *Poultry Science* (1995): 833-43. Print.

Addison WN, Nakano Y, Loisel T, Crine P, McKee MD (October 2008). "MEPE-ASARM peptides control extracellular matrix mineralization by binding to hydroxyapatite: an inhibition regulated by PHEX cleavage of ASARM". *J. Bone Miner. Res.* 23 (10): 1638–49.

Angel NZ, Walsh N, Forwood MR, Ostrowski MC, Cassady AI, Hume DA (2000). "Transgenic mice overexpressing tartrate-resistant acid phosphatase exhibit an increased rate of bone turnover". *J. Bone Miner. Res.* 15 (1): 103–10.

Bai XY, Miao D, Goltzman D, Karaplis AC (2003). "The autosomal dominant hypophosphatemic rickets R176Q mutation in fibroblast growth factor 23 resists proteolytic cleavage and enhances in vivo biological potency". *J. Biol. Chem.* 278 (11): 9843–9.

Bar, A., and S. Hurwitz. "Egg Shell Quality, Medullary Bone Ash, Intestinal Calcium and Phosphorus Absorption, and Calcium-Binding Protein in Phosphorus-Deficient Hens." *Poultry Science* (1984): 1975-979. Print.

Barros, NMT et al. (2013). "Proteolytic processing of osteopontin by PHEX and accumulation of osteopontin fragments in Hyp mouse bone, the murine model of X-linked hypophosphatemia". *Journal of Bone and Mineral Research* 28 (3): 688–699.

Barros, NMT et al. (2013). "Proteolytic processing of osteopontin by PHEX and accumulation of osteopontin fragments in Hyp mouse bone, the murine model of X-linked hypophosphatemia". *Journal of Bone and Mineral Research* 28 (3): 688–699.

Basle MF, Mazaud P, Malkani K, Chretien MF, Moreau MF, Rebel A (1988). "Isolation of osteoclasts from Pagetic bone tissue: morphometry and cytochemistry on isolated cells". *Bone* 9 (1): 1–6.

Bateman TA, Countryman S (April 2002). "Osteoprotegerin and bone loss associated with spaceflight". *Drug Discov. Today* 7 (8): 456–7.

Bauer, R., J. A. Plieschnig, T. Finkes, B. Riegler, M. Hermann, and W. J. Schneider. "The Developing Chicken Yolk Sac Acquires Nutrient Transport Competence by an Orchestrated Differentiation Process of Its Endodermal Epithelial Cells." *Journal of Biological Chemistry* (2012): 1088-098. Print.

Berndt, T. J., S. Schiavi, and R. Kumar. 2005 “Phosphatonins” and the regulation of phosphorus homeostasis. Am. J. Physiol. Renal Physiol. 289;F1170-F1182

Bertazzo, S. & Bertran, C. A. (2006). "Morphological and dimensional characteristics of bone mineral crystals". *Bioceramics*. 309–311 (Pt. 1, 2): 3–10.

Bertazzo, S. *et al*. Nano-analytical electron microscopy reveals fundamental insights into human cardiovascular tissue calcification. *Nature Materials* 12, 576-583 (2013).

Bertazzo, S.; Bertran, C.A.; Camilli, J.A. (2006). "Morphological Characterization of Femur and Parietal Bone Mineral of Rats at Different Ages". *Key Engineering Materials*. 309–311: 11–14.

Biber, J., M. Custer, S. Magagnin, G. Hayes, A. Werner, B. Lotscher, B. Kaissling, and H Murer. 1996. Renal Na/Pi-cotransporters. Kidney Int. 49:981-985

Blair HC, Sun L, Kohanski RA. (2007). "Balanced regulation of proliferation, growth, differentiation, and degradation in skeletal cells". *Ann N Y Acad Sci*. 1116:165-73.

Blair HC, Zaidi M, Huang CL, Sun L. (2008). The developmental basis of skeletal cell differentiation and the molecular basis of major skeletal defects. Biol Rev Camb Philos Soc. 83: 401-15. doi: 10.1111/j.1469-185X.2008.00048

Bloom, William, Margaret A. Bloom, and Franklin C. Mclean. "Calcification and Ossification. Medullary Bone Changes in the Reproductive Cycle of Female Pigeons." *Anat. Rec. The Anatomical Record*: 443-75. Print.

Boling, S.D., M.W. Douglas, R.B. Shirley, C.M. Parsons and K.W. Koelbeck, 2000. The effect of various dietary levels of phytase and available phosphorus on performance of laying hens. Poult. Sci., 79: 535- 583.

Boron WF, Boulpaep EL (2004). "Endocrine system chapter". *Medical Physiology: A Cellular And Molecular Approach*. Elsevier/Saunders.

Bradfield, J. R. G., 1951. Radiographic studies on formation of the hen's egg shell. J. Exp. Biol. 28:125-140.

Brighton, Carl T. and Robert M. Hunt (1997), "Early histologic and ultrastructural changes in microvessels of periosteal callus", *Journal of Orthopaedic Trauma*, 11 (4): 244-253

Broer, S. T., A. Schuster, C. Wager, A. Broer, I. Forster, J. Biber, H. Murer, F. Lang, And A.E Busch. 1998. Chloride conductance and Pi transport are separate functions induced by expression of NaPi-1 in *Xeonpus oocytes*. J. Membr. Biol. 164:71-77.

Bronner, Felix. "Mechanisms of Intestinal Calcium Absorption." *J. Cell. Biochem. Journal of Cellular Biochemistry* (2003): 387-93. Print.

Burley, R.W., D.V. Vadehra (1989) The Avian Egg: Chemistry and Biology. New York, Wiley.

Burmester, B. R., and L. E. Card, 1941. Experiments on the physiology of egg white secretion. Campos M, Couture C, Hirata IY, Juliano MA, Loisel TP, Crine P et al. (2003). "Human recombinant endopeptidase PHEX has a strict S1' specificity for acidic residues and cleaves peptides derived from fibroblast growth factor-23 and matrix extracellular phosphoglycoprotein". *Biochem. J.* 373 (Pt 1): 271–9.

Chandramoni, S., B. Jadhao and R.P. Sinha, 1998. Effect of dietary calcium and phosphorus concentrations on retention of these nutrients by caged layers. Br. Poult. Sci., 39: 544-548.

Cheng, Kui; Weng, Wenjian; Qu, Haibo; Du, Piyi; Shen, Ge; Han, Gaorong; Yang, Juan; Ferreira, J. M. F. (15 April 2004). "Sol-gel preparation andin vitro test of fluorapatite/hydroxyapatite films". *Journal of Biomedical Materials Research* 69B (1): 33–37.

Costoff A. "Sect. 5, Ch. 6: Anatomy, Structure, and Synthesis of Calcitonin (CT)". *Endocrinology: hormonal control of calcium and phosphate*. Medical College of Georgia. Retrieved 2015-07-07.

Curry, J.D. 2006. "The Structure of Bone Tissue" Bones: Structure and Mechanics Princeton U. Press. Princeton, NJ. pps: 12–14

Darden AG, Ries WL, Wolf WC, Rodriguiz RM, Key LL (1996). "Osteoclastic superoxide production and bone resorption: stimulation and inhibition by modulators of NADPH oxidase". *J. Bone Miner. Res.* 11 (5): 671–5.

Deluca, H. F., M. F. Holick, H. K. Schnoes, T. Suda, and R. J. Cousins. "Isolation and Identification of 1,25-dihydroxycholecalciferol. A Metabolite of Vitamin D Active in Intestine." *Biochemistry* (1971): 2799-804. Print.

Eastin, W. C. "On the Control of Calcium Secretion in the Avian Shell Gland (Uterus)." *Biology of Reproduction* (1978): 493-504. Print.

Econs MJ (2000). "Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23". *Nat. Genet.* 26 (3): 345–8.

ed, Russell T. Woodburne ..., consulting (1999). *Anatomy, physiology, and metabolic disorders* (5. print. ed.). Summit, N.J.: Novartis Pharmaceutical Corp. pp. 187–189.

Ekmay, R. D., C. Salas, J. England, S. Cerrate, and C. N. Coon. "The Effects of Pullet Body Weight, Dietary Nonpyhtate Phosphorus Intake, and Breeder Feeding Regimen on Production Performance, Chick Quality, and Bone Remodeling in Broiler Breeders." *Poultry Science* (2012): 948-64. Print.

Ekmay, R.d., and C.n. Coon. "An Examination of the P Requirements of Broiler Breeders for Performance, Progeny Quality and P Balance 1. Non-phytate Phosphorus." *International J. of Poultry Science International Journal of Poultry Science* (2010): 1043-049. Print.

Ek-Rylander B, Flores M, Wendel M, Heinegård D, Andersson G (1994). "Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro". *J. Biol. Chem.* 269 (21): 14853–6.

Ek-Rylander, Barbro, and Goran Andersson. "Modulation Of Osteoclast Adhesion And Migration On Phosphorylated Osteopontin (Opn) By Tartrate-Resistant Acid Phosphatase

(Trap)." *3rd International Conference on Osteopontin and SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) Proteins, 2002*. Print.

Fang, Rejun, Zhifeng Xiang, Manhu Cao, and Jia He. "Different Phosphate Transport in the Duodenum and Jejunum of Chicken Response to Dietary Phosphate Adaptation." *Asian Australas. J. Anim. Sci Asian-Australasian Journal of Animal Sciences* (2012): 1457-465. Print.

Fuchs R, Peterlik M. Intestinal phosphate transport. In: Massry SG, Ritz E, Jahn H, editors. Phosphate and Minerals in Health and Disease. New York: Plenum Press; 1980. p. 381

Fukagawa M, Nii-Kono T, Kazama JJ (2005). "Role of fibroblast growth factor 23 in health and in chronic kidney disease". *Curr. Opin. Nephrol. Hypertens.* 14 (4): 325–9.

G, McDaniel, D. Roland, and M. Coleman. "Poultry Science." *The Effect of Egg Shell Quality on Hatchability and Embryonic Mortality*. 12 June 1978.

Garnero P, Darte C, Delmas PD: A model to monitor the efficacy of alendronate treatment in women with osteoporosis using a biochemical marker of bone turnover. Bone 1999;24(6):603-609

Harada M, Udagawa N, Fukasawa K, Hiraoka BY, Mogi M (February 1986). "Inorganic pyrophosphatase activity of purified bovine pulp alkaline phosphatase at physiological pH". *J. Dent. Res.* 65 (2): 125–7.

Hayman, Alison R. "Tartrate-resistant Acid Phosphatase (TRAP) and the Osteoclast/immune Cell Dichotomy." *Autoimmunity* (2008): 218-23. Print.

Hirst KL, Simmons D, Feng J, Aplin H, Dixon MJ, MacDougall M (Jul 1997). "Elucidation of the sequence and the genomic organization of the human dentin matrix acidic phosphoprotein 1 (DMP1) gene: exclusion of the locus from a causative role in the pathogenesis of dentinogenesis imperfecta type II". *Genomics* 42 (1): 38–45.

Hunton, P (2005). "Research on eggshell structure and quality: an historical overview". *Revista Brasileira de Ciência Avícola* 7: 67–71.

Imel EA, Econs MJ (2006). "Fibroblast growth factor 23: roles in health and disease". *J. Am. Soc. Nephrol.* 16 (9): 2565–75.

Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium; Ross AC, Taylor CL, Yaktine AL, et al., editors. Dietary Reference Intakes for Calcium and Vitamin D. Washington (DC): National Academies Press (US); 2011. 3, Overview of Vitamin D.

Joyner, C.j., M.j. Peddie, and T.g. Taylor. "The Effect of Age on Egg Production in the Domestic Hen." *General and Comparative Endocrinology*: 331-36. Print.

Karsenty G, Oury F (Jan 2014). "Regulation of male fertility by the bone-derived hormone osteocalcin". *Molecular and Cellular Endocrinology* 382 (1): 521–6.

Keshavarz, K., 2000. Nonphytate phosphorus requirement of laying hens with and without phytase on a phase feeding program. Poult. Sci., 79: 748-763.

Keshavarz, K., 2000. Nonphytate phosphorus requirement of laying hens with and without phytase on a phase feeding program. Poult. Sci., 79: 748-763.

Kiela PR, Ghishan FK (January 2009). "Recent advances in the renal-skeletal-gut axis that controls phosphate homeostasis". *Lab. Invest.* 89 (1): 7–14.

Kikuta J, Kawamura S, Okiji F, Shirazaki M, Sakai S, Saito H, Ishii M (Apr 2013). "Sphingosine-1-phosphate-mediated osteoclast precursor monocyte migration is a critical point of control in antibone-resorptive action of active vitamin D.". *Proc Natl Acad Sci U S A* 110 (17): 7009–13.

Kress BC, Mizrahi IA, Armour KW, et al: Use of bone alkaline phosphatase to monitor alendronate therapy in individual postmenopausal osteoporotic women. Clin Chem 1999;45(7):1009-1017

Kruse K, Woelfel D, Strom TM, Storm TM (2002). "Loss of renal phosphate wasting in a child with autosomal dominant hypophosphatemic rickets caused by a FGF23 mutation". *Horm. Res.* 55 (6): 305–8.

Larsson T, Zahradnik R, Lavigne J, Ljunggren O, Jüppner H, Jonsson KB (2003). "Immunohistochemical detection of FGF-23 protein in tumors that cause oncogenic osteomalacia". *Eur. J. Endocrinol.* 148 (2): 269–76. doi:10.1530/eje.0.1480269. PMID 12590648.

Lavelin, I; Meiri, N; Pines, M (2000). "New insight in eggshell formation". *Poultry science* 79 (7): 1014–7

Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C et al. (Aug 2007). "Endocrine regulation of energy metabolism by the skeleton". *Cell* 130 (3): 456–69.

Lee, Na Kyung et al. (10 August 2007). "Endocrine Regulation of Energy Metabolism by the Skeleton" (PDF). *Cell* 130 (3): 456–469.

Lee, T. J.; Jang, J.; Kang, S.; Jin, M.; Shin, H.; Kim, D. W.; Kim, B. S. (2013). "Enhancement of osteogenic and chondrogenic differentiation of human embryonic stem cells by mesodermal lineage induction with BMP-4 and FGF2 treatment". *Biochemical and Biophysical Research Communications* 430 (2): 793–797.

Leske, K., and C. Coon. "A Bioassay to Determine the Effect of Phytase on Phytate Phosphorus Hydrolysis and Total Phosphorus Retention of Feed Ingredients as Determined with Broilers and Laying Hens." *Poultry Science* (1999): 1151-157. Print.

Lindunger, Anders, Carole A. Mackay, Barbro Ek-Rylander, Göran Andersson, and Sandy C. Marks. "Histochemistry and Biochemistry of Tartrate-resistant Acid Phosphatase (TRAP) and

Tartrate-resistant Acid Adenosine Triphosphatase (TrATPase) in Bone, Bone Marrow and Spleen: Implications for Osteoclast Ontogeny." *Bone and Mineral*: 109-19. 1990. Print.
Liu S, Quarles LD (2007). "How fibroblast growth factor 23 works". *J. Am. Soc. Nephrol.* 18 (6): 1637–47.

Liu, S., and L. D. Quarles. "How Fibroblast Growth Factor 23 Works." *Journal of the American Society of Nephrology* (2007): 1637-647. Print.

Long, P. H., S. R. Lee, G. N. Rowland, and W. M. Britton. "Experimental Rickets in Broilers: Gross, Microscopic, and Radiographic Lesions. I. Phosphorus Deficiency and Calcium Excess." *Avian Diseases*: 460. Print.

Luchin A, Purdom G, Murphy K, Clark MY, Angel N, Cassady AI, Hume DA, Ostrowski MC (2000). "The microphthalmia transcription factor reulates expression of the tartrate-resistant acid phosphatase gene during terminal differentiation of osteoclasts". *J. Bone Miner. Res.* 15 (3): 451–460

Luo, Y., P. M. Vassilev, X. Li, Y. Kawanabe, and J. Zhou. "Native Polycystin 2 Functions as a Plasma Membrane Ca2 -Permeable Cation Channel in Renal Epithelia." *Molecular and Cellular Biology* (2003): 2600-607. Print.

Manangi, M. K., and C. N. Coon. "Phytate Phosphorus Hydrolysis in Broilers in Response to Dietary Phytase, Calcium, and Phosphorus Concentrations." *Poultry Science* (2008): 1577-586. Print.

Manolagas, SC (April 2000). "Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis.". *Endocrine Reviews* 21 (2): 115–37.

Martini F., & Nath J. L. (2009). Fundamentals of Anatomy and Physiology 8e. San Francisco, CA: Pearson Education Inc.

Mensenkamp, Arjen R, Joost Gj Hoenderop, and René Jm Bindels. "Recent Advances in Renal Tubular Calcium Reabsorption." *Current Opinion in Nephrology and Hypertension*: 524-29. Print.

Minkin C (1982). "Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function". *Calcif. Tissue Int.* 34 (3): 285–90

Mook, W. (2000) "Chemistry of carbonic acid in water", pp. 143–165 in *Environmental Isotopes in the Hydrological Cycle: Principles and Applications*. INEA/UNESCO: Paris.

Neuman, William F.; Neuman, Margaret W. (1958). The Chemical Dynamics of Bone Mineral. Chicago: The University of Chicago Press.

Nieves, W. Nieves. *Osteoporosis: the role of micronutrients.* Am J Clin Nutr *May 2005 vol. 81* no. 5 1232S-1239S 2005

Nusse, Roel; Varmus, Harold E. (1 June 1992). "Wnt genes". *Cell* 69 (7): 1073–1087.

Nys, Y., and X De Laage. "Effects of Suppression of Eggshell Calcification and of 1,25(OH)2D3 on Mg2 , Ca2 and Mg2 HCO3− ATPase, Alkaline Phosphatase, Carbonic Anhydrase and CaBP Levels—II. The Laying Hen Intestine." *Comparative Biochemistry and Physiology Part A: Physiology*: 839-44. Print.

Nys, Y., T. M. N'guyen, J. Williams, and R. J. Etches. "Blood Levels of Ionized Calcium, Inorganic Phosphorus, 1,25-dihydroxycholecalciferol and Gonadal Hormones in Hens Laying Hard-shelled or Shell-less Eggs." *Journal of Endocrinology* (1986): 151-57. Print.

Nys, Yves; Gautron, Joël; Garcia-Ruiz, Juan M.; Hincke, Maxwell T. (2004). "Avian eggshell mineralization: biochemical and functional characterization of matrix proteins". *Comptes Rendus Palevol* 3: 549–62.

Onyango, E., P. Hester, R. Stroshine, and O. Adeola. "Bone Densitometry as an Indicator of Percentage Tibia Ash in Broiler Chicks Fed Varying Dietary Calcium and Phosphorus Levels." *Poultry Science* (2003): 1787-791. Print.

Orimo, Hideo. "The Mechanism of Mineralization and the Role of Alkaline Phosphatase in Health and Disease." *Journal of Nippon Medical School J Nippon Med Sch* (2010): 4-12. Print.
Plumstead, P. W., H. Romero-Sanchez, R. O. Maguire, A. G. Gernat, J. Brake. 2007. Effects of phosphorus level and phytase in broiler breeder rearing and laying diets on live performance and phosphorus excretion. Poult. Sci. 86; 225-231.

Pote, D. H.,T.C. Daniel, D. J. Nichols, A. N. Sharpley, P. A. Moore, Jr., D. M. Miller, and D.R. Edwards. 1999. Relationship between phosphorus levels in three Ultisols and phosphorus concentrations in run off. J. Environ. Qual. 28:170-175
Poultry Sci. 20: 224-226.

Pratt, Rebecca. "Bone as an Organ". *AnatomyOne*. Amirsys, Inc.

Quarles LD (2003). "FGF23, PHEX, and MEPE regulation of phosphate homeostasis and skeletal mineralization". *Am. J. Physiol. Endocrinol. Metab.* 285 (1): E1–9. doi:10.1152/ajpendo.00016.2003 (inactive 2015-01-13).

Raggatt, L. J. et al. (May 25, 2010). "Cellular and Molecular Mechanisms of Bone Remodeling". *The Journal of Biological* (American Society for Biochemistry and Molecular Biology) 285: p. 25103–25108.

Raisz L, Smith JA, Trahiotism M, et al: Short-term risedronate treatment in postmenopausal women: Effects on biochemical markers of bone turnover. Osteoporos Int 2000;11:615-620

Rao, K. S., and D. A. Roland, 1990. Influence of dietary calcium and phosphorus on urinary calcium in commercial leghorn hens. Poult. Sci. 69; 1991-1997.

Rath, N. C., G. R. Huff, W. E. Huff, and J. M. Balog. "Factors Regulating Bone Maturity and Strength in Poultry." *Poultry Science* (2000): 1024-032. Print.

Rath, N., J. Balog, W. Huff, G. Huff, G. Kulkarni, and J. Tierce. "Comparative Differences in the Composition and Biomechanical Properties of Tibiae of Seven- and Seventy-two-week-old Male and Female Broiler Breeder Chickens." *Poultry Science* (1999): 1232-239. Print.

Raynaud, S.; Champion, E.; Bernache-Assollant, D.; Thomas, P. (2002). "Calcium phosphate apatites with variable Ca/P atomic ratio I. Synthesis, characterisation and thermal stability of powders". *Biomaterials* 23 (4): 1065.

Roberts, R. Juliet. Factors Affecting Egg Internal quality and Egg Shell Quality in Laying Hens. Poultry Science. Vol. 41 (2004) No. 3 P 161-177

Robertson, M. P. *The Egg*. New York: Phyllis Fogelman, 2001. Print.

Robinson, F. E., N. A. Robinson, and T. A. Scott. "Reproductive Performance, Growth Rate and Body Composition of Full-fed versus Feed-restricted Broiler Breeder Hens." *Canadian Journal of Animal Science Can. J. Anim. Sci.* (1991): 549-56. Print.

Romanoff, A.L., A.J. Romanoff (1949) The avian egg. New York, Wiley.

Ross, A. Catharine. *DRI, Dietary Reference Intakes Calcium, Vitamin D*. Washington, D.C.: National Academies, 2011. Print.

S. L. Teitelbaum (2000), "Bone Resorption by Osteoclasts", *Science* 289: 1504–1508,
Saito H, Kusano K, Kinosaki M, Ito H, Hirata M, Segawa H et al. (2003). "Human fibroblast growth factor-23 mutants suppress Na+-dependent phosphate co-transport activity and 1alpha,25-dihydroxyvitamin D3 production". *J. Biol. Chem.* 278 (4): 2206–11.

Salentijn, L. *Biology of Mineralized Tissues: Cartilage and Bone*, Columbia University College of Dental Medicine post-graduate dental lecture series, 2007

Schlesinger, Paul; lair, H.C.; Teitelbaum, S.L.; Edwards, J.C. (1997). "The chloride channel of osteoclast ruffled borders: Molecular characterization and role in bone resorption.". *J. Biol. Chem* 272: 18636–18643.

Schoppet M, Preissner K, Hofbauer L (2002). "RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function". *Arterioscler Thromb Vasc Biol* 22 (4): 549–53.

Sharp, P. J. "Photoperiodic Control of Reproduction in the Domestic Hen." *Poultry Science* (1993): 897-905. Print.

Shimada T, Mizutani S, Muto T, Yoneya T, Hino R, Takeda S et al. (2001). "Cloning and characterization of FGF23 as a causative factor of tumor-induced osteomalacia". *Proc. Natl. Acad. Sci. U.S.A.* 98 (11): 6500–5.

Silve C, Beck L (2003). "Is FGF23 the long sought after phosphaturic factor phosphatonin?". *Nephrol. Dial. Transplant.* 17 (6): 958–61.

Solomon, S. E. *Egg and Eggshell Quality*. S.l.: Wolfe, 1990. Print.

Stamp TC, Haddad JG, Twigg CA. (Jun 1977). "Comparison of oral 25-hydroxycholecalciferol, vitamin D, and ultraviolet light as determinants of circulating 25-hydroxyvitamin D.". *Lancet* 1 (8026): 1341–3.

Straub, D.A. (2007). "Calcium Supplementation in Clinical Practice: A Review of Forms, Doses, and Indications". *NCP- Nutrition in Clinical Practice* 22 (3): 286–96.

Sun, J. M., M. P. Richards, R. W. Rosebrough, C. M. Ashwell, J. P. Mcmurtry, and C. N. Coon. "The Relationship of Body Composition, Feed Intake, and Metabolic Hormones for Broiler Breeder Females." *Poultry Science* (2006): 1173-184. Print.

Tanck, E., G. Hannink, R. Ruimerman, P. Buma, E. H. Burger, and R. Huiskes. "Cortical Bone Development under the Growth Plate Is Regulated by Mechanical Load Transfer." *J Anatomy Journal of Anatomy* (2006): 73-79. Print.

Taylor, T. G. "How an Eggshell Is Made." *Sci Am Scientific American* (1970): 88-95. Print.
Thurner PJ, Chen CG, Ionova-Martin S, Sun L, Harman A, Porter A, Ager JW 3rd, Ritchie RO, Alliston T. (2010) "Osteopontin deficiency increases bone fragility but preserves bone mass". *Bone*. 46: 1564-73.

Tischer, E; Mitchell, R; Hartman, T; Silva, M; Gospodarowicz, D; Fiddes, JC; Abraham, JA (25 June 1991). "The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing.". *The Journal of Biological Chemistry* 266 (18): 11947–54.

Triyuwanta Leterrier, C. and Y. Nys, 1992. Dietary phosphorus and food allowance of dwarf breeders affect reproductive performance of hens bone development of their progeny. Br. Poult Sci., 33: 363-379

Triyuwanta, C. Leterrier, and Y. Nys. 1992. Dietary phosphorus and food allowance of dwarf breeders affect reproductive performance of hens bone development of their progeny. Br. Poult Sci. 33; 363-379.

Tucker, L. A.; Nokes, N.; Adams, T. (2007). "Effect of a Dietary Supplement on Hip and Spine BMD". *Medicine & Science in Sports & Exercise* 39: S230.

Urena, P, Hruby, M, Ferreira, A, Ang, KS, and M.C. de Vernejoul. 1996. Plasma total versus bone alkaline phosphatase as markers of bone turnover in hemodialysis patients. J. Am. Soc. Nephrol. 7; 506-512.

US Dept of Health and Human Services and US Dept of Agriculture *Dietary Guidelines for Americans 2005*. Rockville, MD: 2005

Väänänen H, Zhao H, Mulari M, Halleen J (2000). "The cell biology of osteoclast function". *J Cell Sci* 113 (3): 377–81. p. 378

Valletregi, M. (1997). "Synthesis and characterisation of calcium deficient apatite". *Solid State Ionics*. 101-103: 1279

Van de Velde, J. P., Vermeiden, J. P. W., Touw, J. J. A. and J. P. Andveldhuijzen. 1984. Changes in activity of chicken medullary bone cell populations in relation to the egg-laying cycle. Metab. Bone Dis. Relat. Res. 5(4); 191

Van de Velde, J. P., Vermeiden, J. P. W., Touw, J. J. A. and J. P. Andveldhuijzen. 1984. Changes in activity of chicken medullary bone cell populations in relation to the egg-laying cycle. Metab. Bone Dis. Relat. Res. 5(4); 191

Wada T, Nakashima T, Hiroshi N, Penninger JM (2006). "RANKL-RANK signaling in osteoclastogenesis and bone disease". *Trends Mol Med* 12 (1): 17–25.

Werner, A., M. L. Moore, N. Mantei, J. Biber, G. Semenza, and H. Murer. 1991. Cloning and expression of cDNA for a Na/Pi cotransporter system of kidney cortex. Proc. Natl. Acad. Sci. USA 88:9608-9612

White KE, Jonsson KB, Carn G, Hampson G, Spector TD, Mannstadt M et al. (2001). "The autosomal dominant hypophosphatemic rickets (ADHR) gene is a secreted polypeptide overexpressed by tumors that cause phosphate wasting". *J. Clin. Endocrinol. Metab.* 86 (2): 497–500.

Wideman, R. F., and Y. K. Kirby. "A Pulmonary Artery Clamp Model for Inducing Pulmonary Hypertension Syndrome (Ascites) in Broilers." *Poultry Science* (1995): 805-12. Print.

Wideman, R. F., E. T. Mallinson, and H. Rothenbacher. "Kidney Function of Pullets and Laying Hens During Outbreaks of Urolithiasis." *Poultry Science* (1983): 1954-970. Print.

Wiemeyer, Stanley N., Christine M. Bunck, and Charles J. Stafford. "Environmental Contaminants in Bald Eagle Eggs-1980-84and Further Interpretations of Relationships to Productivity and Shell Thickness." *Archives of Environmental Contamination and Toxicology Arch. Environ. Contam. Toxicol.* (1993): 213-27. Print.

Winget, C. M., A. H. Smith, and G. N. Hoover. "Arterio-venous Differences in Plasma Calcium Concentration in the Shell Gland of the Laying Hen during Shell Formation." *Poultry Science* (1958): 1325-328. Print.

Wojtowicz, J. A. (1998). "Factors affecting precipitation of calcium carbonate" (PDF). *Journal of the Swimming Pool and Spa Industry* 3 (1): 18–23.

Yamada, Takao, Naomichi Matsukawa, Masayuki Matsumoto, Shigeto Morimoto, Toshio Ogihara, Takahiro Ochi, Hideki Yoshikawa, Akihide Nampei, Jun Hashimoto, Kenji Hayashida, Hideki Tsuboi, Kenrin Shi, Isamu Tsuji, and Hideaki Miyashita. "Matrix Extracellular Phosphoglycoprotein (MEPE) Is Highly Expressed in Osteocytes in Human Bone." *Journal of Bone and Mineral Metabolism* (2004): 176-84. Print.

Yamamoto Y, Yoshizawa T, Fukuda T, Shirode-Fukuda Y, Yu T, Sekine K, Sato T, Kawano H, Aihara K, Nakamichi Y, Watanabe T, Shindo M, Inoue K, Inoue E, Tsuji N, Hoshino M, Karsenty G, Metzger D, Chambon P, Kato S, Imai Y (Mar 2013). "Vitamin D receptor in osteoblasts is a negative regulator of bone mass control". *Endocrinology* 154 (3): 1008–20.

Yamashita T, Konishi M, Miyake A, Inui K, Itoh N (2002). "Fibroblast growth factor (FGF)-23 inhibits renal phosphate reabsorption by activation of the mitogen-activated protein kinase pathway". *J. Biol. Chem.* 277 (31): 28265–70.

Yan, F., R. Angel, and C. M. Ashwell. "Characterization of the Chicken Small Intestine Type IIb Sodium Phosphate Cotransporter." *Poultry Science* (2007): 67-76. Print.

Zhang, B., and C. Coon. "The Relationship of Calcium Intake, Source, Size, Solubility in Vitro and in Vivo, and Gizzard Limestone Retention in Laying Hens." *Poultry Science* (1997): 1702-706. Print.

Zhang, Sam; Xianting, Zeng; Yongsheng, Wang; Kui, Cheng; Wenjian, Weng (June 2006). "Adhesion strength of sol–gel derived fluoridated hydroxyapatite coatings". *Surface and Coatings Technology* 200 (22-23): 6350–6354.

Zhuang, Liyan, Ji-Bin Peng, Liqiang Tou, Hitomi Takanaga, Rosalyn M Adam, Matthias A Hediger, and Michael R Freeman. "Calcium-Selective Ion Channel, CaT1, Is Apically Localized in Gastrointestinal Tract Epithelia and Is Aberrantly Expressed in Human Malignancies." *Lab Invest Laboratory Investigation*: 1755-764. Print.

Appendix

APPENDIX A

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Craig N. Coon

FROM: Carol Rodlun, Program Manager
Institutional Animal Care
And Use Committee

DATE: February 15, 2013

SUBJECT: IACUC Modification Request **APPROVAL**
Expiration date: **July 31, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** the modification request (to add the use of metabolism chambers) to Protocol **#13002- "Evaluation of broiler breeder feeding regimes for pure-line and commercial type stock during rearing and production phases and calcium requirement during the production period."** You may implement this modification immediately.

In granting its approval, the IACUC has approved only the modification request provided. Should there be any additional changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request Form] **prior** to initiating the changes.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199