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EVALUATION OF CURRENT AND EMERGING TECHNIQUES FOR MEASURING EGGSHELL INTEGRITY OF THE DOMESTIC FOWL

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EVALUATION OF CURRENT AND EMERGING TECHNIQUES FOR MEASURING EGGSHELL INTEGRITY OF THE DOMESTIC FOWL

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By
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Lexington, Kentucky
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Lexington, Kentucky
2019

ABSTRACT OF DISSERTATION

EVALUATION OF CURRENT AND EMERGING TECHNIQUES FOR MEASURING EGGSHELL INTEGRITY OF THE DOMESTIC FOWL

This dissertation is an investigation into the effect of different zinc sources and levels on eggshell quality and microstructure, as well as keel bone damage. Eggshell function is two-fold; eggshells function to protect the developing embryo, as well as act as a barrier against bacterial penetration, optimizing food safety of the egg for human consumption (Mabe et. Al., 2003). Two small trials were conducted in order to determine differences in eggshell microstructure of eggs produced from hens at peak lay (26 weeks of age) and at the end of lay (88 weeks of age). Two groups of hens were fed a calcium sufficient or a calcium deficient diet. From this it was determined that eggs with higher breaking strengths had some differences in eggshell microstructure. Eggs with greater breaking strengths had a greater density of 'normal' structures, compared to 'abnormal' structures. Additionally, eggs requiring a greater breaking force, had a thicker micro-structure, compared to shells requiring less breaking force. With this knowledge on microstructure, a larger, 36-week study was conducted using different zinc sources. Every four weeks, eggs were collected and standard egg quality measurements were taken and keel bones were scored. At the end of the study, keel bones were collected from randomly selected hens representing each treatment. Picture of these keel bones were taken and measurements were taken to determine type and degree of deformation, in comparison to scores taken on the live bird. Pens selected for keel bone analysis, were the same pens that eggs were taken for imaging by the scanning electron microscope, to determine eggshell microstructure. From this data, it was determined that egg quality differences were detected, as well as differences in eggshell microstructure. Additionally, keel bone scores progressively worsened throughout the 36-week long study, with type and degree of deformation differing depending on zinc source.

KEYWORDS: Bioplex® zinc, egg quality, eggshell microstructure, keel bone deformation

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07/22/2019

Date

EVALUATION OF CURRENT AND EMERGING TECHNIQUES FOR MEASURING EGGSHELL INTEGRITY OF THE DOMESTIC FOWL

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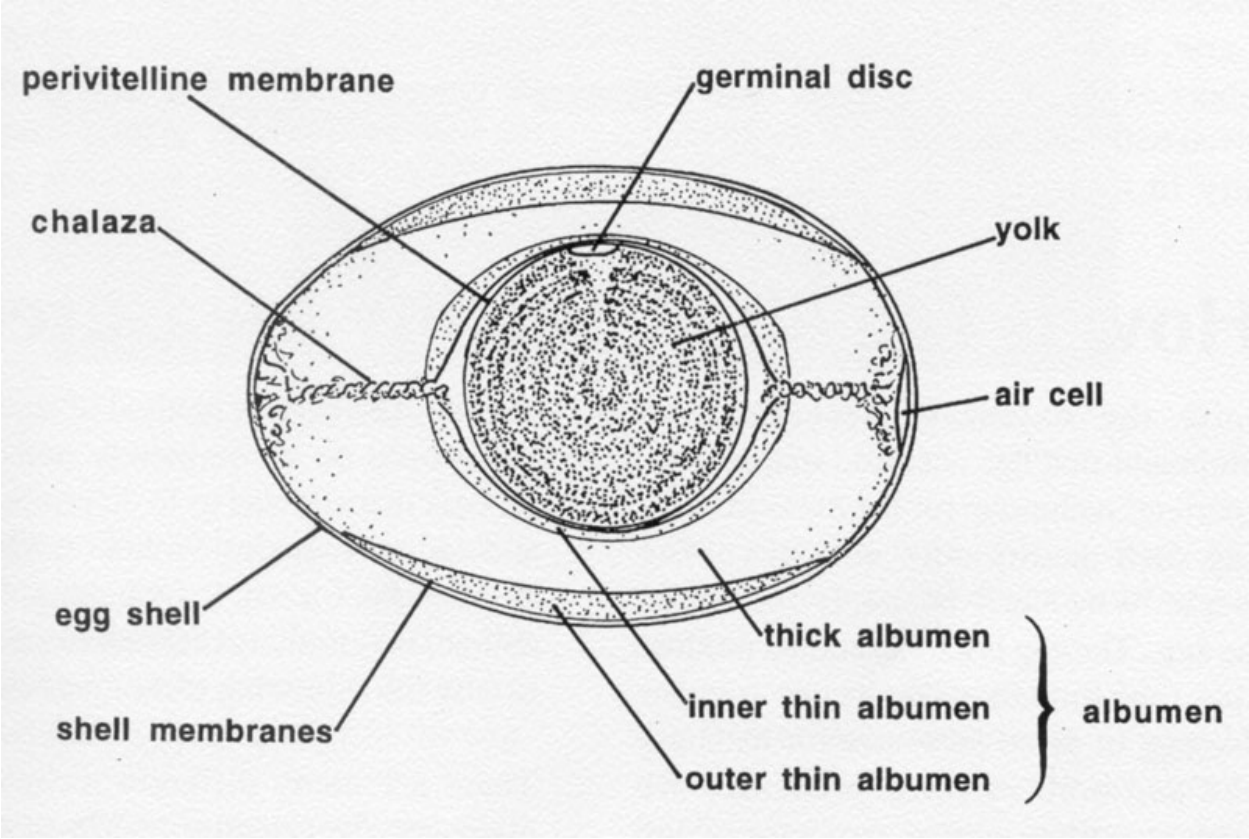
CHAPTER 1. INTRODUCTION

Avian eggshells are thin, mineralized layers that adequately protect egg contents and allow for extra-uterine development of chick embryos (Athanasiadou et al., 2018). In addition to protecting the developing embryo, the eggshell serves as a barrier against bacterial penetration, minimizing defects in the eggshells and optimizes food safety of the egg for human consumption (Mabe et al., 2003). Eighty to 90% of eggs that enter the food chain are routinely downgraded due to being broken or cracked (Mabe et al., 2003) and cost the industry millions of dollars of every year (Roberts, 2004). Regardless of function, formation and composition of the eggshell is formed via the same process, making manipulation of the egg a tedious matter. Eggshell formation and calcification is one of the most rapid bio mineralization processes that occurs (Nys et al., 2001). Ninety-seven percent (by weight) of the domestic chicken's eggshell is calcium carbonate, in the form of calcite, while the remaining 3% (by weight) organic material/matrix (Athanasiadou et al., 2018). Due to the composition of eggshells being primarily calcium carbonate, numerous studies have been conducted to improve eggshell quality in the areas of nutrition, specifically mineral nutrition (Nys et al., 2001). The hen's egg is composed of the yolk (30-33%), albumen (around 60%) and the shell (9-12%) (Stadelman, 1995) and can be seen in Figure 1.1.

Figure 1.1 Internal composition of the egg



(Roberts, 2004)

Eggshells have similar structure, elastic, and mechanical behaviors of ceramic materials, which depend strongly on microstructure (Rodriguez-Navarro et al., 2002). Microstructure is defined by grain size, their shape, and how they are arranged and orientated in the structure (crystallographic texture)" (Rodriguez-Navarro et al., 2002).

CHAPTER 2. LITERATURE REVIEW

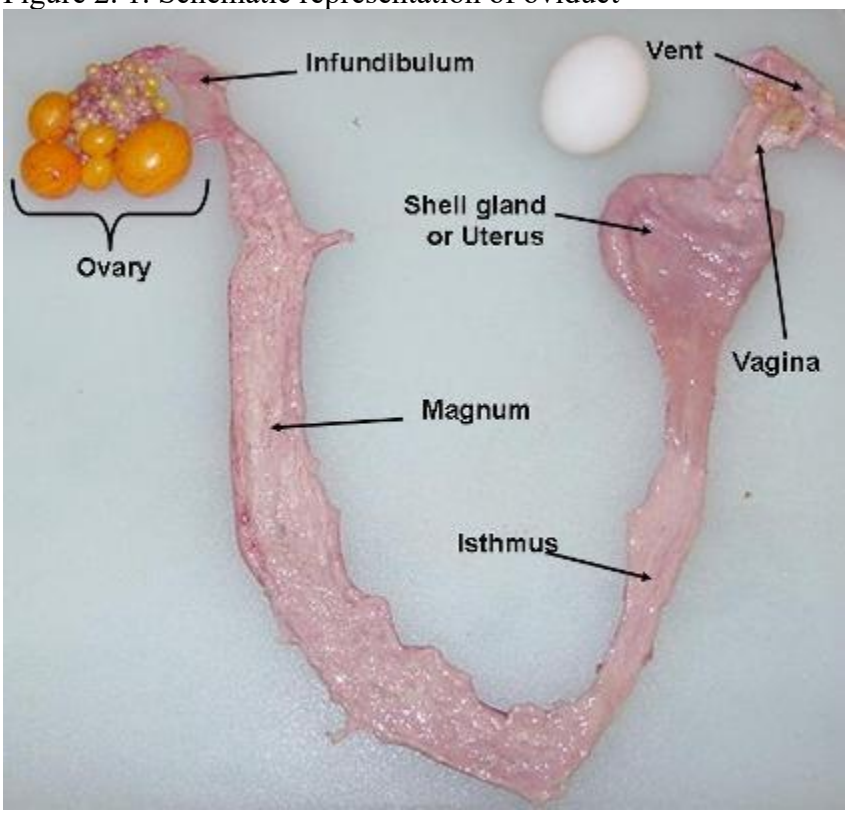
2.1 Eggshell Mineralization

The eggshell has two main functions. Firstly, the shell functions as a protector for the developing embryo, providing protection from physical contact, as well as providing a membrane for gas exchange or breathing (Nys et al., 1999). Secondly, the eggshell protects the contents that will be used for human consumption (Hunton, 2005). Eggshells must be strong enough to prevent cracking, while weak enough for the chick to break through during hatching and thin enough for gas exchange (Altuntaş and Şekeroğlu, 2008). Shell composition is known to be 97% calcium carbonate, that is provided through the diet and bone calcium. Calcium is usually provided in the diet in the form of calcium carbonate, but is broken down by the body and absorbed into the blood stream. Calcium is stored in the bones until needed for eggshell formation or transported to the uterus where it will be synthesized into calcium carbonate for the shell (Hunton, 2005). The structure of the eggshell is perfectly ordered and is the result of sequential deposition of organic and mineral layers within the isthmus and uterus of the oviduct. Eggshell formation (approximately 21 hours) is the most rapid mineralization process occurring in biology and contributes to the ultrastructural and crystallographic characteristics of mineralized tissue by facilitating nucleation and growth control of the crystals (Nys et al., 1999).

2.2 Eggshell Formation

In birds, only the left oviduct is developed, therefore is the only pathway for an egg to be developed. Egg formation, from ovulation to oviposition, takes 25.25 hours (Cutts et al., 2007). An image of the female reproductive tract can be seen in Figure 2.1.

Figure 2. 1. Schematic representation of oviduct



Jacob (2015)

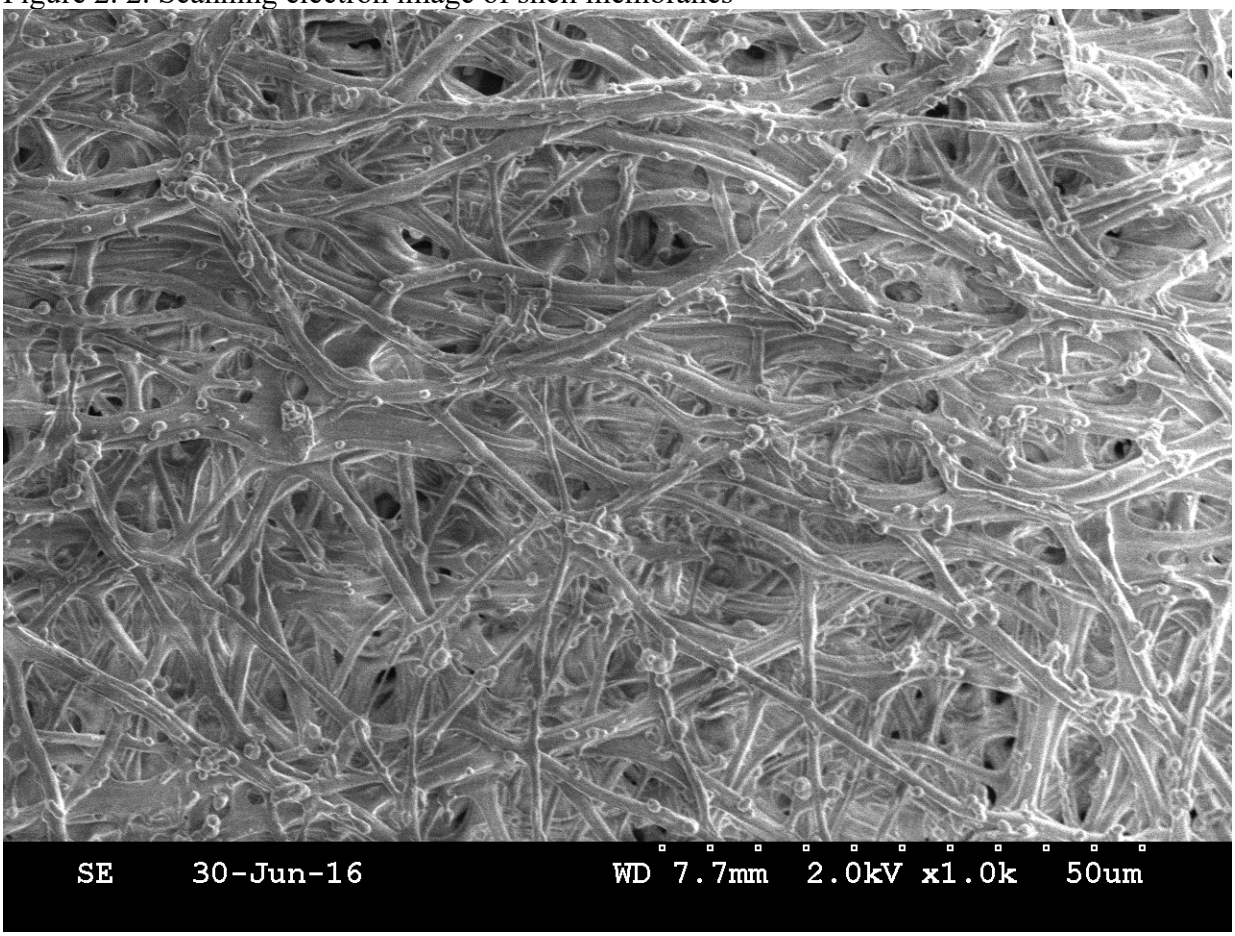
A mature follicle (yolk) is released into the ovary (ovulation) and is engulfed by the infundibulum. The infundibulum is funnel in shape, and is where the first layer of the albumin and the beginning of the chalazae are secreted (Hy-line, 2013a). The yolk spends about 15 minutes in the infundibulum and if fertilization were to occur, this is where fertilization would take place (Cutts et al., 2007). The contents then move to the largest portion of the oviduct, the magnum, and remains here for approximately three hours (Cutts et al., 2007). In this portion of the oviduct, the albumin (egg white) is added to the yolk. The yolk and albumin are then moved to the isthmus, where they spend approximately one hour and have shell membranes added around the egg white. Two layers of membranes are added. The inner and outer shell membranes are proteinaceous woven fibers that surround the albumin and yolk (Parsons, 1982). From the isthmus, the contents are moved to the uterus, or the shell glad, and this is where eggshell formation occurs (Hy-line, 2013a). Eggshell formation takes approximately 21 hours and is where the egg spends the majority of its times (Cutts et al., 2007). In this portion of the oviduct, pigmentation can be added to the eggshell if pigmentation will be added. During eggshell formation, 2 to 3 grams of calcium are added. Calcium and carbonate ions are transferred to uterine fluid, from the blood, bathing the eggshell membranes (Hy-line, 2013a). Upon exiting the uterus, the egg is formed and enters the vagina/cloaca. The vagina has no role in egg development, it simply holds the egg until the hen is ready to release the egg (Hy-line, 2013a).

2.3 Eggshell Microstructure

2.3.1 Shell Membranes

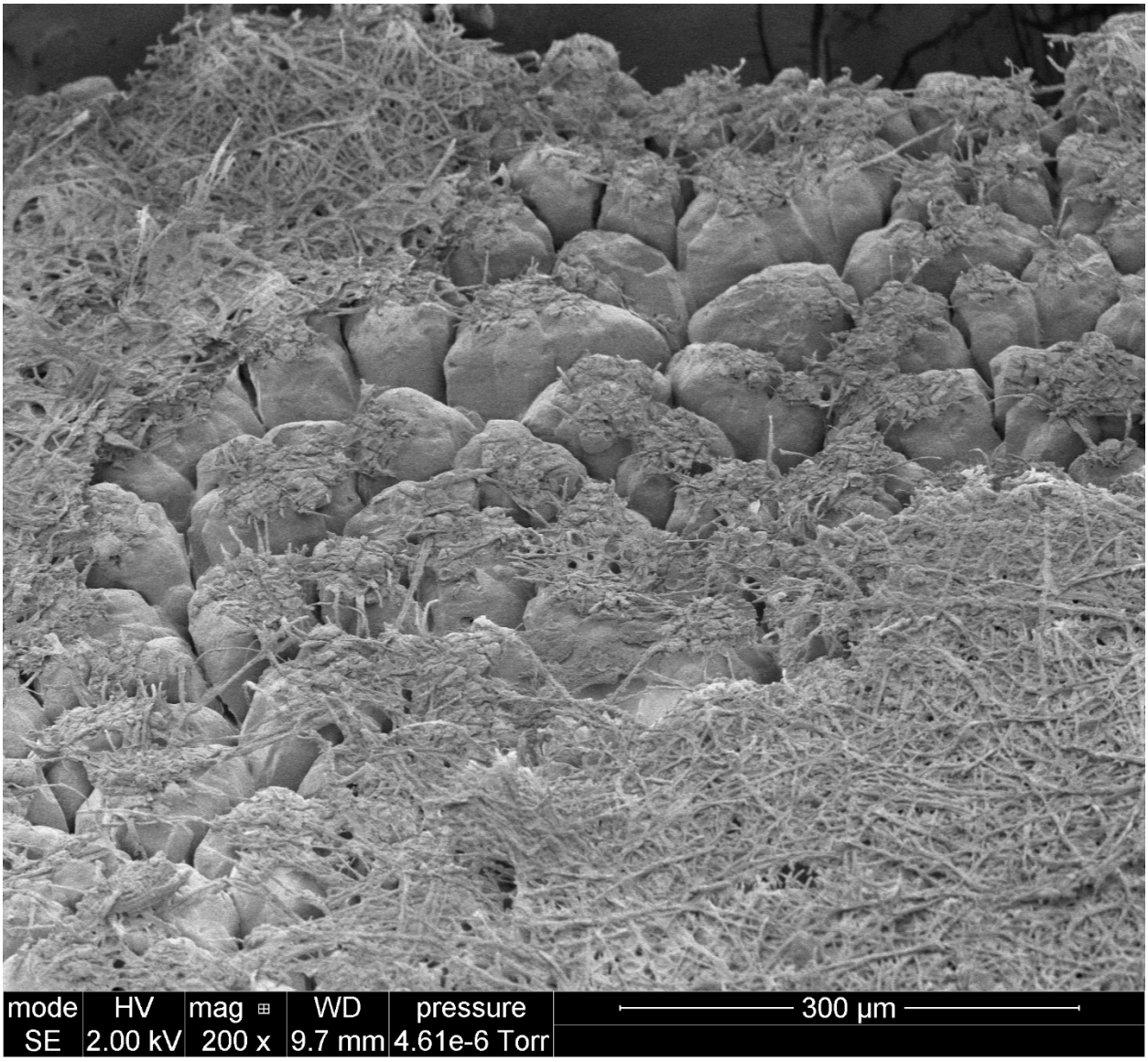
As each portion of the egg is added to the yolk through the different portions of the oviduct, a new layer to the egg's shell microstructure is added. Shell membranes are non-edible by-products of egg production (Nakano et al., 2003). The inner and outer shell membranes are added to the yolk in the isthmus. The membranes are composed of a meshwork of proteinaceous fibers (95% protein) (Nys et al., 1999). These proteinaceous fibers can be seen in Figure 2.2.

Figure 2. 2. Scanning electron image of shell membranes



The membranes adhere tightly to each other, except for at the pole of the egg, where the inner membrane is woven into the egg white, while the outer membrane is interwoven with the calcium portion of the shell, which can be seen in Figure 2.3.

Figure 2. 3. Scanning electron image of shell membrane interwoven with mammillary knobs

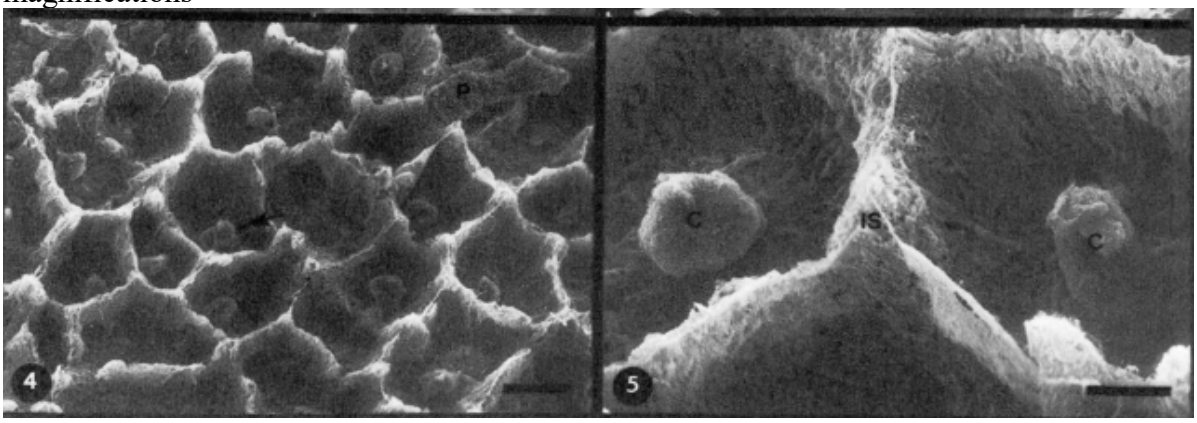


The inner membrane can be identified from the outer membrane, in that its fibers are finer and more tightly woven. In addition, the inner membrane has a smooth appearance due to a thin homogeneous coating found on the inner membrane(Parsons, 1982). Eggshell membranes are prerequisite for shell calcification in laying hens and provide a barrier, preventing inward mineralization (Nys et al., 2001). Numerous studies have shown that disruption of eggshell membrane fibers can severely reduce eggshell strength and quality (Nys et al., 2004).

2.3.2 Mammillary Layer

The next portion of the eggshell's microstructure is the mammillary knob layer, that comes into contact with the outer shell membrane (Parsons, 1982). The mammillary bodies on this layer should cover the shell membranes and are the initiation site for the rest of shell calcification (Hy-line, 2013a). Mammillary body formation takes place in the uterus and provides a latticework where calcium crystallization occurs (Hy-line, 2013a). Inside each mammillary body, Simkiss (1968) demonstrated that an organic core originates here and is thought to the be the starting point for calcium crystal initiation (Parsons, 1982). The organic core of mammillary bodies can be seen in Figure 2.4.

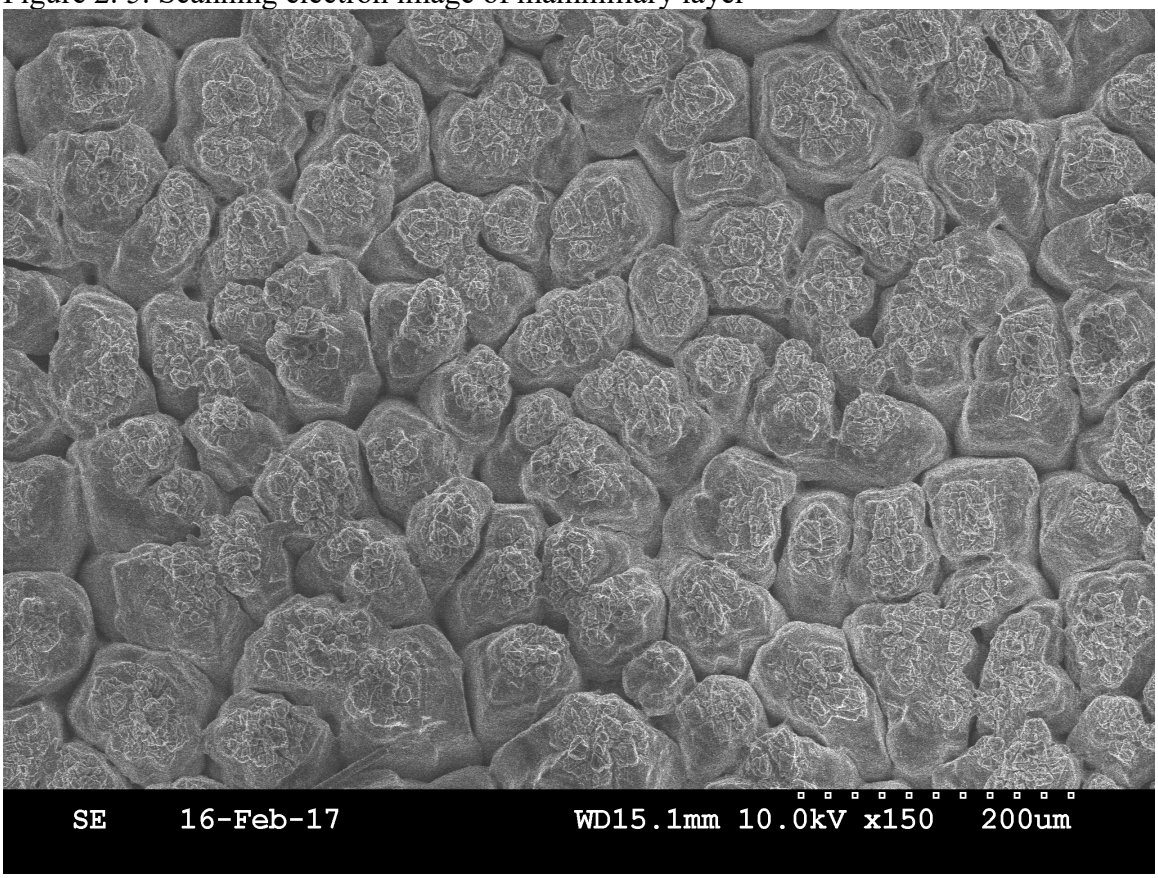
Figure 2. 4. Scanning electron image of organic core of mammillary layer at different magnifications



Parsons (1982)

As these crystals continue to grow, away from the membrane, cones are formed that eventually fuse with other cone formations, creating the mammillary knob layer (Parsons, 1982), which can be seen in Figure 2.5.

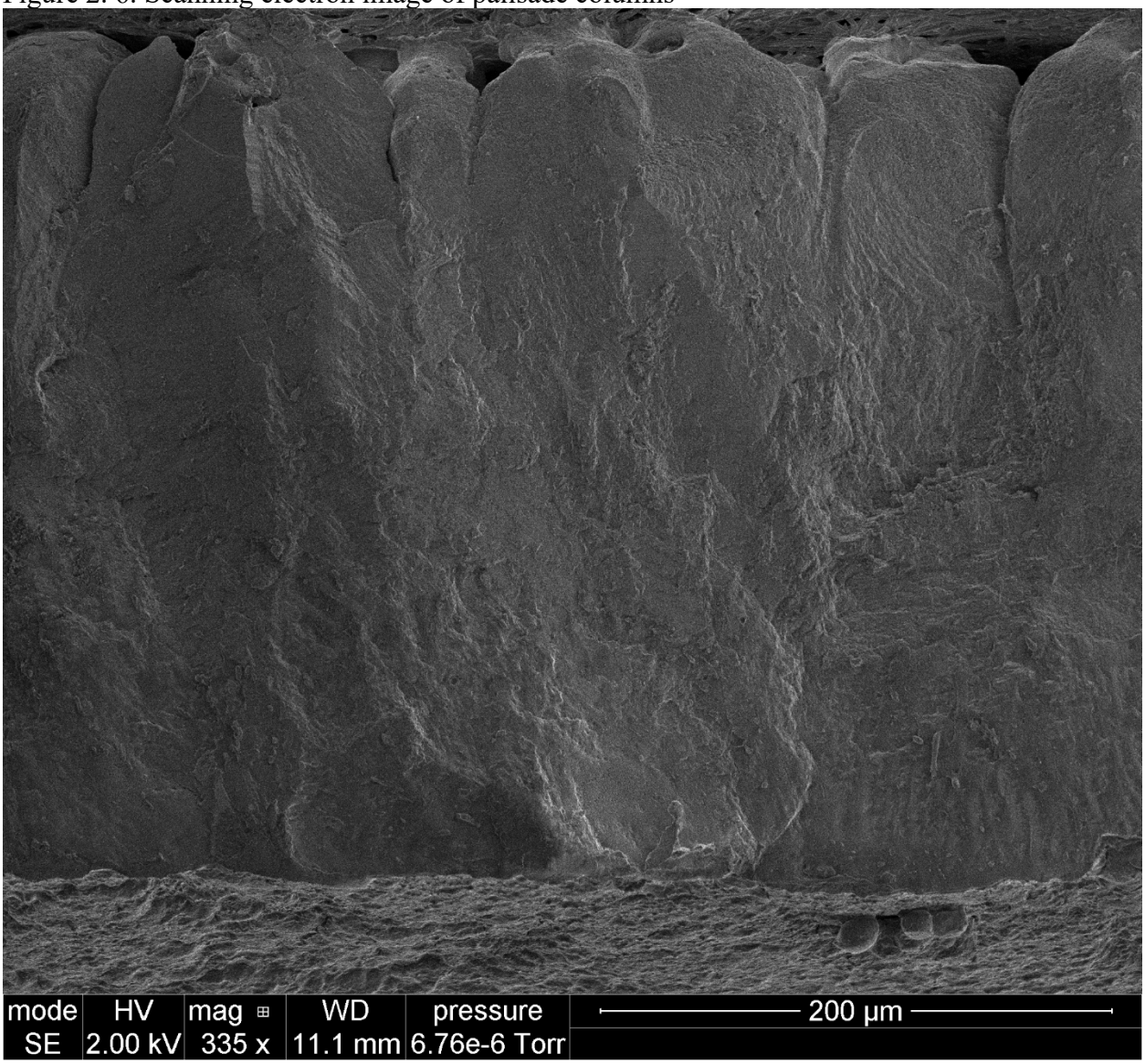
Figure 2. 5. Scanning electron image of mammillary layer



2.3.3 Palisade Layer

Perpendicular to the mammillary knob layer is the palisade layer (Parsons, 1982). This crystalline layer is made of densely packed crystals of calcium in the form of pillars or palisades (Hy-line, 2013a). The end of the mammillary knobs and the start of the palisade layer is somewhat arbitrary (Parsons, 1982). The palisade microstructure can be seen in Figure 2.6.

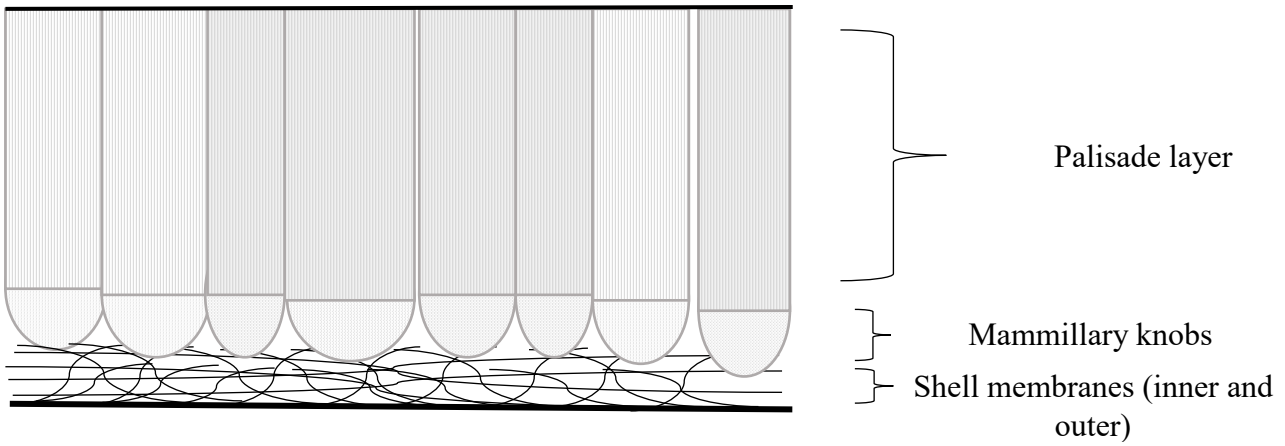
Figure 2. 6. Scanning electron image of palisade columns



The crystalline structure of the palisade layer is typically in an angular pattern, relative to the shell surface, and is commonly referred to as a herring bone cleavage pattern (Parsons, 1982). Simons (1971) studied eggshell quality and microstructure and mentioned, “shell deformation measurements vary with the width of the palisade columns.” Simons hypothesized that eggs with wide crystal columns had high deformation values. From this, he concluded that narrow columns make stronger eggshells (Simons, 1971). Other studies that found similar results with palisade column width hypothesized that column size is regulated by the speed that mammillary knobs coalesce. “Early fusion of the knobs results in shorter interknob spaces that may strengthen the eggshell” (Parsons, 1982).

Above the palisade layer and below the cuticle lies the vertical crystal layer (Parsons, 1982). This layer consists of short narrow crystals that are aligned roughly perpendicular to the shell surface, which can be seen in Figure 2. The role of this layer is still unclear (Parsons, 1982). Covering the vertical crystal layer, a waxy organic layer, known as the cuticle, and covers the calcified portion of the shell (Parsons, 1982). The cuticle protects the egg from microbial invasion and dehydration by lowering permeability. (Belyavin and Boorman, 1980). In addition, Belyavin and Boorman (1980) fond that the cuticle does not contribute to eggshell strength. A schematic drawing of the microstructure of the eggshell can be seen in Figure2.7.

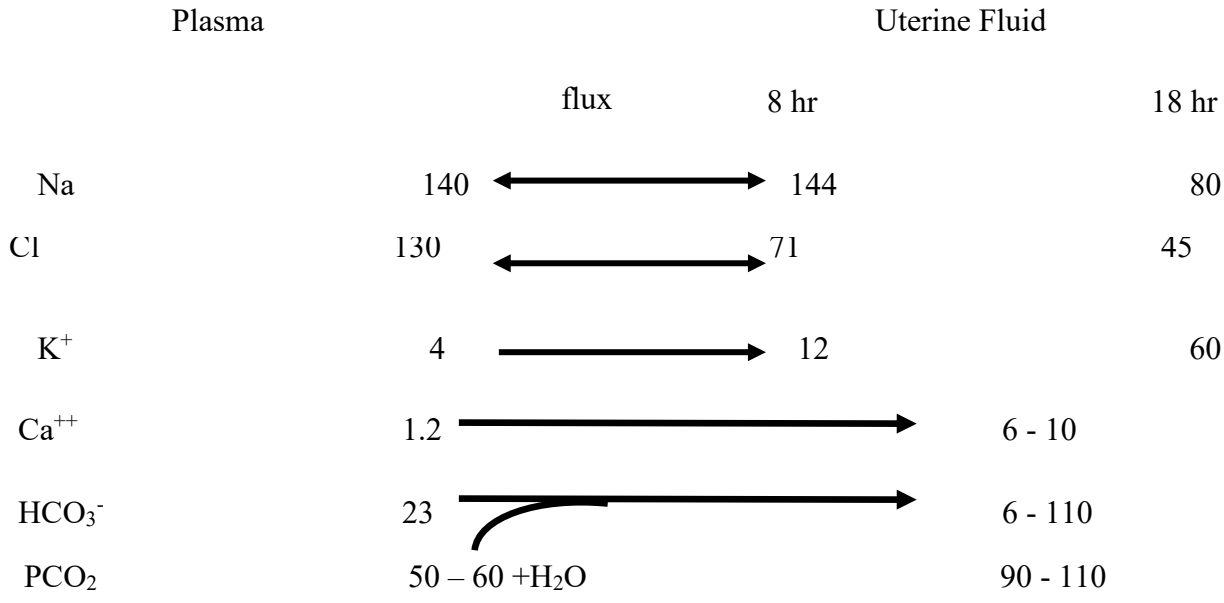
Figure 2. 7. Schematic drawing of eggshell microstructure



2.4 Uterine Fluid Ion Composition

Uterine sodium and chloride concentrations are high at the onset of calcification and decrease throughout calcification, to levels of 2-3 times lower than levels in the plasma. On the other hand, uterine potassium concentrations increase at the end of calcification (Nys et al., 1999). Calcium and bicarbonate concentrations measured in uterine fluid reflect the balance of uterine secretion and calcium carbonate precipitation (Nys et al., 1999). Calcium, which is predominately in the ionized form, increases throughout the active phase of eggshell deposition, however, the rate of precipitation is stable and deposition is linear (10-22 hours of ovulation). Calcium concentration is lower towards the end of calcification but concentration is still higher than that of the plasma (Nys et al., 1999). In addition, bicarbonate is high regardless of shell formation stage and is greater than concentration of bi-carbarbonate in the plasma (Nys et al., 1999). Ion concentrations in the plasma and uterine fluid at initial phase of eggshell formation can be seen in Figure 2.8.

Figure 2. 8. Ion concentrations (mmol/l) in the plasma and in uterine fluid samples at the initial phase of eggshell formation (8 hr) and during the final part of rapid eggshell deposition (18 hr).



Adapted from Nys et al. (1999).

2.5 Minerals

Zinc, manganese, and copper are trace minerals that work as cofactors for enzymes that are involved in eggshell formation (Gupta, 2008). Enzymes related to microelements such as zinc are vital to the mineralization process. Zinc and manganese are cofactors of metaloenzymes which are responsible for carbonate synthesis, which vital in eggshell formation (Swiatkiewicz and Koreleski, 2008). Zinc, as well as other microminerals, can affect mechanical formation, by modifying the crystalline structure of the eggshell (Mabe et al., 2003; Swiatkiewicz and Koreleski, 2008). Zinc is widely available in many different feed sources; however, bioavailability of zinc and manganese often varies. Bioplex® minerals are trace minerals are bound to amino acids and a range of peptides. They are easily absorbed and readily metabolized, optimizing animal performance (Alltech, 2018). Chelating minerals by bonding a metal ion (mineral) and ligand (protein or amino acid), binds the metal at more than one point so the metal atom becomes part of a ring, protecting the mineral from entering unwanted reactions (Swain, 2014).

2.5.1 Zinc

Zinc (Zn) and other micro minerals can affect mechanical properties of eggshells by affecting calcite crystal formation and modifying crystallographic structure of the eggshell (Swiatkiewicz and Koreleski, 2008). Inadequate Zn status in the hen may reduce eggshell quality, hatchability, embryonic development and result in poor chick quality (Mishra et al., 2014). Organic vs inorganic minerals have different bioavailability. Bioavailability is the percentage of nutrients utilized in the body for specific growth measurements. Greater bioavailability indicates greater absorption and deposition of the

mineral (Martin, 2016). Yenice et al. (2015) states that bioavailability of inorganic minerals is low and is often found unutilized in the excreta.

Trace minerals are essential in laying hens diets as they participate in biochemical processes, as well as their catalytic properties to key enzymes, which can be involved in membrane and eggshell formation or through direct interaction with calcite crystals during eggshell formation (Mabe et al., 2003; Yenice et al., 2015). Zinc is a component of the carbonic anhydrase enzyme, which is crucial for supplying the carbonate ions during eggshell formation. Inhibition of this enzyme results in lowered bicarbonate ion secretion and greatly reduces eggshell weight (Zamani et al., 2005). Although Zn has been shown to positively impact carbonic anhydrase activity and ultimately improves eggshell quality, results demonstrating the amount of zinc needed and the importance of zinc source are inconsistent (Zhang et al., 2017).

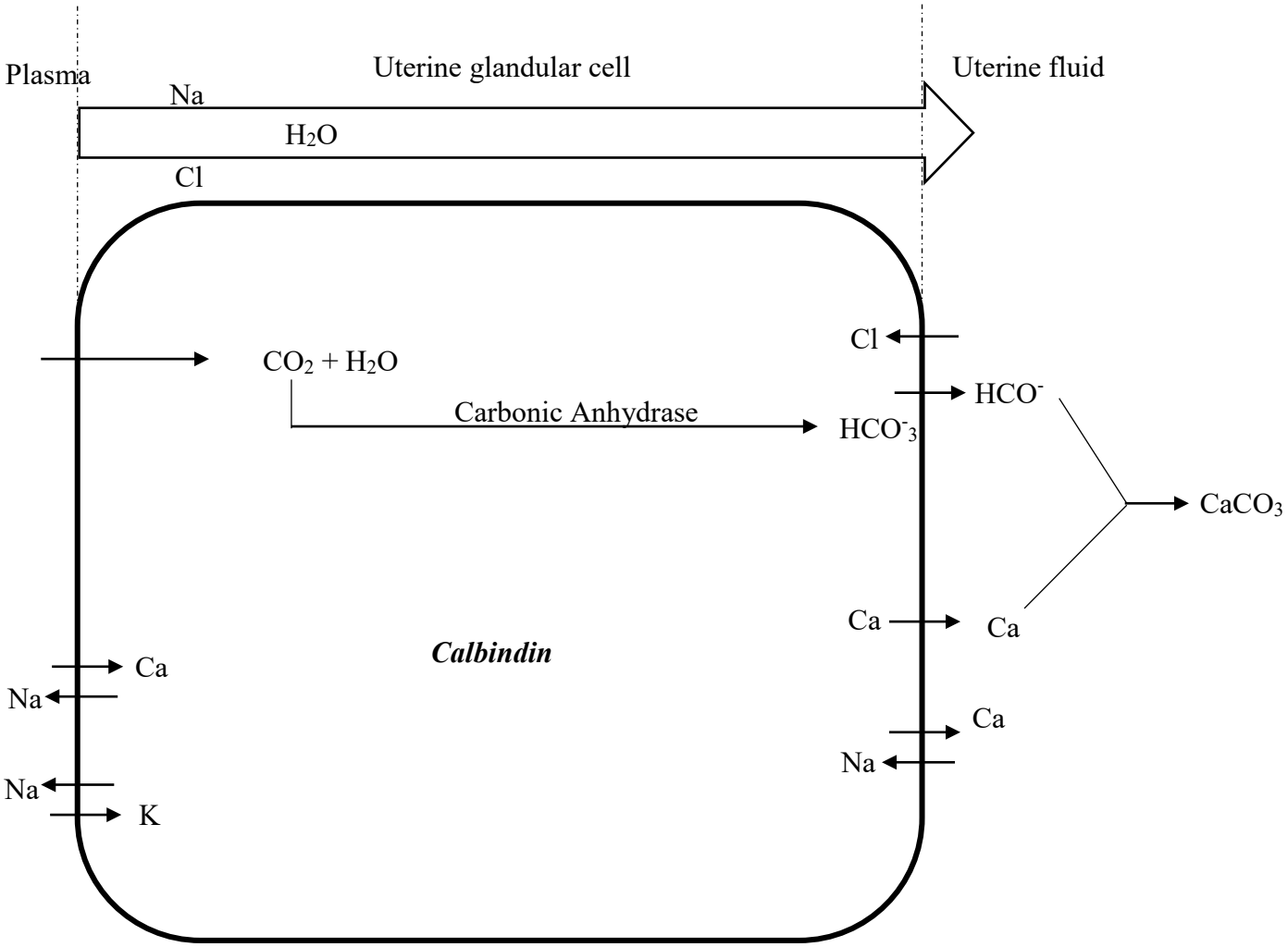
2.5.2 Calcium

The dietary calcium intake of laying hens appears to be 4.2 to 4.6 g/d (based on daily feed intake of approximately 115g/d and recommended calcium content of 3.6 – 4.0%). At zero nutritional balance for calcium and under the theoretical conditions of consistent dietary inflow and shell deposition outflow of calcium absorption in the domestic laying hen should be about 50% of dietary intake. The source of daily deposition of shell calcium, both from diet and bones, in birds with long clutches, comes from the intestine. Carbonic anhydrase and osteopontin are the two proteins that believed to be associate with calcium uptake for eggshell formation in the eggshell gland (ESG) (Bar, 2009).

2.6 Carbonic Anhydrase

Carbonic anhydrase (CA) is a zinc-containing enzyme and is a vital enzyme in calcium carbonate deposition during eggshell formation (Zhang et al., 2017). Carbonic anhydrase stimulates calcium carbonate deposition during eggshell formation and is zinc dependent (Gupta, 2008). Carbonic anhydrase catalyzes the reversible hydration of carbon dioxide and is involved in bone resorption and calcification, ion transport, acid-base metabolism, and movement of respiratory gases (Bar, 2009). Studies have shown that partial or complete inhibition of carbonic anhydrase can result in thin or shell-less egg (Zhang et al., 2017). Improving carbonic anhydrase activity during eggshell formation may help in improving eggshell quality; in addition, zinc can be incorporated into growth during calcite crystal formation (Zhang et al., 2017). The most prominent carbonic anhydrase isoform found in avian tissues is CA-II (Bar, 2009). Carbonic anhydrases are found in the avian kidney, bone osteoclasts, intestine, and eggshell gland (Bar, 2009). Carbonic anhydrases are also found in other cells, where their primary role is to generate H^+ and HCO_3^- during acid-base regulation (Bar, 2009). Bicarbonate formation in the eggshell gland is very important in the deposition of $CaCO_3$, which acts as the sole counter ion for Ca^{2+} (Bar, 2009). Bicarbonate ions required for shell formation are primarily produced in glandular cells from metabolic CO_2, catalyzed by CA (Nys et al., 1999). The reaction of carbonic anhydrase and other reactions occurring in the uterine glandular cells can be seen in Figure 2.9.

Figure 2. 9. Schematic of the ionic fluxes through uterine glandular cells for calcium carbonate deposition



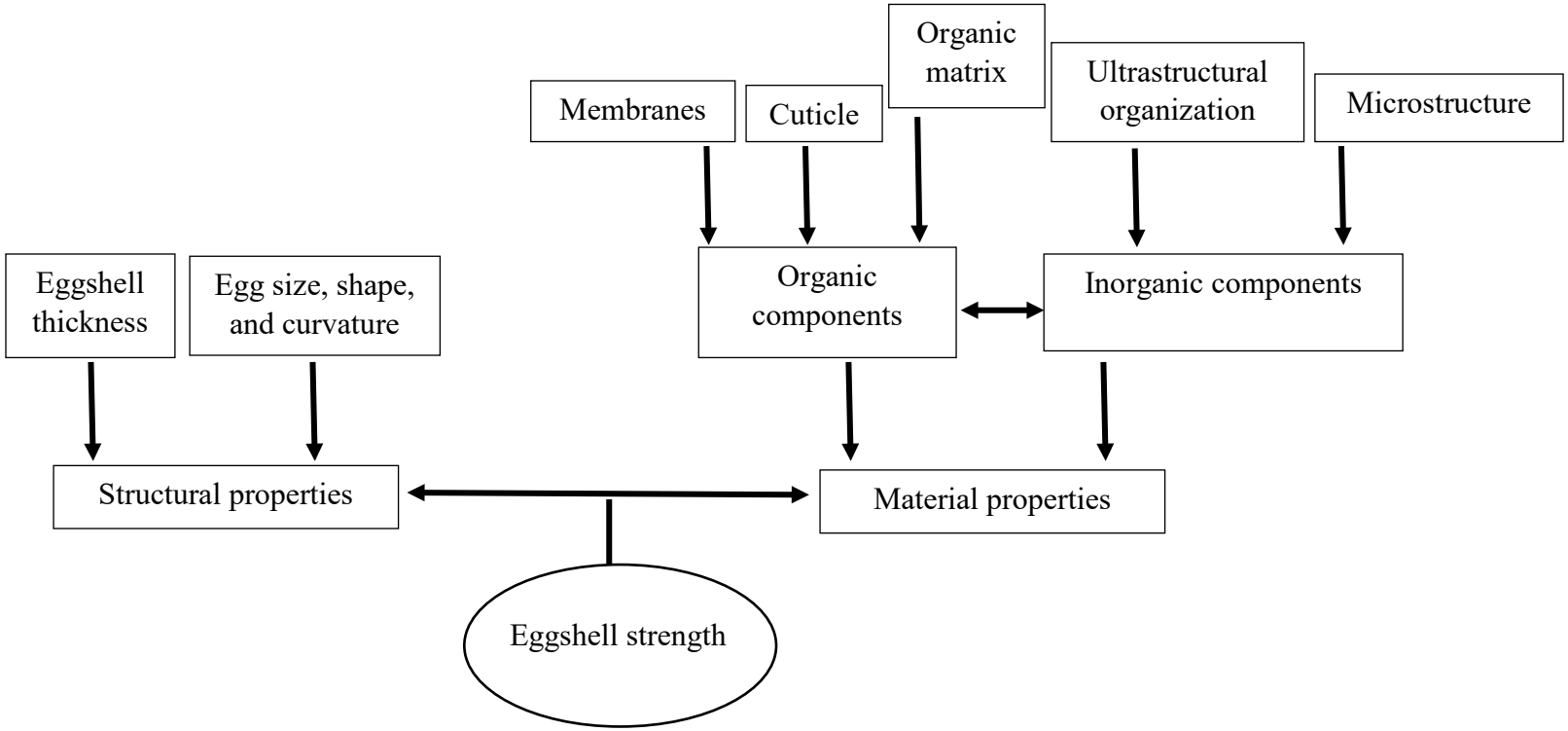
Adapted from Nys et al. (1999)

Changes in carbonic anhydrase are stimulated due to hormonal changes that occur when hens start calcifying eggs. Additionally, calbindin is required to transfer calcium through the uterine wall, indicating that it is also required for calcium carbonate for eggshell formation (Nys et al., 1999).

2.7 Eggshell Quality Measurements

Different measurements can be taken to indicate shell quality and relay information about shell quality and formation to producers and researchers. There are some destructive as well as non-destructive methods that are commonly used in the poultry industry to determine eggshell quality. The most common destructive method used is quasi-static compression or breaking strength of the egg (Bain, 2005). This test is completed by placing an egg between two plates and compressing the egg at a constant speed until a fracture occurs. The force required to fracture the egg is recorded (Bain, 2005). The greater the force required to break the egg, the thicker the shell. Macleod et al. (2006) found that micro cracks are formed by high stress levels which develop on the inner surface of eggshells and often form at the contact zone of the plate used for breaking strength measurement. Crack detection devices relying on mechanical excitation are unable to detect the presence of these micro cracks in eggs. It is through these cracks that potentially harmful bacteria can enter the egg, compromising egg safety (Macleod et al., 2006). Although breaking strength is commonly used to assess eggshell quality and strength, Bain (2005) suggests that other structural as well as material properties affect eggshell strength (Figure 2.10.).

Figure 2. 10. Schematic of various parameters influencing eggshell strength.



Adapted from Bain (2005)

Material properties are dependent on the inorganic and organic components of the eggshell, and how they interact with one another. On the other hand, structural properties are dependent on the thickness of the eggshell, as well as the distribution of shell material over the egg surface, and, the size and shape of the egg (Bain, 2005; Nedomova et al., 2009).

Shell thickness is another method used to determine eggshell quality. Stadelman (1990) stated that it has been estimated that a shell thickness of at least 0.33mm is required for the egg to have a better that 50% chance of moving through normal market handling without breaking. Shell thickness is measured using a paper-thickness gauge. However, it is important to avoid measuring the shell along its curvature, resulting in an inaccurate shell thickness measurement (Stadelman, 1990). Specific gravity has also been used to estimate eggshell quality. Determination of specific gravity requires a series of sodium chloride solutions varying in specific gravity, no more than 0.005 g/mL, to accurately estimate values. Eggs are submerged in the solution. Eggs that float are removed and marked for that buckets specific gravity, while eggs that sunk are submerged in the subsequent solution until they float and specific gravity is determined (Stadelman, 1990). Specific gravity measurements can be affected by the number of days after being collected. All eggs should be of the same age and stored using the same methods to measure specific gravity. Table 2.1. shows the correlation coefficients between these different methods of eggshell quality determination.

Table 2. 1 Simple Correlation Coefficients among Several Measures of Shell Quality

	B	C	D	E	F	G
Shell thickness (A)	0.78	0.80	0.78	0.26	0.73	0.54
Specific Gravity (B)		0.81	0.69	0.14	0.70	0.61
Percent egg as shell (C)			0.76	0.08	0.78	0.37
Shell Weight (D)				0.67	0.62	0.55
Egg Weight (E)					0.10	0.45
Force to crush shell (F)[a]						
Impact device force (G)[a]						

Source: Frank et al. (1964)

[a] Relationship between F and G not calculated, as both measures are destructive and cannot be evaluated on the same egg

2.8 Shape Index

Individual eggs have different egg shapes and studies have shown that egg shape, or shape index, as well as shell thickness and affect the proportion of damaged eggs during handing and transport (Altuntaş and Şekeroğlu, 2008). Shape index is measured by dividing the diameter of the egg into the height of the egg then multiplied by 100 (Anderson et al., 2004). Eggs are placed into one of three categories based on their shape index. Sharp (<72), normal or standard (72-76), and round (>76) (Nedomova et al., 2009). Eggs that are within the normal shape tend to withstand more pressure during handling, fit properly into packing, and require a greater force to break compared to eggs that are more round and narrow/long eggs (Ebubekir and Sekeoglu, 2008)

2.9 Bone Health

Laying hens have three types of bone tissue: cortical, trabecular (cancellous), and medullary bone (Riczu et al., 2004). Cortical (hard) bone is the outer surface of round bones, including the femur, and flat bones, such as the pelvis (Hyline, 2013b). Trabecular or spongy bone is less dense compared to cortical bone and helps support the structure of cortical bones (Hyline, 2013b). Cortical and trabecular bone are formed during growth, until puberty (Whitehead and Fleming, 2000). Medullary bone is a woven bone where calcium is reserved for eggshell formation. This bone is easily created as well as reabsorbed, and is the first source of calcium to be mobilized when dietary calcium is deficient (Hyline, 2013b). At sexual maturation, medullary bone, a nonstructural bone, is formed (Whitehead and Fleming, 2000). Cortical and spongy bone contribute to skeletal strength (Wistedt, 2013). Indirectly, both cortical and spongy bone contribute to eggshell formation. These bones are reabsorbed to maintain medullary bone, which provides 1/3

of the total calcium required for eggshell formation (Wistedt, 2013). Medullary bone is a woven bone, which provides a source of calcium that can be used for eggshell formation (Whitehead and Fleming, 2000). Cortical and trabecular bone provide the majority of structural strength for the bone and the skeletal system (Whitehead and Fleming, 2000). Medullary bone is a readily available source of calcium when insufficient calcium is available in the diet (Fleming et al., 1998). Medullary calcium is replaced when calcium from the diet is in excess of the hens requirement, and is an ongoing cycle, that can occur on a daily basis (Fleming et al., 1998). Medullary bone amount is constantly changing due to the constant remodel and increase in volume throughout the egg-laying period, at the cost of cortical and spongy bone degradation (Wistedt, 2013). Break down for cortical and spongy bone can begin as early as puberty in laying hens (Wilson and Thorp, 1998). If medullary bone calcium is insufficient, cortical and trabecular bone calcium can be used to meet the requirement of the hen (Riczu et al., 2004). However, this method of calcium utilization can be detrimental, because the hen has no method to replace it throughout the laying cycle. This use of calcium throughout egg production can predispose the hen to bone weakness and ultimately bone breakage (Riczu et al., 2004).

Bone health and egg performance often go hand-in-hand. These issues often arise from deficiencies, imbalance or malabsorption of calcium, phosphorus, and/or Vitamin D_3 (Hy-Line, 2013b). Skeletal issues within a flock can typically be seen by decreased production, crooked keels, fractures, and poor eggshell quality. The skeleton of the laying hen is strongly influenced by the level of egg production, diet formulation in relation to feed consumption and disease status. Hens that are well grown will typically not face skeletal issues until after peak lay (25-28wks of age), even when mild to moderately

deficient diets are being fed. Deficiencies will usually cause skeletal and/or shell quality issues soon to follow (Hy-Line, 2013b).

2.10 Keel Bone Function and Damage

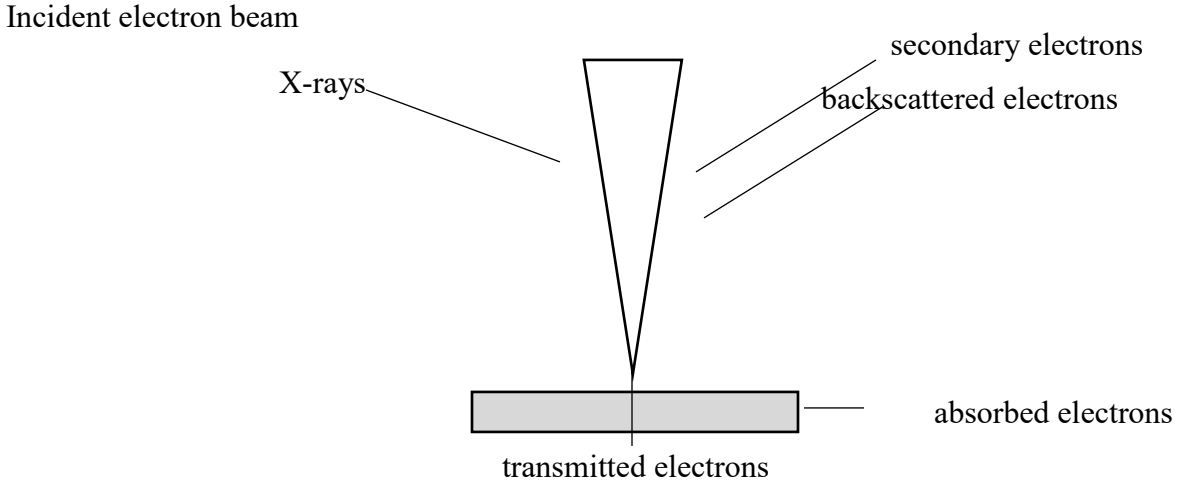
The incidence of broken and weak bones is an increasing problem as hens progress through the lay cycle in the table egg industry (Riczu et al., 2004). The effect of bone fractures on bird welfare is unknown, however bone fractures cause pain and is likely to cause the same effect in birds (Nasr et al., 2012). Pain caused by keel bone fractures may have an effect on egg production and eggshell quality (Nasr et al., 2012). A total of 28-30 times the hen's total body calcium reserve is used during egg production, throughout the entire lay cycle, resulting in weak bones and increased bone breakage (Riczu et al., 2004). Medullary bone is the first source of calcium mobilization, however, if a hen is deficient in calcium, cortical and trabecular calcium stores will be mobilized as a source of calcium (Hy-Line, 2013b). The keel bone is an extension of the ventral surface of the sternum and spans from the cranial to the caudal tip, with the spine of the keel tapering as it approaches the caudal end (Casey-Trott, 2016).

2.11 Scanning Electron Microscopy

The scanning electron microscope (SEM) is a powerful and frequently used instrument, and is used to study topography, composition, crystallography and properties on a local scale (Methods, 2013). The spatial resolution is better than an optical microscope and has extremely large depth of focus and is suited for topographic imaging (Methods, 2013). During SEM, the specimen is bombarded by an electron beam, which is scanned across the surface. The electron beam generates different signals, which are

emitted from the area on the specimen where the electron beam is pinging, which can be seen in Figure 2.11.

Figure 2. 11. Example of some of the different types of signals produced when high-energy electrons pinging on a material.



Methods (2013)

During imaging, the specimen resides in a high vacuum chamber to prevent scattering of the electron bean and damage to the microscope (Methods, 2013). Specimens that are not electrically conductive may result in electron build-up, due to the inability to dissipate the charge, causing an unclear image. In addition, the sample must be completely dry and free of water or other volatile components that could destroy the vacuum and damage the microscope (Methods, 2013). In order to image samples that do not meet the criteria, samples can undergo preparation such as metal coating with a conductive substance to reduce collection of electrons are charging and drying to remove water from the sample (Methods, 2013).

2.12 Conclusion

Currently the layer industry is experiencing a shift in housing, from conventional cages to "cage-free" and aviary systems. The USDA has predicted that 75% of US hens must be housed in cage-free production in order to meet consumer demand by 2026 (United Egg Producers, 2019). Understanding the microstructure of the eggshell, how it is put together, factors that affect the eggshell, as well as factors that can affect laying hens, are important to improve eggshell quality. The aim of this dissertation was to understand if microstructure of the eggshell was affected by nutritional changes, such as calcium depletion and zinc source, while evaluating current and emerging techniques to estimated eggshell quality. The hypothesis of this dissertation was different levels of dietary calcium and zinc would create differences in eggshell quality, as well as eggshell microstructure.

CHAPTER 3. EVALUATION OF SHELL QUALITY OF HENS RECEIVING CALCIUM REDUCED DIETS AT THE END OF THE LAYING CYCLE

3.1 Introduction

Eggshell formation takes approximately 21 hours and the majority of this time is spent in the uterus (Cutts et al., 2007). In this portion of the oviduct, pigmentation can be added to the eggshell, if pigmentation will be added. During eggshell formation, 2 to 3 grams of calcium (Ca) are added. In order for this amount of calcium to be added to the eggshell, adequate dietary calcium needs to be available. During eggshell formation, different layers are added that compose the eggshell. Shell membranes are non-edible by-products of egg production, but contains biologically active compounds (Nakano et al., 2003). The inner and outer shell membranes are added to the yolk in the isthmus. The membranes are composed of a meshwork of proteinaceous fibers (95% protein) (Nys et al., 1999). The next portion of the eggshell's microstructure is the mammillary knob layer, that comes into contact with the outer shell membrane (Parsons, 1982). The mammillary bodies on this layer should be cover the shell membranes and are the initiation site for the rest of shell calcification (Hy-line, 2013a). Perpendicular to the mammillary knob layer is the palisade layer (Parsons, 1982). This crystalline layer is made of densely packed crystals of calcium in the form of pillars or palisades (Hy-line, 2013a). Additionally, the makeup of the eggshell will differ and have different eggshell parameters depending on the stage of lay the hen is in. Eggshells will be of highest quality at peak lay (26-29 weeks of age) and of lowest quality at the end of lay (80-90 weeks of age). Different measurements can be taken to indicate shell quality and relay information about shell quality and formation to producers and researchers. There are

some destructive as well as non-destructive methods that are commonly used in the poultry industry to determine eggshell quality. The aim of this study was to look at these egg quality measurements in relation to the microstructure of the shell, determined through scanning electron imaging, at the end of a laying cycle (66 weeks of lay; 82 weeks of age).

3.2 Materials and Methods

Twelve 82-week old White Leghorn laying hens were placed on either a diet deficient (n=6), 0.88% Ca, or sufficient in Calcium (n=6) (Ca), 4.96% Ca. These values were based off of values from NRC for laying hens, at the end of a lay cycle. All procedures were conducted under protocols approved by Institutional Animal Care and Use Committee (IACUC). Six hens were randomly allotted to treatment 1, sufficient in Ca; and treatment 2, deficient in Ca. The calculated ingredient composition for each treatment can be seen in Table. 3.1 and dietary component composition for each treatment in Table. 3.1. Diets were analyzed by the University of Missouri.

Table 3. 1 Ingredient Composition of Diets

Ingredient (%)	Diet 1 Sufficient Ca	Diet 2 Reduced Ca
Corn	54.00	76.10
Soybean Meal	28.10	11.60
Alfalfa	2.30	9.40
Blended Fat	3.30	-
Salt	0.47	0.40
Limestone	5.50	1.60
Oyster Shell	3.60	-
Dicalcium phosphate	1.40	1.60
Vitmain-mineral premix	0.20	0.20
DL- Methionine	1.30	-
Integral[1]	1.00	-
Total	**100**	**100**

[1]Integral is a yeast based product to aid in reducing mycotoxin toxicity (Alltech)

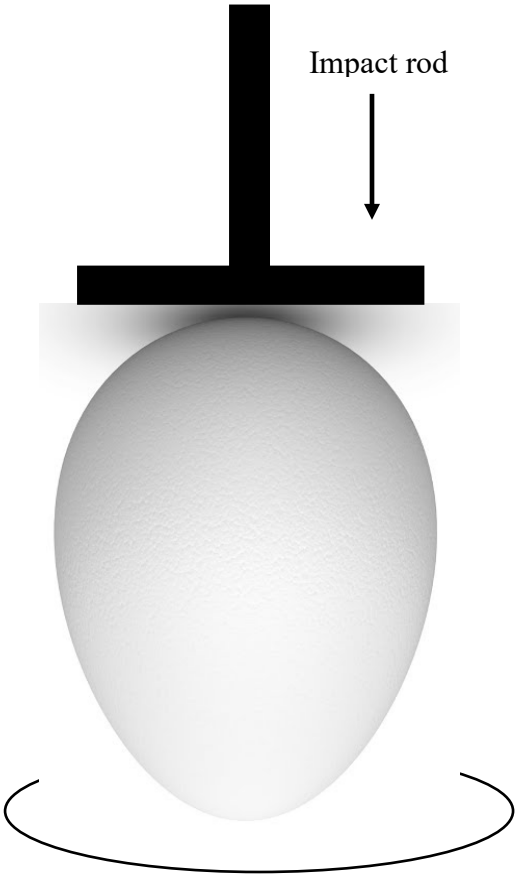
Table 3. 2 Analyzed diet composition

Component (%)	Diet 1 Sufficient Ca	Diet 2 Reduced Ca
Crude Protein	18.11	13.21
Crude Fat	4.30	2.94
Crude Fiber	2.85	4.86
Calcium	4.96	0.88
Phosphorus	0.66	0.64

It is important to note that other dietary differences were detected between diet 1 and diet 2, aside from calcium concentrations. However, the overall object of this study was to create eggshells that were different in eggshell quality, as well as microstructure.

Hens were housed in individual cages and supplied *ad libitum* access to feed and water. Hens were on dietary treatments for 20 days. Egg collection began on week 66 of lay, from each hen daily for 20 days. After collection, eggs were weighed, and then subjected to specific gravity, breaking strength (kgf), Haugh unit was calculated from albumen height, and dry shell weight was determined, from which percent shell was calculated. For specific gravity, eggs were submerged in different gradational salt solutions with predesignated densities for specific gravity determination (Roberts, 2004). Breaking strength was determined using quasi-static compression where the egg is compressed under controlled conditions using a 5kg load cell (Tyler, 1961). Eggshell breaking strength is recorded as the minimum force required to break, or cause eggshell failure (Roberts, 2004). In Figure 3.1

Figure 3. 1. Schematic of impact loading along long axis at the blunt end of the egg.



(Eissa and Alghannam, 2011)

Eggs were loaded so that the egg was broke along the long axis, with the blunt end of the egg receiving the pressure of the impact rod (Nedomová et al., 2009). Albumen height is the measurement of the viscosity of the thick albumen, typically 1 cm from the edge of the yolk (Roberts, 2004). Albumen height was then converted to Haugh Unit (H.U), which was then used to give the egg an internal quality grade. The calculation to determine H.U can be found below and is the same equation used by Roberts (2004):

$$H.U = 100LOG\ (H - \frac{\sqrt{G(30W^{0.37} - 100}}{100} + 1.0)$$

H = albumen height in mm

G= 32.3

W = weight of whole egg in grams

Shell weight is measured by breaking open the egg, removing the contents, rinsing the shell, and drying the shell. Shell weight is then recorded and used to calculate percent shell of the total egg (Roberts, 2004).

Eggshells from the first and last egg by each hen was laid were used for imaging on the Scanning Electron Microscope (SEM). The first egg from each bird was considered the baseline for the experiment, while eggshell differences should be detected by 20 days on experiment diets. Eggshells selected for the SEM were soaked in a 6% sodium hypochlorite. 4.12% sodium chloride, and 0.15% sodium hydroxide solution (12.5% bleach solution) overnight, rinsed with water, and dried for 48hrs, to remove shell membranes. Membrane-free shells were then coated in gold and platinum, mounted on a metal stub, and read using the SEM. Scanning electron imaging was conducted on the

Hitachi S-4300, using a high-vacuum mode at 5 kV and samples were sputter-coated using an Emscope gold sputter coater. From this, the number of 'normal' and 'b' bodies on the mammillary layer were counted and averaged within a 32.35in^2 image for 3 different areas on each eggshell.

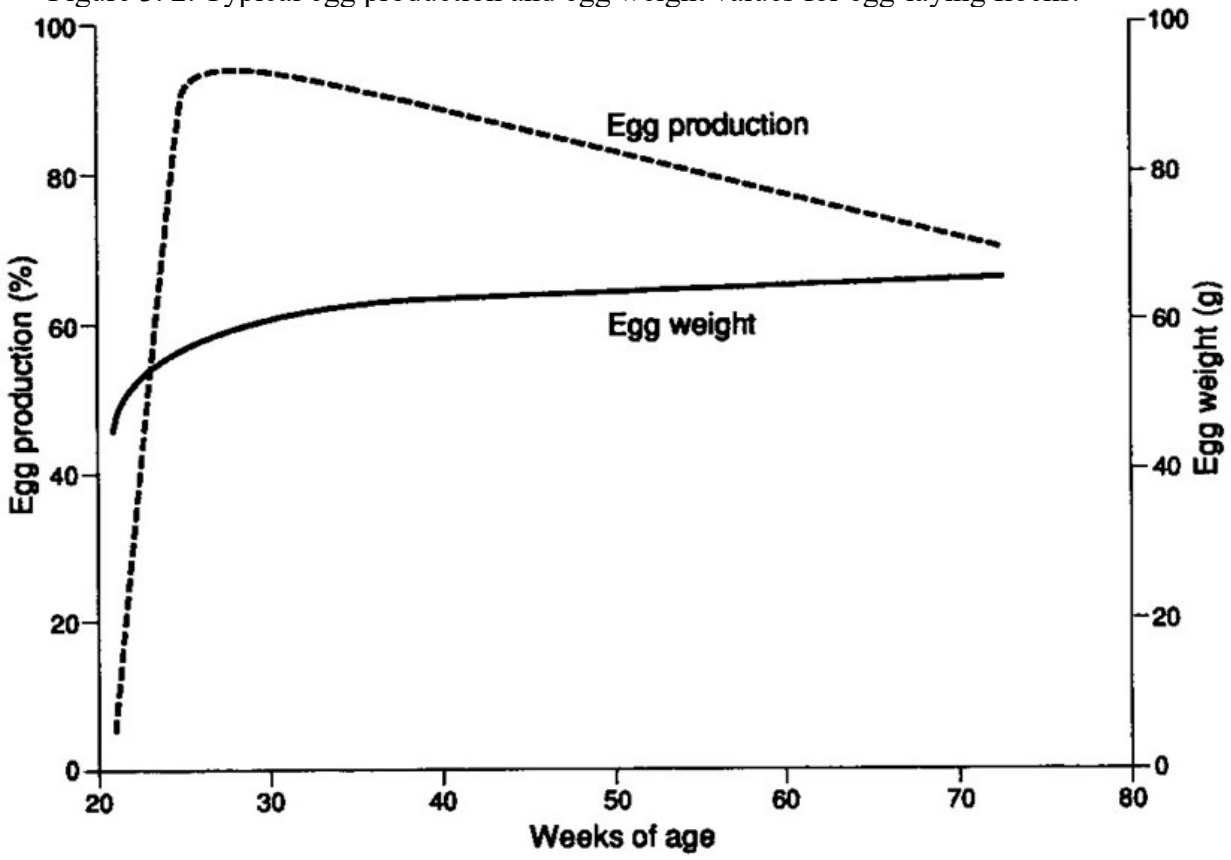
3.3 Statistical Analysis

Data was analyzed using the GLM procedure in SAS 9.4, using a univariate approach with treatment as the dependent variable. Significance was detected at $P < 0.05$.

3.4 Results and Discussion

Different studies have shown that eggshell quality decreases as the hen ages (Roland et al., 1975; Roberts, 2004). The purpose of this study was to create eggshell structures that were microstructurally opposite and to determine if shells with a lower breaking strength will have a different microstructure compared to the microstructure of eggshells with a higher breaker strength. In this study, no significant differences were detected between treatments concerning egg weights (P = 0.90). This is as expected, as eggs used in this study were collected from hens in the same stage of production and same age. The graph published by Jacob (2015) (Figure 3.2.) demonstrates the projected weight of an egg based on the hen's age and stage of production.

Figure 3. 2. Typical egg production and egg weight values for egg-laying flocks.



(Jacob, 2015)

Breaking strength differences were detected between the two different treatments. Diet 1 (sufficient Ca) had significantly higher breaking strength compared to eggshells from diet 2. Data collected from this study agrees with results found from An et al. (2016), who found that eggs from hens on 3.5% Ca had significantly weaker eggs (2.25kg/cm^2) cmpared to eggs from hens on 4.7% Ca (2.46kg/cm^2) ($P < 0.05$). These results also agree with results found by Jiang et al. (2013), who found that low Ca diets produced thinner weaker eggshells, compared to high Ca diets. Internal egg quality concerning Haugh unit was significantly higher for diet 2 (insufficient Ca) compared to internal quality of eggs from diet 1. Similar to data found by An et al. (2016), this study found a numerical decrease in Haugh unit with increasing Ca in the diet. Roberts (2004) explains that differences in HU from different Ca levels may be caused by the amount of time the egg spends in the shell gland, or the uterus. Leeson and Caston (1997) speculate that albumen becomes less viscous due to an increase of water uptake, resulting from increased time spent in the uterus, leading to a thinner albumen and HU. On the other hand, eggs that pass through the uterus quicker, have a thinner eggshell, but higher albumen due to less water uptake, resulting from less time being spent in the shell gland (Roberts, 2004). Shell weight of eggs from diet 1 were higher than shells from eggs of diet 2 meaning that shell percentage was higher from treatment 1 eggs compared to treatment 2 eggs. This is to be expected because Ca is the primary component in eggshells, so eggs produced from hen's receiving greater dietary Ca would have a greater percent shell compared to eggs from hen's receiving insufficient Ca. Specific gravity is conducted with solutions that are differently by 0.005g/mL, so specific gravity was significantly different between diets 1

and 2, those numbers would not be biologically different from each other. Results can also be seen in Table 3.3.

Table 3. 3 Effect of diet on eggshell quality across days

	Diet[1] ± SE[2]		
	1	**2**	**P-value**
	Sufficient Ca	**Reduced Ca**	
Egg Weight (g)	64.17[a] ± 0.56	64.06[a] ± 0.80	0.9050
Breaking strength (kgf)	2.67[a] ± 0.08	1.68[b] ± 0.13	<0.0001
Haugh Unit	58.30[a] ± 0.99	63.11[b] ± 1.66	0.00001
Shell Weight (g)	4.92[a] ± 0.09	3.71[b] ± 0.13	<0.0001
Percent Shell (%)	7.63[a] ± 0.13	5.82[b]± 0.18	<0.0001
Specific gravity	1.069[a] ± 0.001	1.062[b] ± 0.001	<0.0001

[1]Diets were: 1 = Ca sufficient diet (4.30% Ca), 2 = Ca deficient diet (0.88% Ca)
[2]Standard error of the mean
Different letters within a row indicate differences between diets

In addition to internal and external egg quality parameters, Scanning Electron Microscopy (SEM) was performed on the first egg laid by each hen (day 0 of the trial) and the last egg laid by each hen (day 20 of the trial). Images taken on the SEM were taken using a 10kv current at a magnification at 150 - 180, reading an image of 200µm - 250µm. These images were then used to determine the number of 'normal' and 'b' bodies present as Ca deposition decreased in the Ca deficient eggs (diet 2) compare to the eggs from hens being fed sufficient Ca treatment 1). 'Normal' bodies and 'b' bodies were identified using criteria outlined by Solomon (1994). 'Normal' bodies are circled in Figure 3.3, and 'b' bodies are circled in Figure 3.4.

Figure 3. 3. ‘Normal’ bodies on mammillary layer.

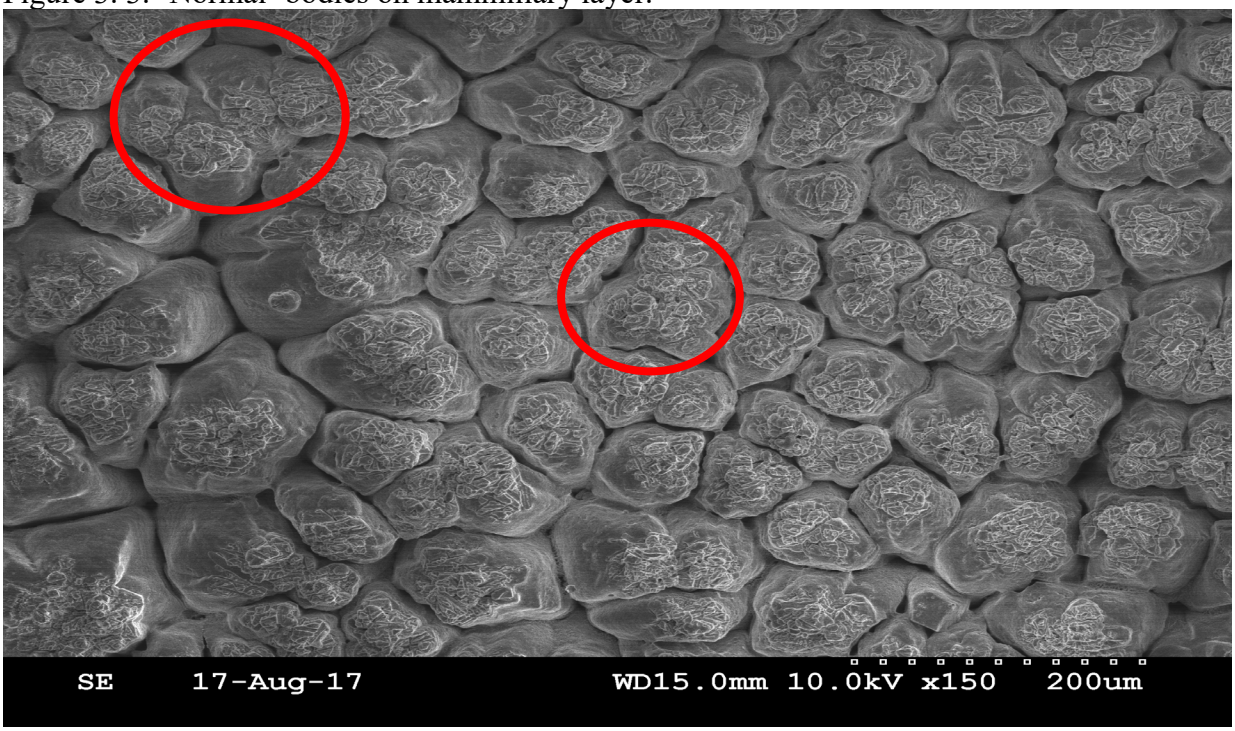
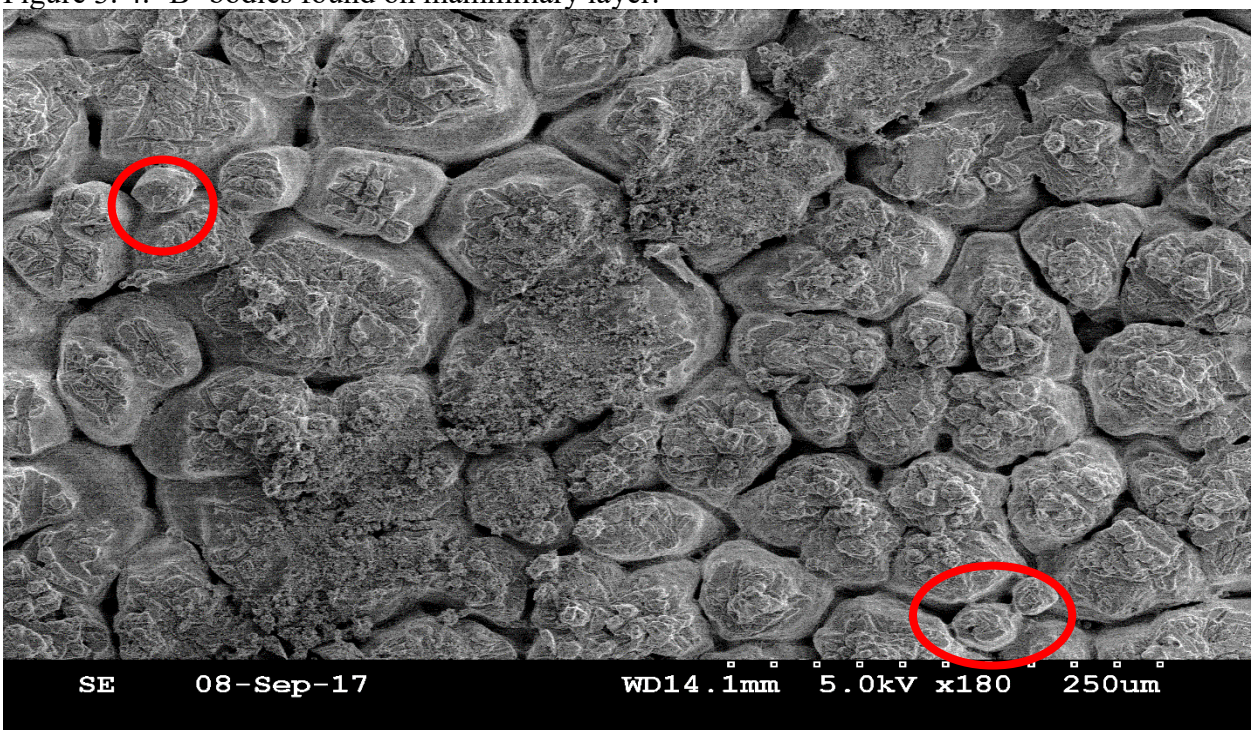


Figure 3. 4. ‘B’ bodies found on mammillary layer.



Over the 20 day period, hens on diet 1 produced eggs with a higer ratio of 'normal:b' bodies (40) compared to eggs produced by hens on diet 2 (14). Although not significant, this data agrees with work done by Solomon (1997), who concluded that "'b' bodies make no contribution to the thickness of the 'true'shell". Higher 'normal:b' ratios could indicate a stronger shell compared to shells with a lower 'normal:b' ratio. Totals for 'normal' and 'b' bodies for each hen on day 1 and day 20 can be see in the appendix, table 1 and 2.

A study conducted by Van Toledo and colleagues (Van Toledo et al., 1982), found that the density, or total number, of mammillary knobs was significantly greater in low eggshell strength (LES) compared to mammillary knob density of high eggshell strength (HES). Data from this study supports that shells with a great amount of mammillary knobs per unit of surface have allows for greater intersitial area between mammillary formations, allowing cracks to occur along these "natural fracture lines" (Van Toledo et al., 1982). Data trends from this data set disagrees with data found during this study, where greater mammillary knobs were found on eggshells with a higher breaking strength, but this data may differ from Van Toledo et al. (1982), due to being in ratio form compared to the number of 'b' bodies found on the mammillary layer. On the other hand, this data does agree with Hincke et al. (2012) that found that eggshells composed of smaller, less mutually aligned mammillary knobs have higher breaking strength. This is due to strong calcite crystal formation by a larger, higher oriented mamillary layer.

3.5 Conclusions

Based on the data collected from this experiment two extremes of eggshell quality were reached during late stages of production. Six hens were fed a diet sufficient in calcium (diet 1) produced eggs with significantly higher breaking strengths and greater percent shells compared to eggs from hens fed a diet insufficient in calcium (diet 2). Eggs from hens receiving diet 2 had significantly higher Haugh units compared to the haugh units from eggs produced by hens on diet 1. Althought specific gravity was significantly greater for eggs on treatment 1, there is no biological difference between the specific gravity from eggs on treatment versus treatment 2. Indicating that specific gravity is not a good indicator for shell quality.

Scanning electron imaging showed that there was an increased ratio of 'normal:b' bodies on the mammillary layer of eggs from hens receiving sufficient calcium compared to the ratios of 'normal:b' bodies of eggs from hens receiving insufficient calcium. However, not enough eggs were imaged to determine any significance of this value.

CHAPTER 4. EVALUATION OF SHELL QUALITY OF HENS RECEIVING CALCIUM REDUCED DIETS AT PEAK LAY

4.1 *Introduction*

Eggshell formation takes approximately 21 hours and spends the majority of its time in the uterus (Cutts et al., 2007). In this portion of the oviduct, pigmentation can be added to the eggshell, if pigmentation will be added. During eggshell formation, 2 to 3 grams of calcium are added. In order for this amount of calcium to be added to the eggshell, adequate dietary calcium needs to be available. During eggshell formation, different layers are added that compose the eggshell. Shell membranes are non-edible by-products of egg production, but contains biologically active compounds (Nakano et al., 2003). The inner and outer shell membranes are added to the yolk in the isthmus. The membranes are composed of a meshwork of proteinaceous fibers (95% protein) (Nys et al., 1999). The next portion of the eggshell's microstructure is the mammillary knob layer, that comes into contact with the outer shell membrane (Parsons, 1982). The mammillary bodies on this layer should be cover the shell membranes and are the initiation site for the rest of shell calcification (Hy-line, 2013a). Perpendicular to the mammillary knob layer is the palisade layer (Parsons, 1982). This crystalline layer is made of densely packed crystals of calcium in the form of pillars or palisades (Hy-line, 2013a). Additionally, the makeup of the eggshell will differ and have different eggshell parameters depending on the stage of lay the hen is in. Eggshells will be of highest quality at peak lay (26-29 weeks of age) and of lowest quality at the end of lay (80-90 weeks of age). Different measurements can be taken to indicate shell quality and relay information about shell quality and formation to producers and researchers. There are some destructive as well as non-destructive methods that are commonly used in the poultry industry to determine eggshell quality.

The aim of this study was to look at these egg quality measurements in relation to the microstructure of the shell, determined through scanning electron imaging, at the beginning of a laying cycle (10 weeks of lay; 26 weeks of age).

4.2 Materials and Methods

Twelve 26-week old White Leghorn laying hens were placed on either a diet deficient (n=6) or sufficient in calcium (n=6) (Ca). Six hens were randomly allotted to diet 1, sufficient in calcium; and diet 2, deficient in calcium. All procedures were conducted under protocols approved by Institutional Animal Care and Use Committee (IACUC). The ingredient composition for each treatment can be seen in Table. 4.1 and component composition for each treatment in Table. 4.2. Diets were were sampled and sent for analysis to University of Missouri.

Table 4. 1 Ingredient composition of diets

Ingredient (%)	Diet 1 Sufficient Ca	Diet 2 Reduced Ca
Corn	54.00	76.10
Soybean Meal	28.10	11.60
Alfalfa	2.30	9.40
Blended Fat	3.30	-
Salt	0.47	0.40
Limestone	5.50	1.60
Oyster Shell	3.60	-
Dicalcium phosphate	1.40	1.60
Vitmain-mineral premix	0.20	0.20
DL- Methionine	1.30	-
Integral[1]	1.00	-
Total	**100**	**100**

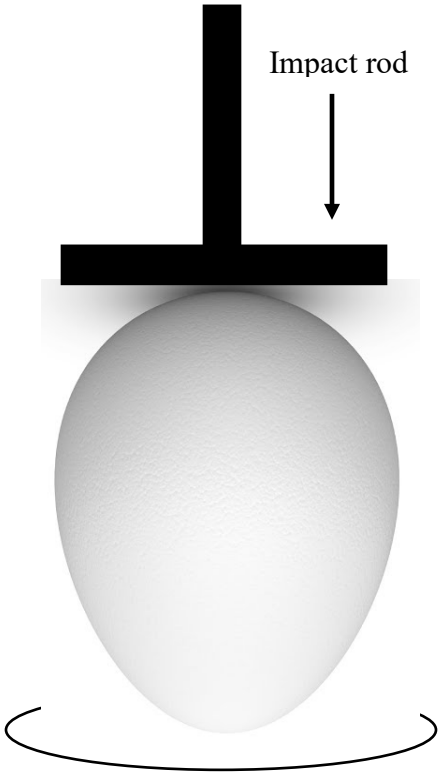
[1]Integral is a yeast based product to aid in reducing mycotoxin toxicity (Alltech)

Table 4. 2 Analyzed diet composition

Component (%)	Diet 1 Sufficient Ca	Diet 2 Reduced Ca
Crude Protein	18.11	13.21
Crude Fat	4.30	2.94
Crude Fiber	2.85	4.86
Calcium	4.55	0.88
Phosphorus	0.66	0.64

Hens were housed in individual cages and supplied *ad libitum* access to feed and water. Hens were started on experimental diets and egg collection began on week 10 of lay, from each hen daily for 20 days. After collection, eggs were weighed, and then subjected to breaking strength (kgf), Haugh unit was calculated from albumen height, and dry shell weight was determined, from which percent shell was calculated. Breaking strength was determined using quasi-static compression where the egg is compressed under controlled conditions using a 5kg load cell (Tyler, 1961). The minimum force required to cause failure of the shell is recorded and considered the breaking strength of the eggshell (Roberts, 2004). Figure 4.1 demonstrates the set up used to determine eggshell breaking strength.

Figure 4. 1 Schematic of impact loading along the long axis at the blunt end of the egg.



(Eissa and Alghannam, 2011)

Eggs were loaded so that egg was broke along the long axis, with the blunt end of the egg receiving the pressure of the impact rod (Nedomová et al., 2009). Albumen height is the measurement of the viscosity of the thick albumen, typically 1 cm from the edge of the yolk (Roberts, 2004). Albumen height was then converted to Haugh Unit (HU), which was then used to give the egg an internal quality grade. The calculation to determine HU can be found below and is the same equation used by Roberts (2004):

$$HU = 100LOG\ (H - \frac{\sqrt{G(30W^{0.37} - 100}}{100} + 1.0)$$

H = albumen height in mm

G= 32.3

W = weight of whole egg in grams

Shell weight was measured by breaking open the egg, removing the contents, rinsing the shell, and drying the shell. Shell weight is then recorded and used to calculate percent shell of the total egg (Roberts, 2004).

Eggshells from the first and last egg by each hen was laid were used for imaging on the Scanning Electron Microscope (SEM). Eggshells selected for the SEM were soaked in a 6% sodium hypochlorite. 4.12% sodium chloride, and 0.15% sodium hydroxide solution (12.5% bleach solution) overnight, rinsed with water, and dried for 48hrs, to remove shell membranes. Membrane-free shells were then coated in gold and platinum, mounted on a metal stub, and read using the SEM.

4.3 Statistical Analysis

Data was analyzed using the GLM procedure in SAS 9.4, using a univariate approach with treatment as the dependent variable. Significance was detected at $P < 0.05$.

4.4 Results and Discussion

This study was conducted to determine the effectiveness of standard egg quality measurements and scanning electron microscopy (SEM) for determining changes in shell quality and structure; specifically, the thickness of the mammillary and palisade layer. Variables measured in this experiment were egg weight (g), breaking strength (kgf), Haugh unit, and percent shell. Similar to the previous study, there were many differences in diet composition, including dietary calcium. The purpose of this experiment was to produce eggshells that were different structurally to see differences with the scanning electron microscope. Table 4.3. demonstrates the effect of diet on eggshell quality.

Table 4. 3 Effect of dietary calcium on eggshell quality across 20 day trial

	Diet [1] ± SE [2]		
	1 **Sufficient Ca**	**2** **Reduced Ca**	**P-value**
Egg Weight (g)	56.80[a] ± 0.433	50.17[b] ± 0.606	0.0001
Breaking Strength (kgf)	3.860 [a] ± 0.265	2.976 [b] ± 0.413	0.1000
Haugh Unit	76.49 [a] ± 0.554	78.24 [b] ± 0.773	0.1000
Percent Shell (%)	9.26 [a] ± 0.118	7.10 [b] ± 0.170	0.0001

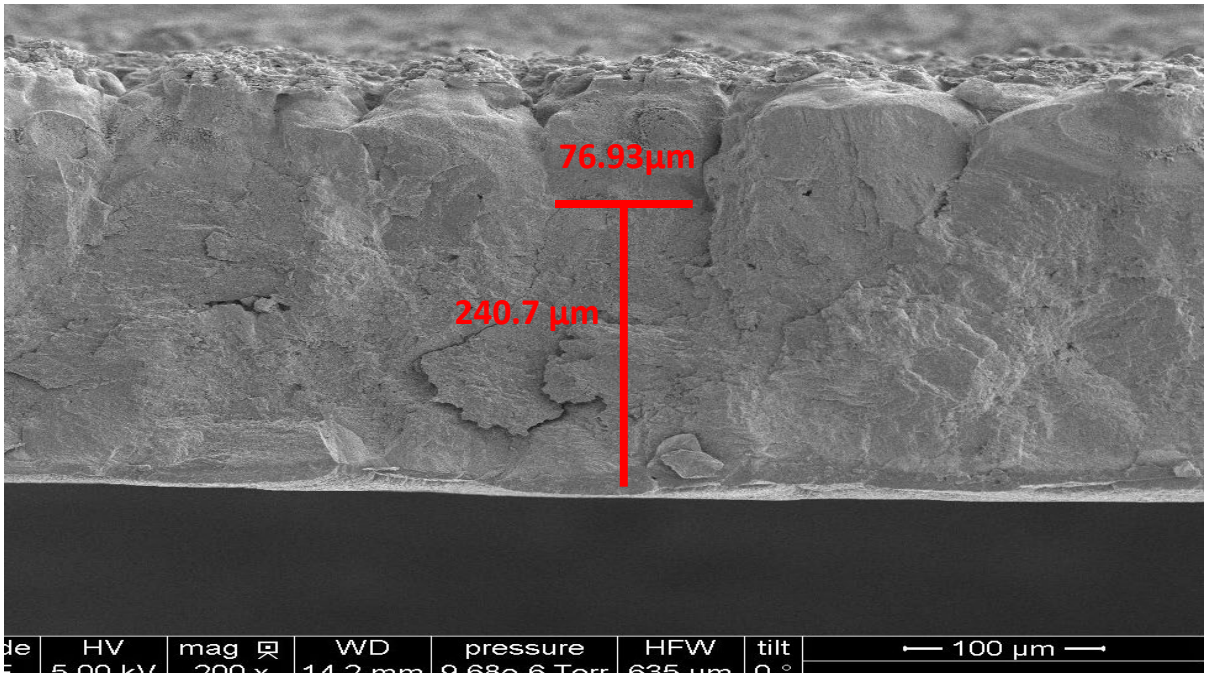
[1]Diets were: 1 = Ca sufficient (4.96% Ca), 2 = Ca deficient diet (0.88% Ca)
[2] Standard error of the mean
Different letters within a row indicate differences between diets

Significant differences were seen concerning egg weights, with eggs from hens on diet 1 producing significantly higher egg weights compared to eggs from hens on diet 2. This data differs from results found by Castillo et al. (2004), who found no significant differences in egg weights with five levels of calcium for laying hens at 23 weeks of age. Breaking strength differences were found, with the eggs from hens on diet 1 producing eggs that required significantly greater force to break their eggs, compared to eggs from hens on diet 2. This data agrees with An et al. (2016) and Roland (1988), who found a linear increase of breaking strengths with increasing dietary calcium. Similar to data produced by An et al. (2016) and in the previous chapter of this dissertation, differences were seen concerning Haugh Unit. Eggs from hens on diet 1 having lower HU compared to eggs from diet 2. Roberts (2004) explains that differences in HU from different Ca levels may be caused by the amount of time the egg spends in the shell gland, or the uterus. Leeson and Caston (1997) speculate that albumen becomes less viscous due to an increase of water uptake, resulting from increased time spent in the uterus, leading to a thinner albumen and HU. On the other hand, eggs that pass through the uterus quicker, have a thinner eggshell, but higher albumen due to less water uptake, resulting from less time being spent in the shell gland (Roberts, 2004). Shell percentage was significantly higher of eggs from diet 1, 9.26%, compared to the percent shell of eggs produced from diet 2, 7.10%. Calcium is the primary component in eggshells, so diets higher in Calcium content should produce a higher eggshell percentage compared to diets with lower Calcium concentrations.

In addition to egg quality parameters, scanning electron microscopy (SEM) was performed on all eggs produced from one hen receiving each treatment over the 20-day

period that eggs were collected. Images on the SEM were taken using 5kv current at a magnification of 200. Cross sections of each egg were imaged, measuring the thickness of the palisade and mammillary layer. Hamilton (1986) stated that the palisade layer accounts for about two-thirds of overall thickness of the eggshell. For this study, a palisade layer ≥ 200 μm was considered thick. Below is an image of an egg produced on day 2 from a hen receiving sufficient calcium in her diet.

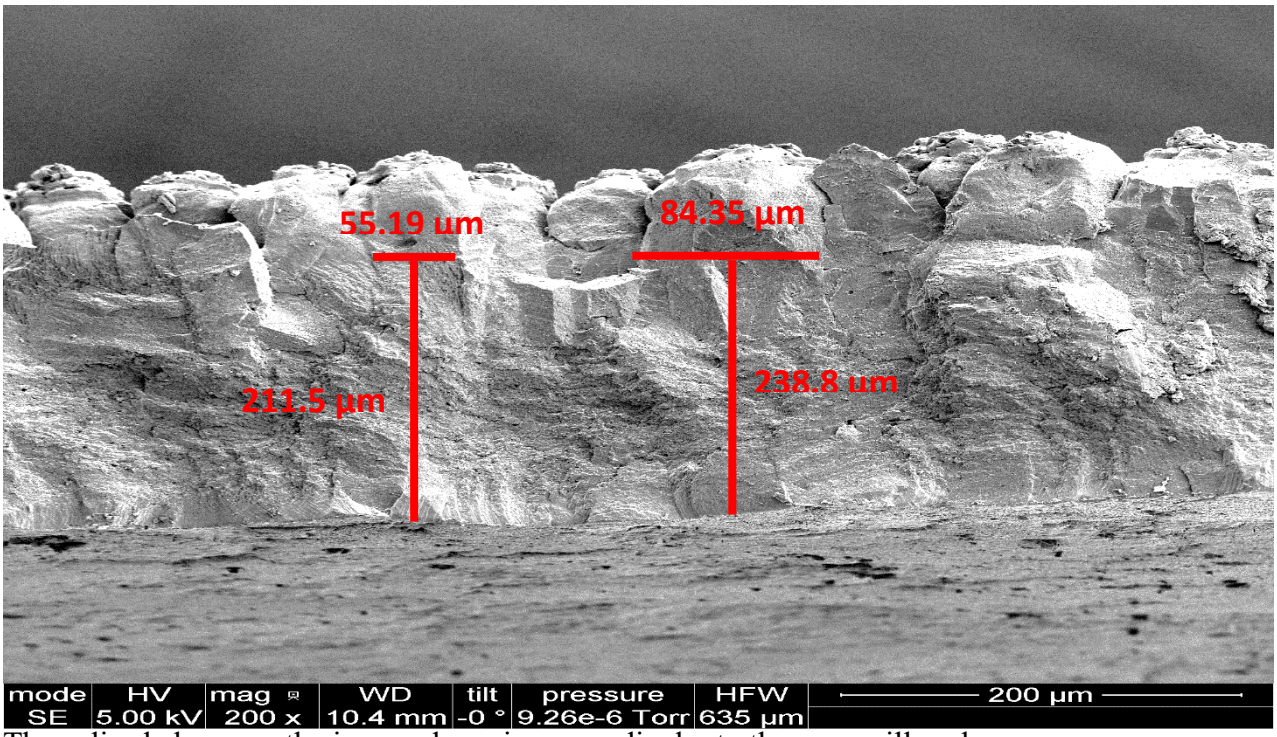
Figure 4. 2 Cross section of egg from diet 1 on day 2



The palisade layer on the image above is perpendicular to the mammillary layer. The palisade layer for this egg was 240.7 µm in thickness, while the mammillary layer was 76.93 µm wide.

From this image, it can be seen that a narrow mammillary body (76.93 μm) sits on a thick palisade layer (240 μm). Additionally, the breaking strength for this egg was 3.579 kgf. From this information, it can be assumed that this is a microstructurally sound egg, with a thick palisade sitting atop a narrow mammillary. Twenty days later, an egg from the same hen on the calcium sufficient diet was imaged and shown in figure 4.3.

Figure 4. 3 Cross section of egg on diet 1 on day 20.



The palisade layer on the image above is perpendicular to the mammillary layer.

Similar to figure 4.2, a narrow mammillary body, 55.19 µm and 84.34 µm, is the base for the thick palisade layer (211.5 µm and 238.8 µm respectively), that sits perpendicular to the mammillary layer. The breaking strength for this egg was 3.981 kgf. This agrees with the data collected on day 2 of the experiment, that a narrow mammillary cap and a thick palisade layer creates a microstructurally sound egg, with a high breaking strength. Eggs were also imaged from one hen receiving a diet reduced in calcium, and after one day of receiving reduced calcium, microstructural changes were already detected. Figure 4.4 is the scanning electron image of the egg on day 2 from hen receiving diet 2.

Figure 4. 4 Cross Section of Egg from Diet 2 on day 2.

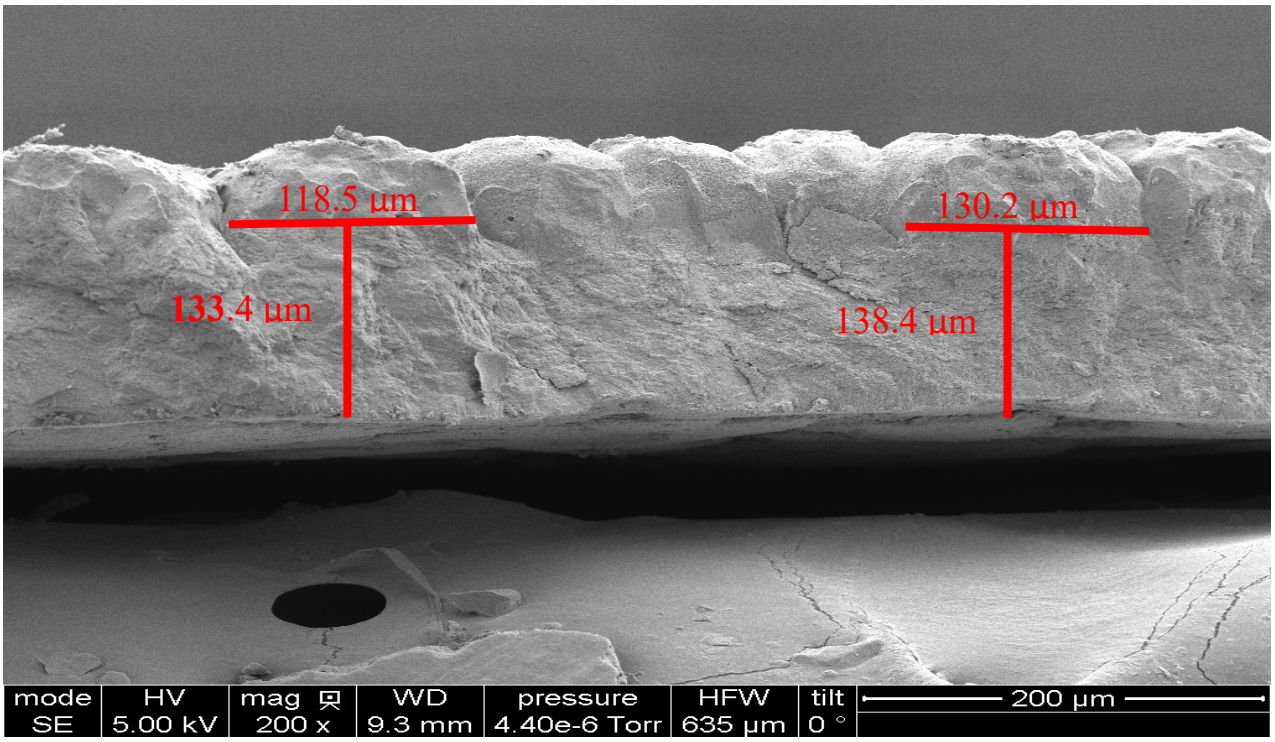
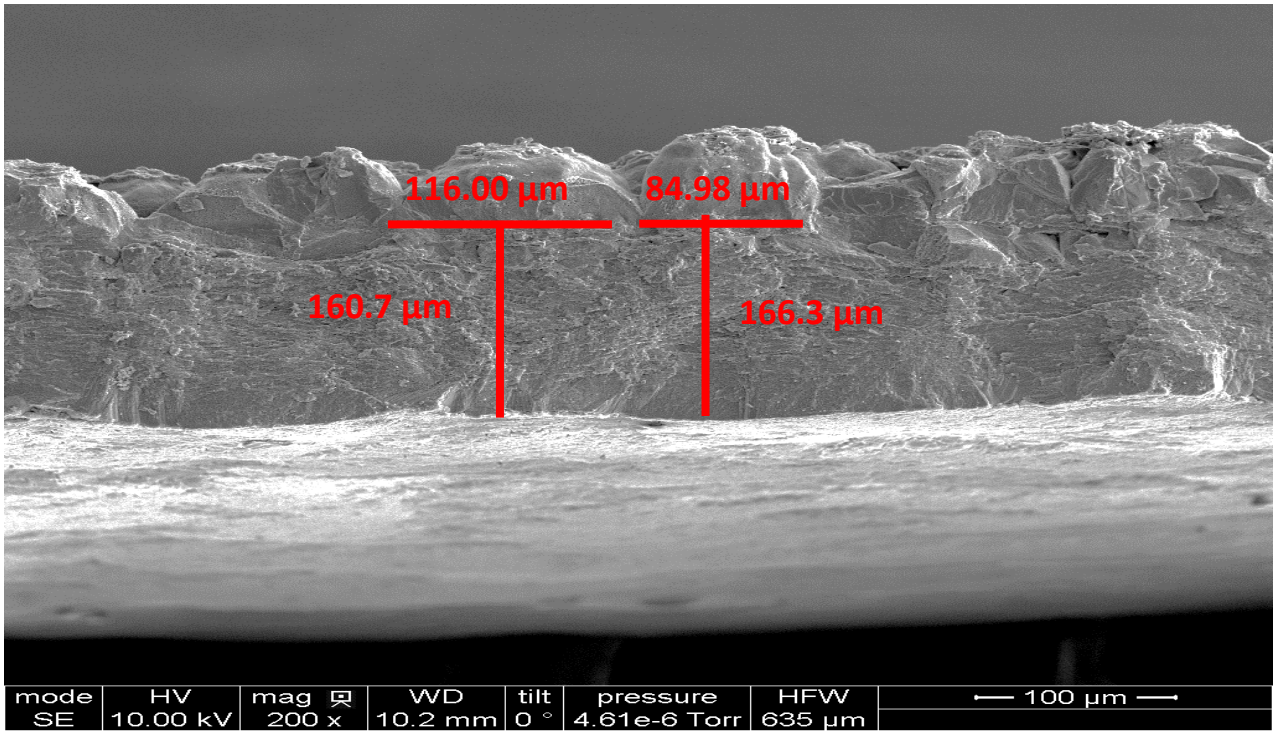


Figure 4.4. is an image from an egg the day after the hen's diet was changed to the reduced calcium diet. The egg was fairly sound with a breaking strength of 2.995kgf. However, microstructurally this egg was a little different from the egg of the hen on the sufficient calcium diet. The mammillary cap was very wide, 118.5 µm and 130.2 µm, while the palisade layer was thinner, 133.4 µm and 138.4 µm respectively. The palisade from this egg was numerically smaller compared to the eggs, both at day 1 and day 20, had a considerably thicker palisade layer. On day 20, the egg was more microstructurally similar to the egg on day 2. The scanning electron image for the egg on day 20 from diet 2 can be seen in figure 3.5.

Figure 4. 5 Cross section of egg from diet 2, day 20.



Similar to the microstructure of the egg on day 2 from diet 2, the mammillary bodies are narrow, 116 µm and 84.89 µm, which is the starting point for the palisade layer, which was thin at 160.7 µm and 166.3 µm. Additionally, this egg was broke during the laying process, which was directly correlated to the thin palisade layer. From this data it be concluded that a thin palisade layer leads to an egg requiring less force to be broken, possibly leading to be egg breakage during the laying process. Values for each egg produced by each hen produced over the 20 day period can be seen in the appendix, table 3.

4.5 Conclusions

From this data, it can be concluded that hens fed a diet with sufficient calcium had significantly higher breaking strength and shell percentage than eggs from hens receiving a calcium deficient diet. Microstructure changes were detected in eggshells from hens being fed a diet sufficient in calcium, with thicker palisade layers, compared to thinner palisade layers of eggs from hens being fed a diet reduced in calcium. Additionally, microstructure changes in the eggshell were detected the day after the hens diet was switched to the reduced calcium diet, indicating that scanning electron imaging can be used to determine the microstructural integrity of the eggshell.

CHAPTER 5. EFFECT OF ZINC SOURCE ON DIFFERENT EGGSHELL QUALITY PARAMETERS AND EGGSHELL MICROSTRUCTURE

5.1 Introduction

Trace minerals play a vital role to poultry layer diets, because they are required for growth and performance, as well as eggshell development (Fernandes, 2008). The eggshell is a mineralized structure, that requires numerous minerals and enzymes for formation, one of which is carbonic anhydrase (Zhang et al., 2017). Carbonic anhydrase is an enzyme that requires zinc, and is vital for calcium carbonate deposition during eggshell formation (Zhang et al., 2017). It has been accepted that organic forms of minerals are more bioavailable and have higher absorption, compared to the inorganic forms of minerals (Zhang et al., 2017). Form and amount of zinc supplementation on eggshell quality in laying hens has been studied in numerous studies, but results and the effect on shell quality are inconsistent. In these studies, eggshell quality parameters were evaluated using different levels and sources of zinc, including organic versus inorganic zinc. Solomon and Bain (2012), conducted a study looking at organic forms of zinc and selenium (Bioplex™ and Selplex™) and its effect on eggshell quality and microstructure. From this, it was concluded that organic sources of zinc and selenium produced an egg with a significantly sound microstructure, producing a stronger, higher quality eggshell. Zamani et al (2005) found that dietary zinc supplementation at 50 ppm created thicker egg, while Guo et al (2002) reported that 80 ppm was required to create a thicker eggshell. The aim of this study was to determine the effect of organic (Bioplex™) versus inorganic zinc sources (ZnO), at 30 and 80ppm, on egg production, eggshell quality and microstructure, as white and brown egg layers' progress through the laying cycle.

5.2 Materials and Methods

Two hundred and forty Hy-line W36 layers and 240 Hy-line Brown layers were used during this study. Birds were placed on one of five treatments, with six birds per replicate and 8 replications per color of bird. All procedures were conducted under protocols approved by Institutional Animal Care and Use Committee (IACUC). Birds were place on their experimental diet at 29 weeks of age and were remained on the experimental diet for 36 weeks of lay (65 weeks of age). Hens were housed in conventional cages, with 2 birds per pen and supplied *ad* libitum access to feed and water. Eggs were collected every 4 weeks. Feed was weighed in at the start of the experiment. Feed was added as need and weighed every 4 weeks. On the next collection date, orts were weighed back and new feed was weighed in. In addition, birds were weighed every 4 weeks throughout the experiment. The ingredient composition for each treatment can be seen in Table 5.1 and component composition for each treatment in Table 5.2. Premix composition for each diet can be found in the appendix. Diets were sampled and sent to University of Missouri for diet analysis composition.

Table 5. 1 Ingredient composition of diets

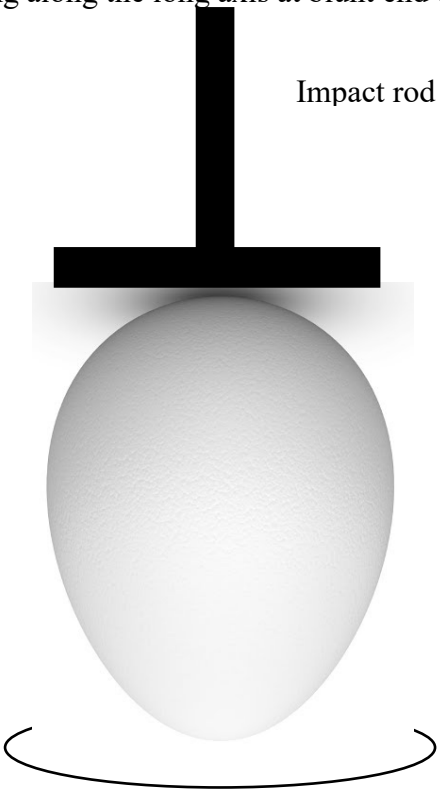
Ingredient (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Corn	56.00	56.00	56.00	56.00	56.00
SBM, dehulled (48% cp)	28.00	28.00	28.00	28.00	28.00
Soy oil	3.75	3.75	3.75	3.75	3.75
Dical (23-18)	1.00	1.00	1.00	1.00	1.00
Limestone	7.20	7.20	7.20	7.20	7.20
Oyster shell	3.00	3.00	3.00	3.00	3.00
Salt, iodized	0.38	0.38	0.38	0.38	0.38
DL-methionine	0.17	0.17	0.17	0.17	0.17
Vitamin premix (No Mineral)	0.25	0.25	0.25	0.25	0.25
Mineral premix 1 (No Zn)	0.25	-	-	-	-
Mineral premix 2 (80 ppm Zn as ZnO)	-	0.25	-	-	-
Mineral premix 3 (30 ppm Zn as ZnO)	-	-	0.25	-	-
Mineral premix 4 (80 ppm Zn as Bioplex)	-	-	-	0.25	-
Mineral premix 5 (30 ppm Zn as Bioplex)	-	-	-	--	0.25
Total	100	100	100	100	100

Table 5. 2 Analyzed diet composition

Component (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Crude Protein	16.30	17.72	17.43	17.82	17.05
Crude Fat	5.13	5.47	5.52	5.47	5.46
Crude Fiber	4.67	3.61	2.79	3.09	2.51
Calcium	4.13	5.12	4.73	5.30	5.12
Phosphorus	0.57	0.61	0.52	0.49	0.52
Zinc (ppm)	26.7	107.0	55.9	105.0	54.3

All eggs per replication were collected on day 0, at 29 weeks of age, and every 4 weeks after. All eggs were scanned using the Volscan Profiler, creating a three-dimensional image of each egg. The Volscan also measured egg length and the width of the egg, which were used to calculate shape index. Eggs were weighed, then subjected to breaking strength (kgf), Haugh unit was calculated from albumen height, and dry shell weight was determined, from which percent shell was calculated. Breaking strength was determined using quasi-static compression. The egg was compressed under controlled conditions using a 5kg load cell (Tyler, 1961). The minimum force required to cause failure of the shell was recorded and considered the breaking strength of the eggshell (Roberts, 2004). The method used to determine eggshell breaking strength can be seen in Figure 5.1.

Figure 5.1 Schematic of impact loading along the long axis at blunt end of the egg
Schematic of impact loading along the long axis at blunt end of the egg



(Eissa and Alghannam, 2011)

Eggs were loaded so that egg was broke along the long axis, with the blunt end of the egg receiving the pressure of the impact rod (Nedomová et al., 2009). Albumen height was the measurement of the viscosity of the thick albumen, typically 1 cm from the edge of the yolk (Roberts, 2004). Albumen height was converted to Haugh Unit (HU), which was then used to give the egg an internal quality grade. The calculation to determine HU can be found below and is the same equation used by (Roberts, 2004):

$$H.U = 100LOG\ (H - \frac{\sqrt{G(30W^{0.37} - 100}}{100} + 1.0)$$

H = albumen height in mm

G= 32.3

W = weight of whole egg in grams

Shell weight was measured by breaking open the egg, removing the contents, rinsing the shell, and drying the shell. Shell weight was then recorded and used to calculate percent shell of the total egg (Roberts, 2004). Eggshells from the middle of the trial (week 16) and the end of the trial (week 36) were randomly selected and were used for imaging on the Scanning Electron Microscope (SEM). Pens used selected for SEM can be seen in Table 5.3.

Table 5. 3 Pens used for scanning electron microscopy of eggshells

	Diet	Pen Number
White Egg Layers	1	29
	2	5
	3	2
	4	30
	5	23
Brown Egg Layers	1	24
	2	7
	3	22
	4	10
	5	8

Eggshells selected for the SEM were soaked in a 6% sodium hypochlorite, 4.12% sodium chloride, and 0.15% sodium hydroxide solution (12.5% bleach solution) overnight, rinsed with water, and dried for 48hrs, to remove the shell membranes. Membrane-free shells were then coated in gold and platinum, mounted on a metal stub, and read using the SEM. The SEM was set to a magnification of 150 for aerial images and 225 for cross section images. All images were taken using a voltage of 5.00kv.

5.3 Statistical Analysis

Bird production data and egg quality data were analyzed using PROC GLM function in SAS 9.4. Significant differences were detected at $P \leq 0.05$. Data from the first two collections periods, week 0 and week 4 were combined at considered period 1, while data from weeks 32 and 36 were combined and considered period 2. Egg shape index was calculated using length and width of the eggs, measured using the volscan, at weeks 24, 28, 32, and 36. Shape index was also analyzed using PROC GLM in SAS 9.4 and significant differences were detected at $P \leq 0.05$.

5.4 Results and Discussion

5.4.1 Production Results

Feed efficiency for white layers was numerically higher in period 2, compared to feed efficiency at the beginning of the experiment, period 1. Feed efficiency values can be seen in table 5.4.

Table 5. 4 White layer feed efficiency per gram of egg produced (kg/kg)

	Diet				
Period	**1 No suppl. Zn**	**2 80 ppm ZnO**	**3 30 ppm ZnO**	**4 80ppm Bioplex® Zn**	**5 30ppm Bioplex® Zn**
1	1.67^{c}	1.67^{c}	1.73^{bc}	1.74^{bc}	1.73^{bc}
2	1.82^{ab}	1.79^{b}	1.87^{a}	1.86^{a}	1.83^{ab}

Period 1 = week 0 and 4 of trial; period 2 = week 32 and 36 of trial
Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg as ZnO; 3=corn-soy diet 30mg Zn/kg as ZnO; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and period interaction; significance detected at $P \leq 0.05$

Additionally, feed efficiency for brown layers was numerically higher in period 2, compared to feed efficiency at the beginning of the experiment, period 1. Feed efficiency values can be seen in table 5.5.

Table 5. 5 Brown layer feed efficiency per gram of egg produced (kg/kg)

Period	Diet				
	1 No suppl. Zn	**2 80 ppm ZnO**	**3 30 ppm ZnO**	**4 80ppm Bioplex® Zn**	**5 30ppm Bioplex® Zn**
1	1.67^{c}	1.67^{c}	1.73^{bc}	1.74^{bc}	1.73^{bc}
2	1.82^{ab}	1.79^{b}	1.87^{a}	1.86^{a}	1.82^{ab}

Period 1 = week 0 and 4 of trial; period 2 = week 32 and 36 of trial
Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and period interaction; significance detected at $P \leq 0.05$

Feed efficiency was significantly higher in period 2 for diet 1, 2, 3, and 4. However, no significant differences were seen between period 1 and 2 for diet 5, 1.73 g and 1.82 g respectively. Between diets, diet 2 had significantly lower feed efficiency compared to diet 3, 30ppm ZnO, and diets 4 and 5, Bioplex™ Zn. The control diet had a feed efficiency intermediate to diet 2 and diets 3, 4, and 5. These values differ from results found by Zhang et al. (2017) and Fernandes et al. (2008), who saw no significant differences between feed efficiency based on zinc source. Values for feed efficiency for this experiment are similar to values found in the Hy-line Production manual.

Pounds of feed required to produce a dozen eggs was calculated from feed consumed between each collection date, divided by the number of eggs produced between collection period and converted from grams to pounds. Values for pounds of feed required to produce a dozen eggs at each time period for each diet can be seen in table 5.6.

Table 5. 6 Feed conversion per dozen of white shelled eggs (kg)

Period	Diet				
	1 No suppl. Zn	**2 80 ppm ZnO**	**3 30 ppm ZnO**	**4 80ppm Bioplex® Zn**	**5 30ppm Bioplex® Zn**
1	1.38^{c}	1.36^{b}	1.37^{c}	1.42^{c}	1.40^{c}
2	1.70^{ab}	1.64^{ab}	1.67^{ab}	1.67^{ab}	1.77^{a}

Period 1 = week 0 and 4 of trial; period 2 = week 32 and 36 of trial
Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and period interaction; significance detected at $P \leq 0.05$

From these values it can be seen that pounds of feed required to produce a dozen eggs was numerically lower at period 1 compared to period 2. This can be attributed to eggs becoming heavier in weight as the hen progresses through lay. Additionally, diet 1, 3, 4, and 5 required significantly greater feed to produce a dozen eggs in period 2 compared to period.

Values for pounds of feed required to produce a dozen brown eggs at each time period for each diet can be seen in table 5.7.

Table 5. 7 Feed consumption per dozen of brown shelled eggs (kg)

	Diet				
Period	**1 No suppl. Zn**	**2 80 ppm ZnO**	**3 30 ppm ZnO**	**4 80ppm Bioplex® Zn**	**5 30ppm Bioplex® Zn**
1	1.27^{b}	1.35^{b}	1.33^{b}	1.39^{ab}	1.33^{b}
2	1.80^{ab}	1.84^{ab}	1.91^{a}	1.81^{ab}	1.77^{ab}

Period 1 = week 0 and 4 of trial; period 2 = week 32 and 36 of trial
Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and period interaction; significance detected at $P \leq 0.05$

From these values it can be seen that pounds of feed required to produce a dozen eggs was numerically lower at period 1 compared to period 2. This can be attributed to eggs becoming heavier in weight as the hen progresses through lay. Additionally, diet 3 required significantly greater feed to produce a dozen eggs in period 2 compared to period 1.

Egg production was numerically higher at the start of the experiment, period 1, for all diets compared to egg production at the end of the experiment, period 2. Values for egg production per week can be seen in table 5.8.

Table 5. 8 White layer egg production (eggs/hen/week)

Period	Diet				
	1 No suppl. Zn	**2 80 ppm ZnO**	**3 30 ppm ZnO**	**4 80ppm Bioplex® Zn**	**5 30ppm Bioplex® Zn**
1	5.55^{abc}	5.83^{ab}	5.86^{a}	5.86^{ab}	5.80^{ab}
2	5.23^{c}	5.55^{abc}	5.35^{bc}	5.47^{abc}	5.20^{c}

Period 1 = week 0 and 4 of trial; period 2 = week 32 and 36 of trial Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and period interaction; significance detected at $P \leq 0.05$

Egg production was only significant between period 1 and 2 for diet 5, 5.80 and 5.20 eggs respectively. From this experiment, it was determined that brown egg production did not change from period 1 to period 2. Values for egg production of brown egg layers can be seen in table 5.9.

Table 5. 9 Brown layer egg production per week

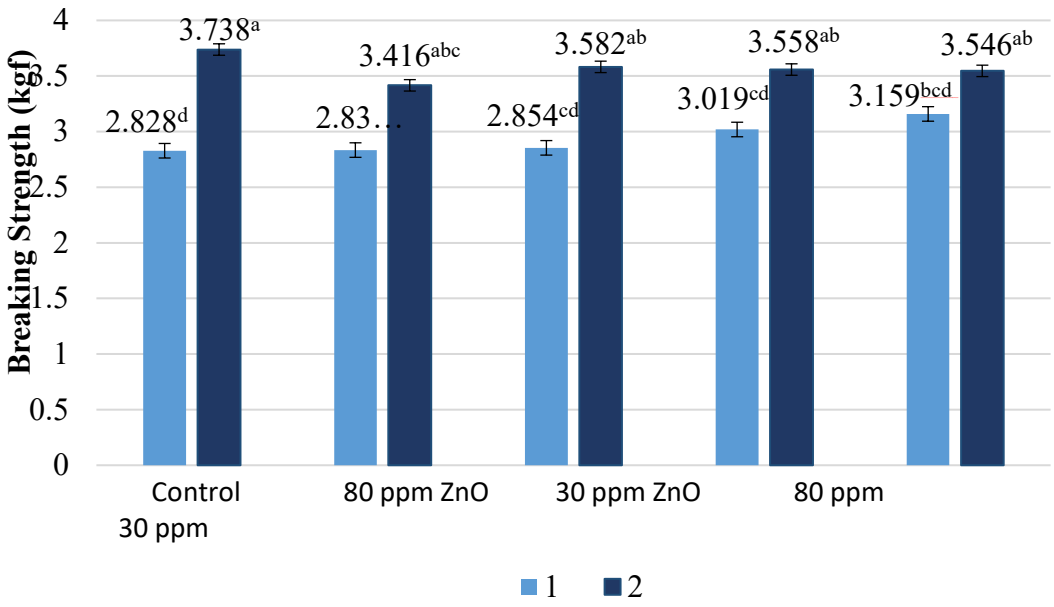
Period	Diet 1 No suppl. Zn	2 80 ppm ZnO	3 30 ppm ZnO	4 80ppm Bioplex® Zn	5 30ppm Bioplex® Zn
1	5.43[ab]	5.49[b]	5.60[ab]	5.38[ab]	5.81[ab]
2	5.37[ab]	5.17[b]	5.28[b]	5.37[ab]	5.27[b]

Period 1 = week 0 and 4 of trial; period 2 = week 32 and 36 of trial
Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and period interaction; significance detected at $P \leq 0.05$

5.4.2 Egg Quality Data

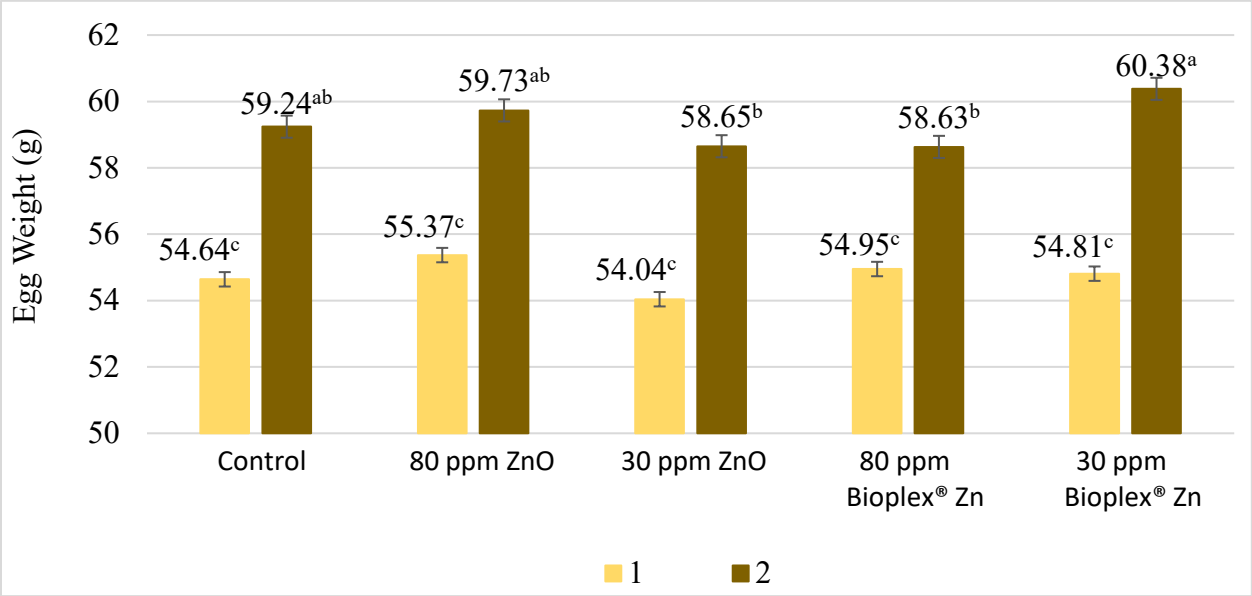
At the start of the experiment, period 1, eggs for all 5 diets were significantly lower ($P < 0.05$) compared to eggs from all diets at the end of the experiment, period 2. Values for each diet for white and brown eggs can be seen in figure 5.2and 5.3, respectively.

Figure 5.2 White shelled egg weight



Period 1 = week 0 and 4; period 2 = week 32 and 36
Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within a row indicates differences between diets; significance detected at $P \leq 0.05$

Figure 5.3 Brown shelled egg weight



Period 1 = week 0 and 4; period 2 = week 32 and 36

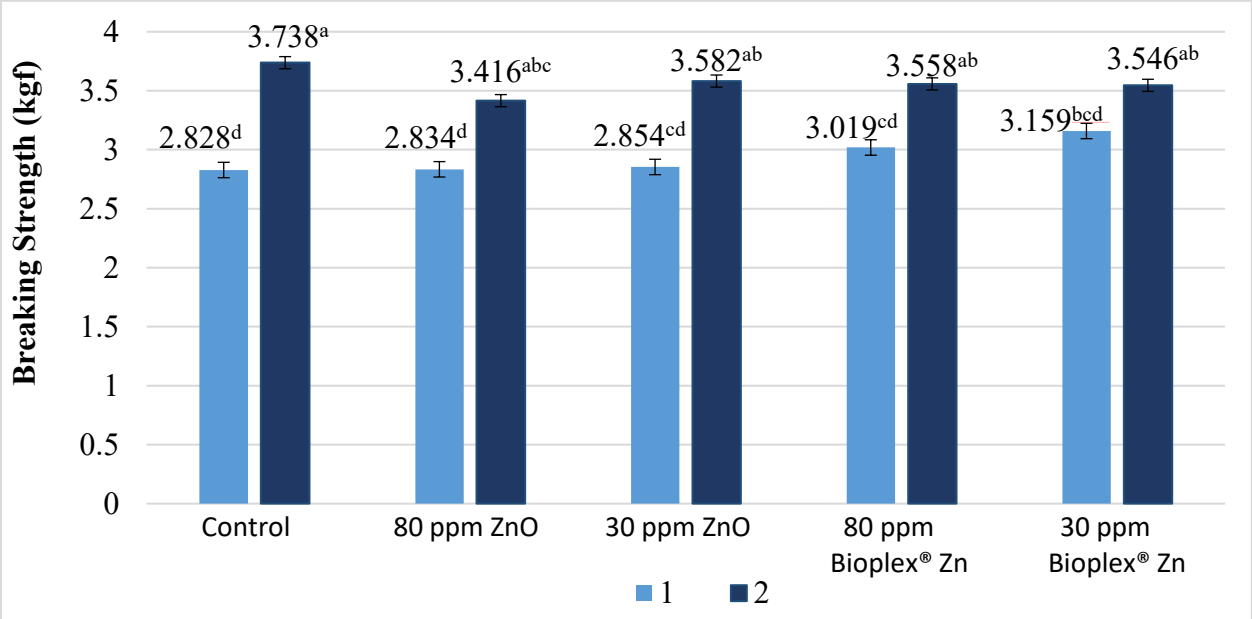
Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.

Different letters within a row indicates differences between diets; significance detected at $P \leq 0.05$

Similar to results found by Plaimast et al. (2008) and Swiatkiewicz and Koreleski (2008), no significant differences were found for egg weights between diets. However, egg weights from this experiment were numerically smaller than the egg weights found by Plaimast et al. (2008) and Swiatkiewicz and Koreleski (2008). Results for egg weights from this trial were also similar to results found by Zamani et al. (2005) and Solomon and Bain (2012), where average egg weight for older birds was significantly higher than egg weights from younger birds. Data from Zamani et al. (2005) found that additional zinc (ZnO) did not influence egg weight, which is similar to results found in this study. Egg weights during period 1 and period 2 were not affected by treatment. Similarly, Solomon and Bain (2012) found that birds supplemented with Bioplex™ did not have significantly higher egg weights compared to diets without Bioplex™. Additionally, white shell egg weights for this experiment were similar to values indicated by Hy-line's Management Guide at time 1 (29 weeks of age), 58.6 g, as well as at time period 2, 63.5 g (Hy-line).

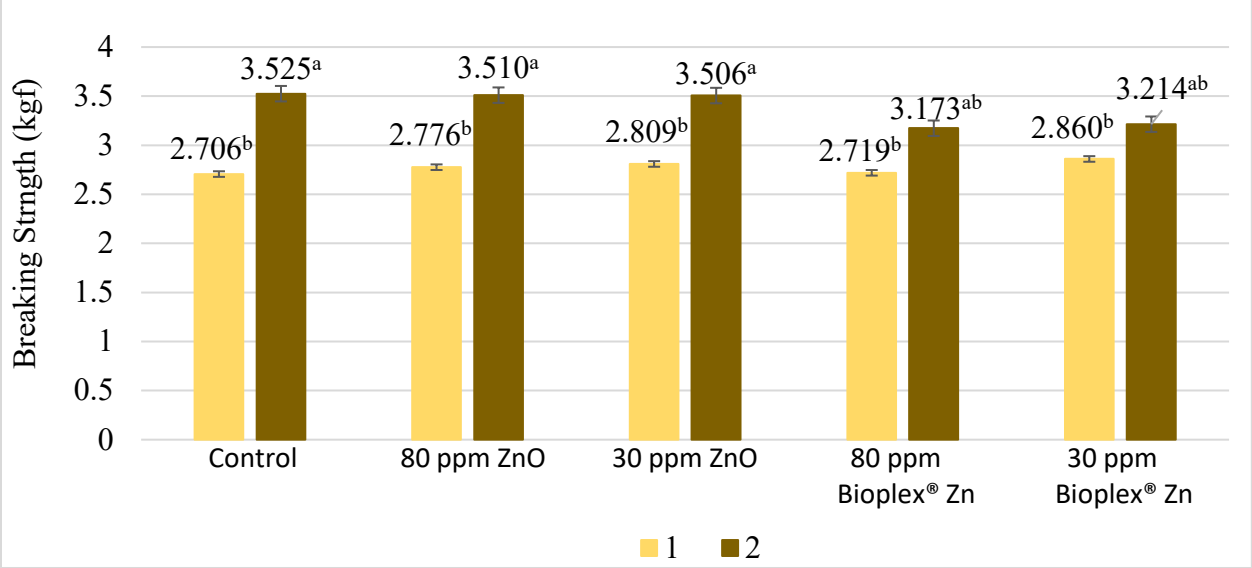
Breaking strength was the force required to break the egg along the long axis and was recorded. Statistically, all values were higher at period 2 for all diets compared to values for all diets at period 1, for both white and brown eggs. These values can be seen in table 5.12 and 5.13.

Figure 5.4 White shelled eggs breaking strength (kgf)



Period 1 = week 0 and 4 of trial; period 2 = week 32 and 36 of trial
Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and period interaction; significance detected at $P \leq 0.05$

Figure 5.5 Brown shelled eggs breaking strength (kgf)



Period 1 = week 0 and 4 of trial; period 2 = week 32 and 36 of trial
Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and period interaction; significance detected at $P \leq 0.05$

From figure 5.4, it can be seen that diet 1 had a significantly higher breaking strength at period 2, 3.738 kgf, compared to period 1, 2.828 kgf ($P< 0.05$). Diet 2 had significantly higher breaking strength at period 2 (3.416 kgf) compared to period 1 (2.834 kgf). Additionally, diet 3 and 4 had significantly higher breaking strengths at period 2, 3.582 and 3.558 kgf respectively, than period 1, 2.854 and 3.019 kgf respectively. However, there was no significant differences detected between period 1 and 2 for diet 5.

Similar to white shelled eggs, breaking strengths for brown shelled eggs were numerically greater in period 2, breaking strength was only significantly higher in period 2 compared to period 1 for diets 1, 2, and 3. These values and their significance can be seen in figure 5.5. This data differs from results found by Swiatkiewicz and Koreleski (2008), that saw a decrease in breaking strength as birds aged regardless of treatment. Additionally, results found by Swiatkiewicz and Koreleski (2008) found that organic zinc and manganese at 100% had significantly higher breaking strength compared to organic minerals at lower levels.

Breaking strength was recorded by Solomon and Bain (2012), where eggs were analyzed from hens who received minerals in the form of Selplex™ + Bioplex™ had significantly greater breaking strengths *(P = 0.008)* (37.44N) compared to eggs from hens receiving inorganic selenium (sodium selenite), 36.52N. Additionally, eggs from hens receiving just Selplex™ had breaking strengths intermediate to breaking strength from eggs of hens receiving Selplex™ + Bioplex™ and eggs from sodium selenite, 36.10N (Solomon and Bain, 2012). In this experiment, eggs were analyzed from hens receiving just Bioplex™ zinc or inorganic zinc. Data from this experiment were similar to data by Solomon and Bain (2012). Eggshells from Bioplex™, regardless of supplementation amount (30 or 80ppm), were similar to breaking strength values produced by eggs from hens receiving inorganic zinc at 30 and 80ppm. This data, along with data from Solomon and Bain (2012) may suggest that Bioplex™ + Selplex™ need to be paired to produce eggshells with higher breaking strengths compared to eggs from hens receiving inorganic trace minerals.

Haugh unit (HU), for brown and white shelled eggs, were calculated from albumen height. It was determined that HU at period 2, for all diets, was significantly lower for all diets at period 1. However, all eggs at period 2 were still considered AA quality eggs, as they were for all diets at period 1. These values can be seen in table 5.10 and 5.11.

Table 5. 10 White egg Haugh unit

	Diet [1] ± SE [2]				
Period[3]	**1 No suppl. Zn**	**2 80 ppm ZnO**	**3 30 ppm ZnO**	**4 80ppm Bioplex® Zn**	**5 30ppm Bioplex® Zn**
1	82.48[a] ± 1.66	82.09[a] ± 1.49	83.41[a] ± 1.68	82.45[a] ± 1.45	82.85[a] ± 1.58
2	76.33[b] ± 1.23	75.09[b] ± 1.25	75.38[b] ± 1.29	75.88[b] ± 1.22	77.00[b] ± 1.25

[1]Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and period interaction; significance detected at $P \leq 0.05$
[2]SE = Stand error of the mean
[3]Period 1 = week 0 and 4 of trial; period 2 = week 32 and 36 of trial

Table 5. 11 Brown egg Haugh unit

	Diet[1] ± SE[2]				
Period[3]	**1 No suppl. Zn**	**2 80 ppm ZnO**	**3 30 ppm ZnO**	**4 80ppm Bioplex® Zn**	**5 30ppm Bioplex® Zn**
1	$84.07^{a} \pm 1.55$	$84.47^{a} \pm 1.51$	$83.78^{a} \pm 1.53$	$84.51^{a} \pm 1.54$	$84.35^{a} \pm 1.49$
2	$74.21^{c} \pm 1.31$	$74.92^{bc} \pm 1.33$	$77.72^{b} \pm 1.34$	$76.36^{bc} \pm 1.37$	$74.68^{bc} \pm 1.36$

[1]Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and period interaction; significance detected at $P \leq 0.05$
[2]SE = Stand error of the mean
[3]Period 1 = week 0 and 4 of trial; period 2 = week 32 and 36 of trial

Results from this trial concerning, white eggs, determined HU significantly decreased from the start of the experiment, period 1, to the end of the experiment, period 2; regardless of treatment. These results are similar to findings by Trinidad Neto et al. (2011) and Martin (2016), who found that HU decreased with time. Additionally, this data found significant decrease in HU in period 2 for diet 1 compared to diet 3. Martin (2016) found that hens fed inorganic zinc had significantly higher HU compared to eggs from hens being fed organic zinc sources. Although, values were significantly lower at the end of the experiment, HU values were still representative of a double AA egg.

Percent shell was calculated from dry eggshell weight and egg weight. From this data it was determined that percent shell was significantly higher, for white shelled eggs, at period 1 for diet 4 compared to period 2 (11.14 and 8.89% respectively). However, no other differences were detected between diets at period 1 and 2. These values can be seen in figure 5.6 and 5.7.

Figure 5.6 White egg percent shell

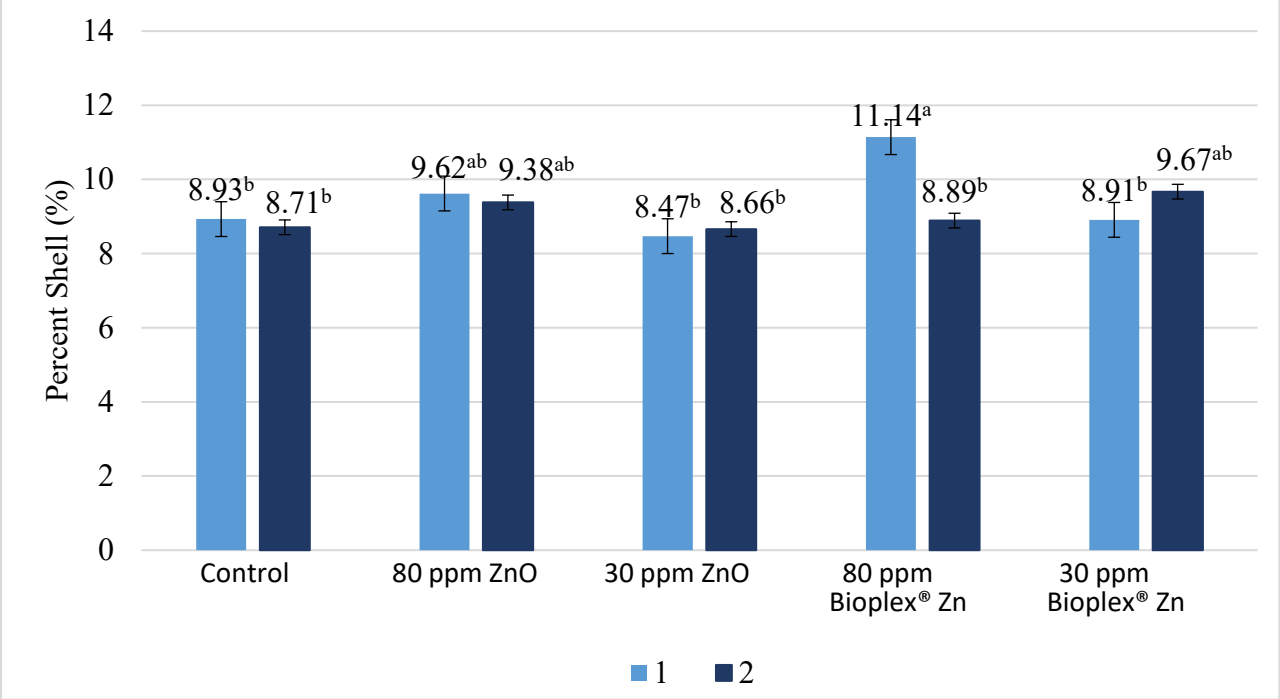
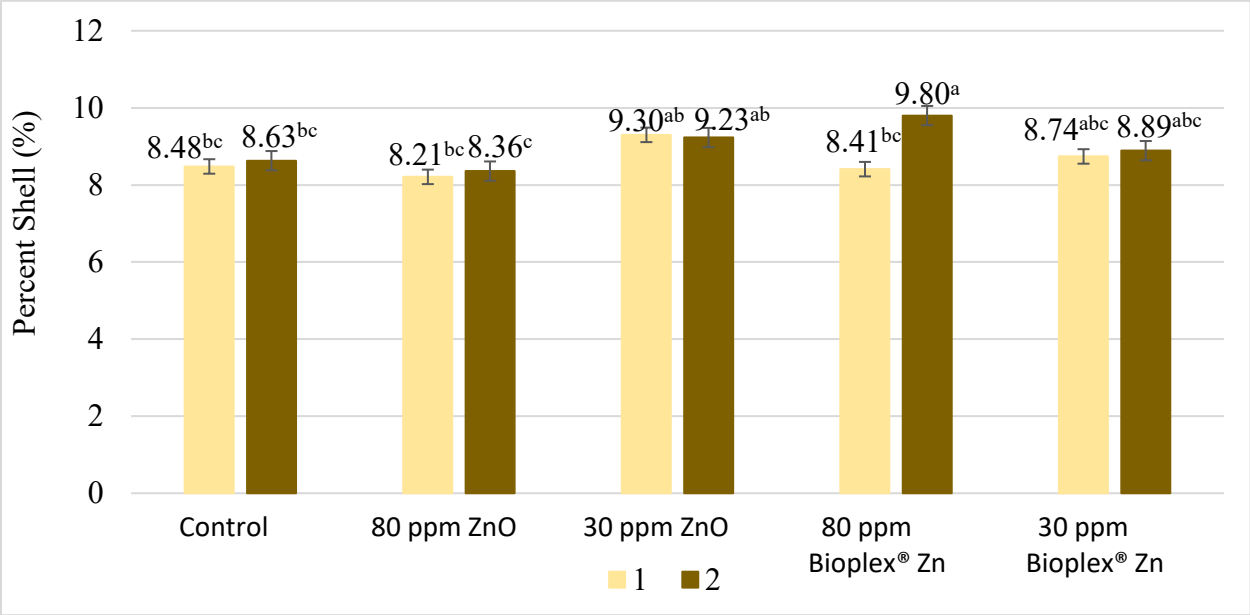


Figure 5.7 Brown egg percent shell



From this data, for white shell eggs, it can be seen that no significant differences were detected between period 1 and 2 for diets 1, 2, 3, and 5. However, percent shell, for white shelled eggs, was significantly higher at the start of the trial compared to the end of the trial. Egg weights were significantly higher for white eggs at the start of the trial compared to the end, which could explain differences found for percent shell for diet 4. For brown shell eggs, it can be seen that significant differences were only detected with shells from diet 4, with significantly greater percent shell at period 2 with 9.80% compared to 8.41% shell at period 2. The relationship between these two time periods for each diet can be seen in figure 5.5. Values from this trial, concerning white shelled eggs, were unlike values found by Zamani et al. (2005) and Swiatkiewicz and Koreleski (2008). Zamani et al. (2005) found that percent shell was significantly higher at the start of their experiment compared to the end of the experiment; while Swiatkiewicz and Koreleski, 2008 found differences in eggshell percentage concerning inorganic versus organic zinc supplementation. Although values for this experiment were not significantly higher than eggshell percentage from period 1 to period 2, values reported by Zamani et al. (2005) for 50 and 100ppm ZnO were similar to the values found in this experiment. Results for brown shelled from this experiment match results found by Mabe et al. (2003) and Swiatkiewicz and Koreleski, 2008, who found that percent shell from inorganic trace minerals was lower compared to the average of percent shell from eggs of hens receiving organic trace minerals. Brown eggs from diet 4 had a significantly higher percent shell at period 2, 9.80%, compared to percent shell at the start of the trial, 8.41%. This can be attributed to the bioavailability of organic zinc, as Bioplex® zinc at 80ppm, compared to the other diets.

Percent yolk was calculated from yolk weight and egg weight. These values can be seen in figure 5.8 for white shelled eggs and 5.9 for brown shelled eggs.

Figure 5.8 White egg percent yolk

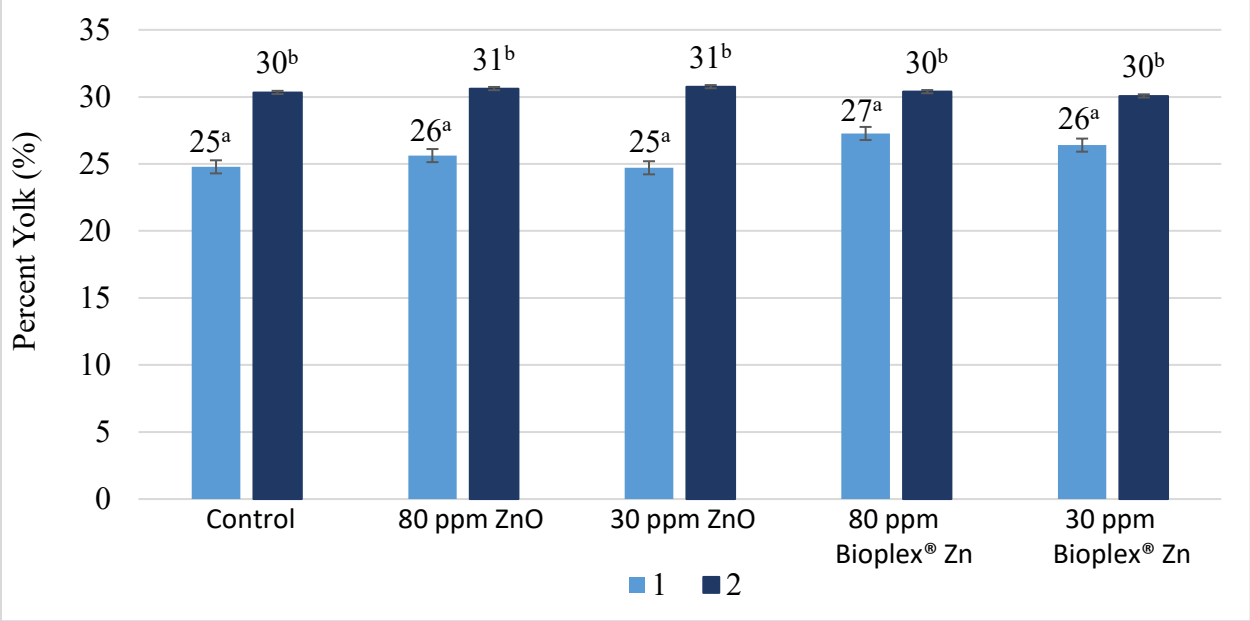
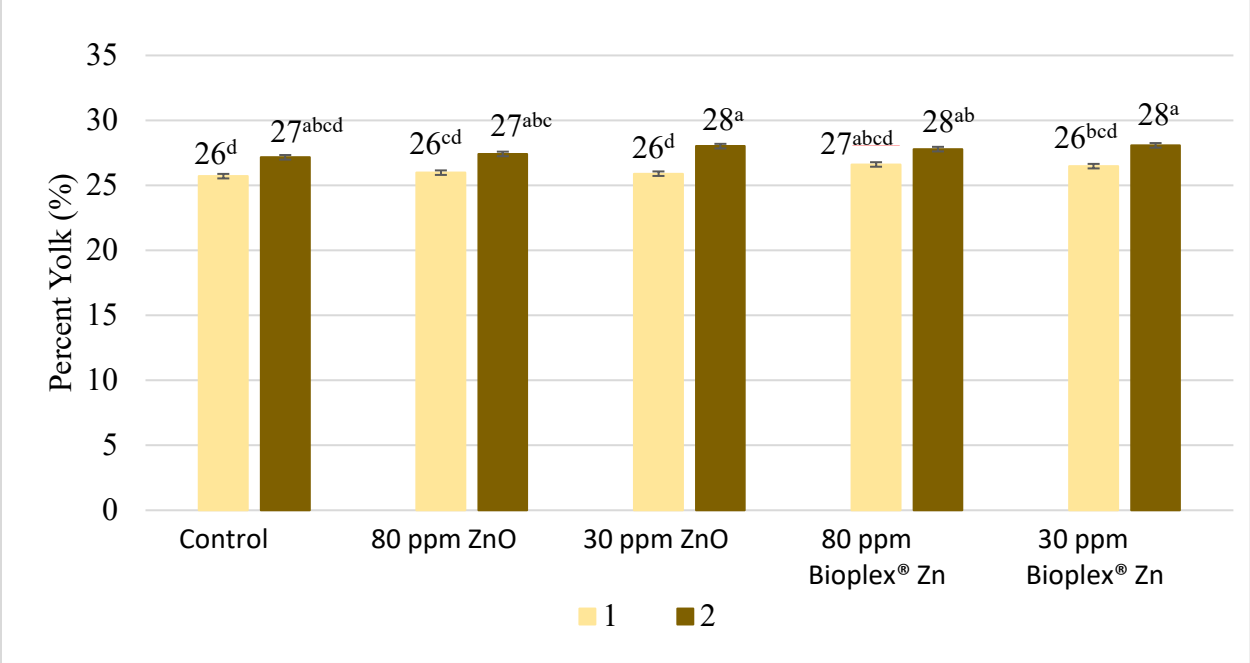


Figure 5.9 Brown egg percent yolk



From this it was determined that percent yolk from white shelled eggs, for all diets, at period 2 was significantly higher than percent yolk at period 1. Additionally, percent yolk was significantly higher for diet 4 at period 1 (27.27%) compared to the other diets at period 1 ($P<0.05$). Similar to white shelled eggs, percent yolk for brown shelled eggs was numerically higher for all diets in period 2 compared to period one. Additionally, percent yolk for diet 4 and 5 had significantly higher percent yolk at period 2 (28.02 and 28.08% respectively) compared to period 1 (25.91 and 26.48%). Data from this experiment differed from results found by Martin (2016) who found that hens fed inorganic zinc produced eggs with significantly higher yolk percentage, compared to hens being fed organic zinc. However, inorganic from the Martin (2016) study was zinc sulfate, while in this current study, inorganic zinc was fed in the form of zinc oxide, which could explain some of the differences seen between the two studies. This data suggests organic zinc, as Bioplex® Zn, produced a higher percent yolk, compared to eggs produced by hens receiving inorganic zinc, as zinc oxide.

Shape index has shown promise, with heritage breed eggs, as a nondestructive test to predict eggshell quality (Nolan 2017). Shape index was calculated using length and width of the egg $(\frac{w}{l}) * 100$. These values can be seen in table 5.20 and 5.21, for white and brown eggs respectively.

Table 5. 12 White egg shape index

Week	Diet 1 No suppl. Zn	2 80 ppm ZnO	3 30 ppm ZnO	4 80ppm Bioplex® Zn	5 30ppm Bioplex® Zn
24	74.47^{bc}	74.56^{abc}	74.75^{ab}	74.60^{abc}	75.70^{abc}
28	74.38^{c}	74.45^{bc}	74.47^{bc}	74.39^{c}	74.61^{abc}
32	74.56^{abc}	74.52^{abc}	74.70^{abc}	74.54^{abc}	74.51^{abc}
36	74.83^{a}	74.69^{abc}	74.68^{abc}	74.60^{abc}	74.56^{abc}

Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and week interaction; significance detected at $P \leq 0.05$

Table 5. 13 Brown Egg Shape Index

Week	Diet				
	1 No suppl. Zn	**2 80 ppm ZnO**	**3 30 ppm ZnO**	**4 80ppm Bioplex® Zn**	**5 30ppm Bioplex® Zn**
24	75.86^{abc}	75.78^{abc}	75.84^{abc}	75.83^{abc}	75.86^{abc}
28	76.00^{a}	75.86^{abc}	75.84^{abc}	75.89^{ab}	75.71^{bc}
32	75.78^{abc}	75.87^{abc}	75.85^{abc}	75.81^{ab}	75.91^{ab}
36	75.64^{c}	75.76^{abc}	75.77^{abc}	75.95^{ab}	75.77^{abc}

Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.
Different letters within table indicates differences between diet and week interaction; significance detected at $P \leq 0.05$

Although these values are significantly different, shape indices fall into one of three categories with any value less than 72 is considered a long narrow egg, a normal or standard egg has a shape index value of 72-75, while round eggs have a value great than 76 (Nedomova et al., 2009). Shape index values for all brown eggs, regardless of week and diet, were a 76, indicating a more rounded egg shape. Shape index values for all white eggs, regardless of week and diet, had a normal shape egg (74-75), while shape index for brown shelled eggs were on the border for a round egg, it is still very close to a "normal" shape egg. This is most likely due to the genetic selection of white and brown layers. Nolan et al. (2019) found that shape index could be used to predict eggshell quality in heritage breeds, due to the lack of genetic selection for those birds compared to the genetic makeup of Hy-line layers.

5.4.3 Scanning Electron Microscopy

One pen of each diet was randomly selected and all eggs produced by that pen were imaged for date 5 (16 weeks, 45 weeks of age) and date 10 (36 weeks, 65 weeks of age). Solomon and Bain (1996) stated that decline in eggshell quality, due to an increase in bird age, begins to occur between 60 and 27 weeks of age. These dates were chosen to see any shifts in microstructure of the eggshell as hens progressed throughout lay. Each egg was imaged from above, looking down on the mammillary layer, and from a cross section of the egg, to examine the palisade layer. All images of the mammillary layer were taken at 150 magnification with 5.00kv voltage, while cross sections were imaged at a magnification of 225 and 5.00kv voltage. From the mammillary layers, the density of the 'normal' caps were counted; as well as the density of 'b' bodies. From the cross section images, the width of the mammillary cap, as well as the length of the palisade

layer were measured using the software on the scanning electron microscope. From this data, a trend of narrower mammillary caps with thicker palisade layers on eggs requiring greater strength to break the egg. Additionally, eggs with a lower breaking strength, had wider mammillary caps with thinner palisade layers. This data was consistent to data found in Chapter 4 of this dissertation.

Due to the amount of time required to image eggs, only a small sample of eggs were imaged, leading to no significant differences between treatments or collection dates. However, Solomon and Bain (1996) developed a score sheet that can be used to quantify eggshell quality through SEM. This score sheet can be seen in figure 5.8.

Figure 5. 1 Score sheet to quantify shell quality through scanning electron microscopy

Area:	Area 1	Area 2	Area 3	Mean: S.D:
				Score
CONFLUENCE:	None (3)	Isol. (4)	Mod. (6)	Ext. (1)
CAPS:	G (1) G- (3)	P+ (6)	P(8)	P-(10)
EARLY FUSION:	Ext. (1)	Mod. (2)	Isol. (4)	
LATE FUSION:	Ext. (6)	Mod. (3)	Isol. (1)	
MAMM. ALIGNMENT:	None (1)	Isol. (2)	Mod. (4)	Ext. (7)
TYPE B's:	None (1)	Isol. (2)	Mod. (4)	Ext. (8)
PITTED:	None (1)	Dep. (5)	Eros. (7)	Hole (12)
ARAGONITE	None (1)	Isol. (2)	Mod. (5)	
TYPE A'	None (1)	Isol. (2)		
CUBICS	None (1)	Isol. (2)	Mod. (5)	
CUFFING	None (5)	Isol. (4)	Mod. (1)	
CHANGED MEMBRANE	None (1)	Isol. (4)	Mod. (8)	Ext. (14)

Isol. = Isolated
Mod. = Moderate
Ext. = Extensive
Dep. = Depression
Eros. = Erosion

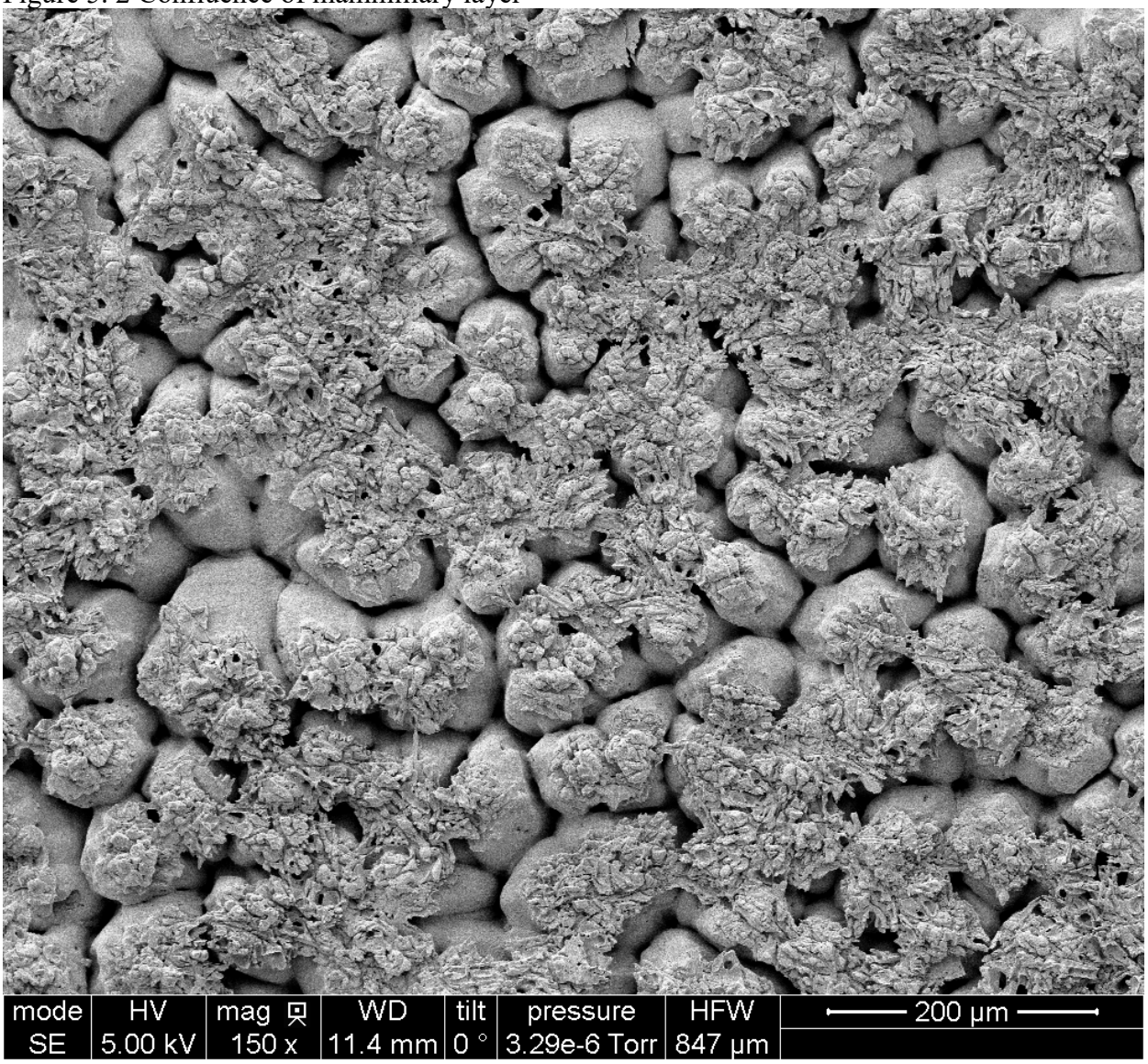
Based on the score sheet, eggshells with a lower score should have a stronger shell strength, and shell images with higher score will have a weaker egg.

Eggshell microstructure composition is dependent upon location; with some areas of the eggshell being structurally stronger than others. It has been determined that the egg is stronger at ends of the egg, with weak points being found along the equator. All images were taken from the equator of the eggshells. For this experiment, anything below 3.000kgf was considered a weaker egg, while an eggshell with a breaking strength of 3.00kgf or greater was considered a strong egg.

Confluence

Confluence creates a layer that connects mammillary caps to one another, which ultimately influences the formation of the palisade (Solomon, 1997). Ideally, an eggshell with a greater degree of confluence should have a stronger eggshell, that is able to withstand greater pressure. Figure 5.9 demonstrates the most extensive confluence seen throughout this 36 week experiment.

Figure 5. 2 Confluence of mammillary layer

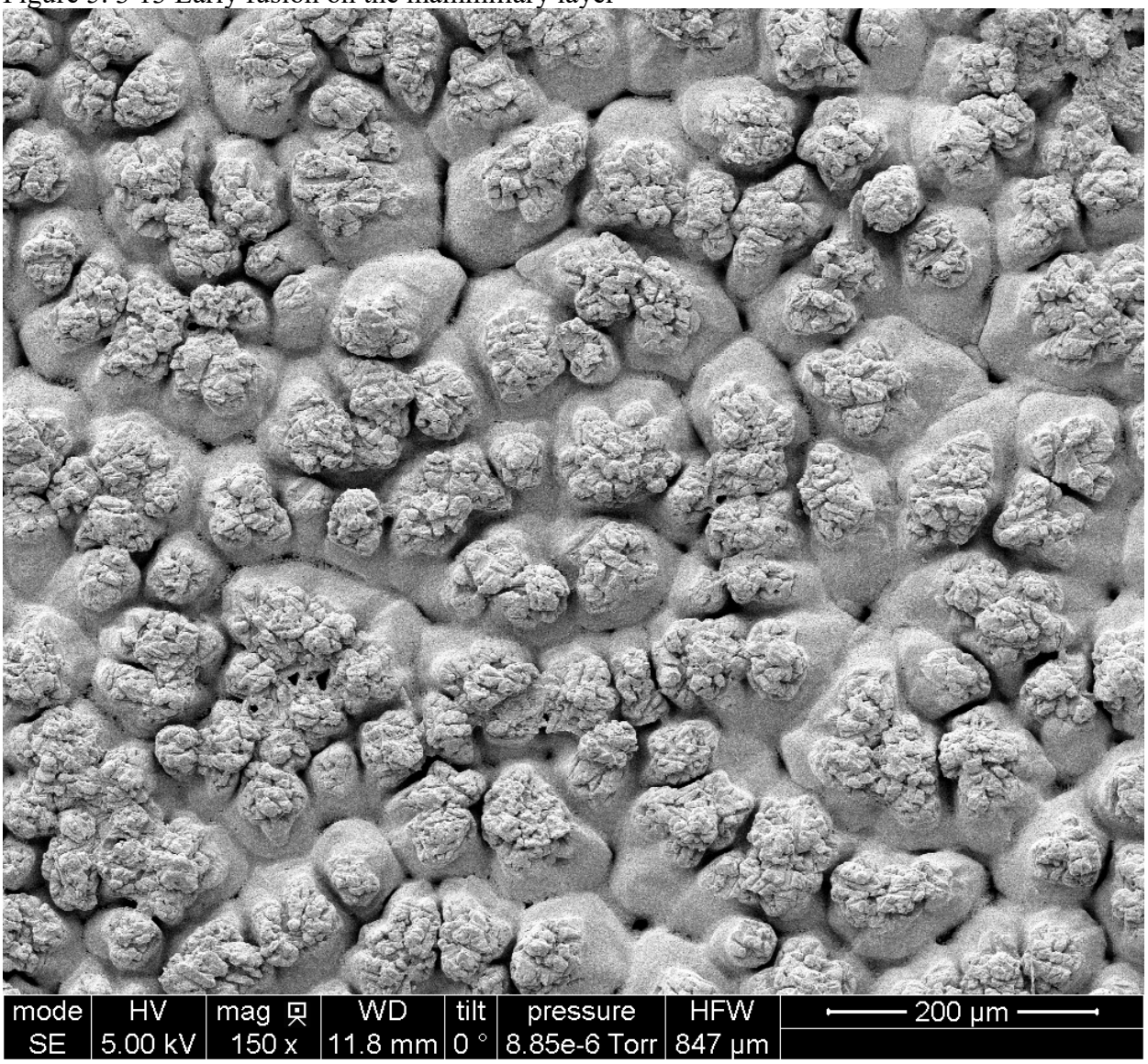


This egg was at 45 weeks of age, half way through the experiment. This egg was from a hen receiving diet 5, 30ppm Bioplex® Zn. The force required to break this egg was 2.675 kgf. From this image and the force required to break the egg, it can be determined that if a force were to occur that this point of the egg, a fracture or break would be less likely to occur.

Early Fusion

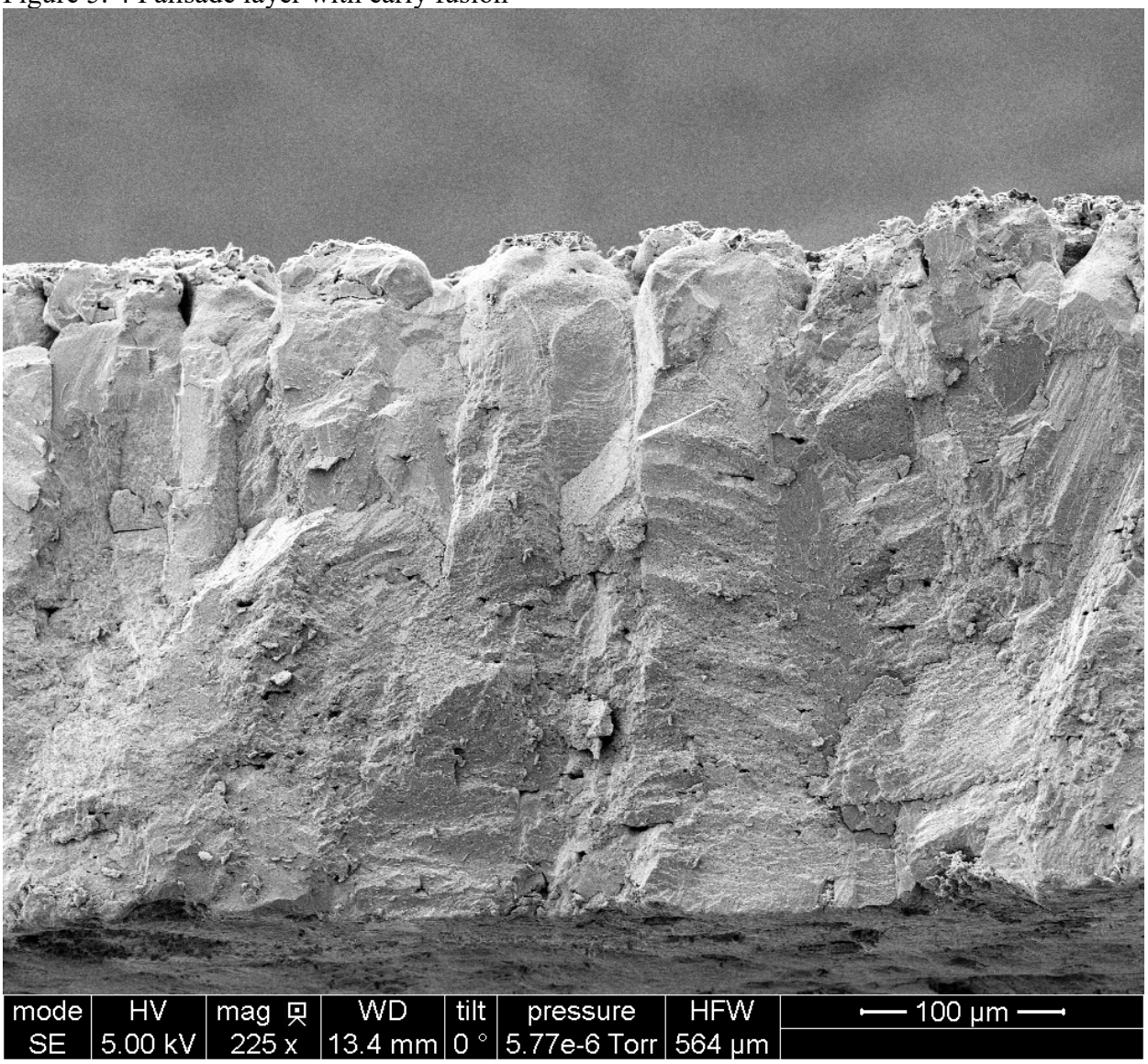
Early fusion occurs when adjacent palisade columns fuse early during the shell formation process and increased the thickness of the palisade layer, ultimately creating a microstructurally sound egg (Solomon and Bain, 1996). Figure 5.10 demonstrates early fusion.

Figure 5. 3 13 Early fusion on the mammillary layer



From figure 5.10 it can be seen that there is very little space between mammillary caps. This reduces the surface area available for fault lines to propagate and move through the eggshell. Additionally, early fusion can be seen in the palisade layer. The overall thickness of the palisade, before mammillary cap begins, increasing overall thickness. This can be seen in figure 5. 11.

Figure 5. 4 Palisade layer with early fusion

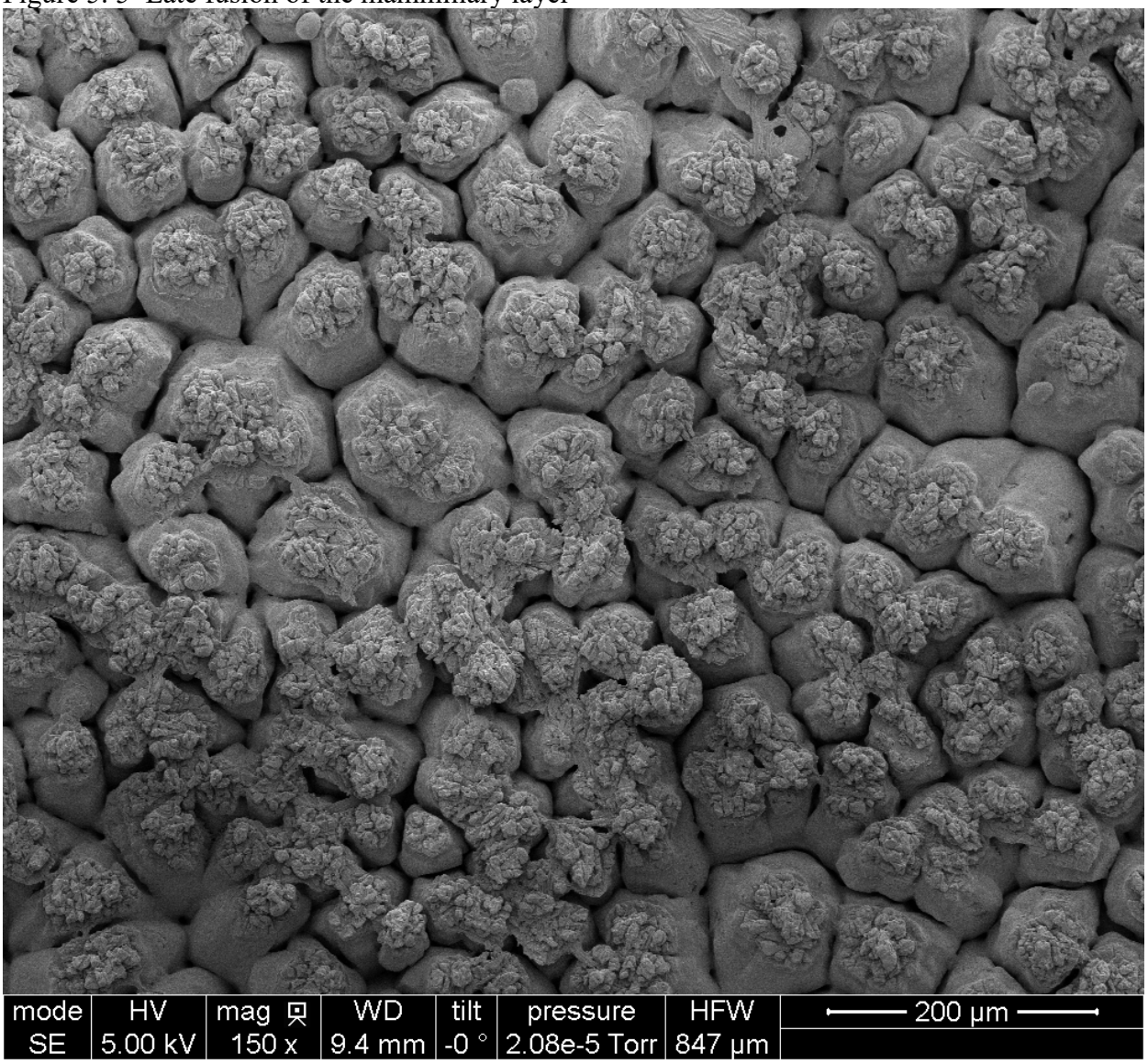


This egg was from collection date 5, 45 weeks of age, and the was receiving diet 1, the control diet. The breaking strength of this egg was 2.214kgf. This may have been a strong point on the shell, indicating that if force were to be applied at this point, a crack would not have occurred here. Additionally, although a lower score is given to shells with extensive early fusion (1), a score of (4) is given to isolated early fusion, which isn't as a high of a score for other "unfavorable" conditions of the eggshell.

Late fusion

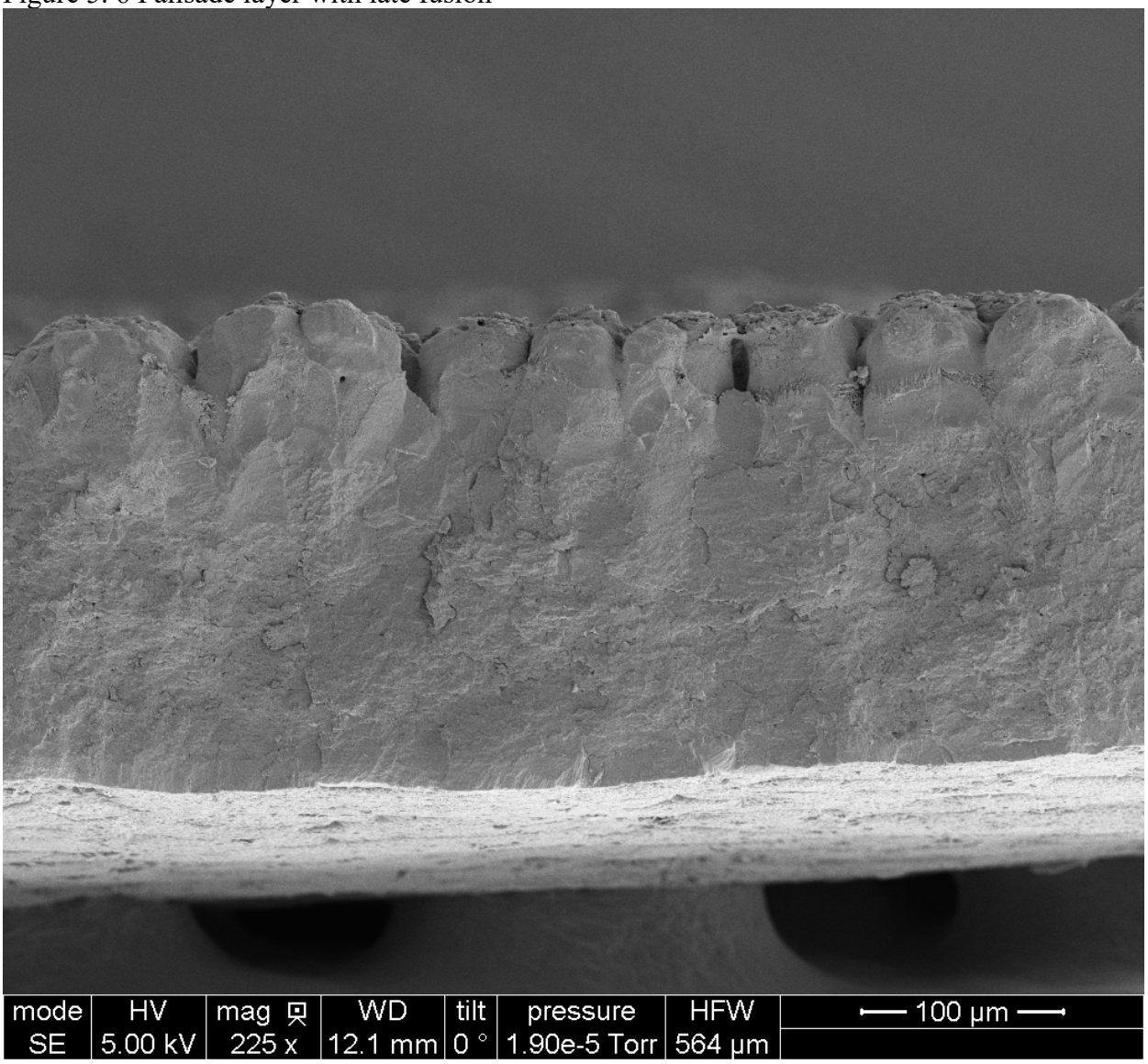
Opposite of early fusion, late fusion occurs later during eggshell formation, resulting in a thinner palisade layer, and a microstructurally weaker egg Solomon and Bain (1996). Figure 5.12 is a SEM image of late fusion.

Figure 5. 5 Late fusion of the mammillary layer



This egg was from collection date 10, 65 weeks of age, and the was receiving diet 3, 30ppm ZnO. The breaking strength of this egg was 2.046kgf. This portion of the shell was microstructurally weak, which is in agreement with the breaking strength recorded for this egg. Additionally, late fusion can be seen in the palisade layer. The overall thickness of the palisade, before mammillary cap begins, decreases overall thickness. This can be seen in figure 5.13.

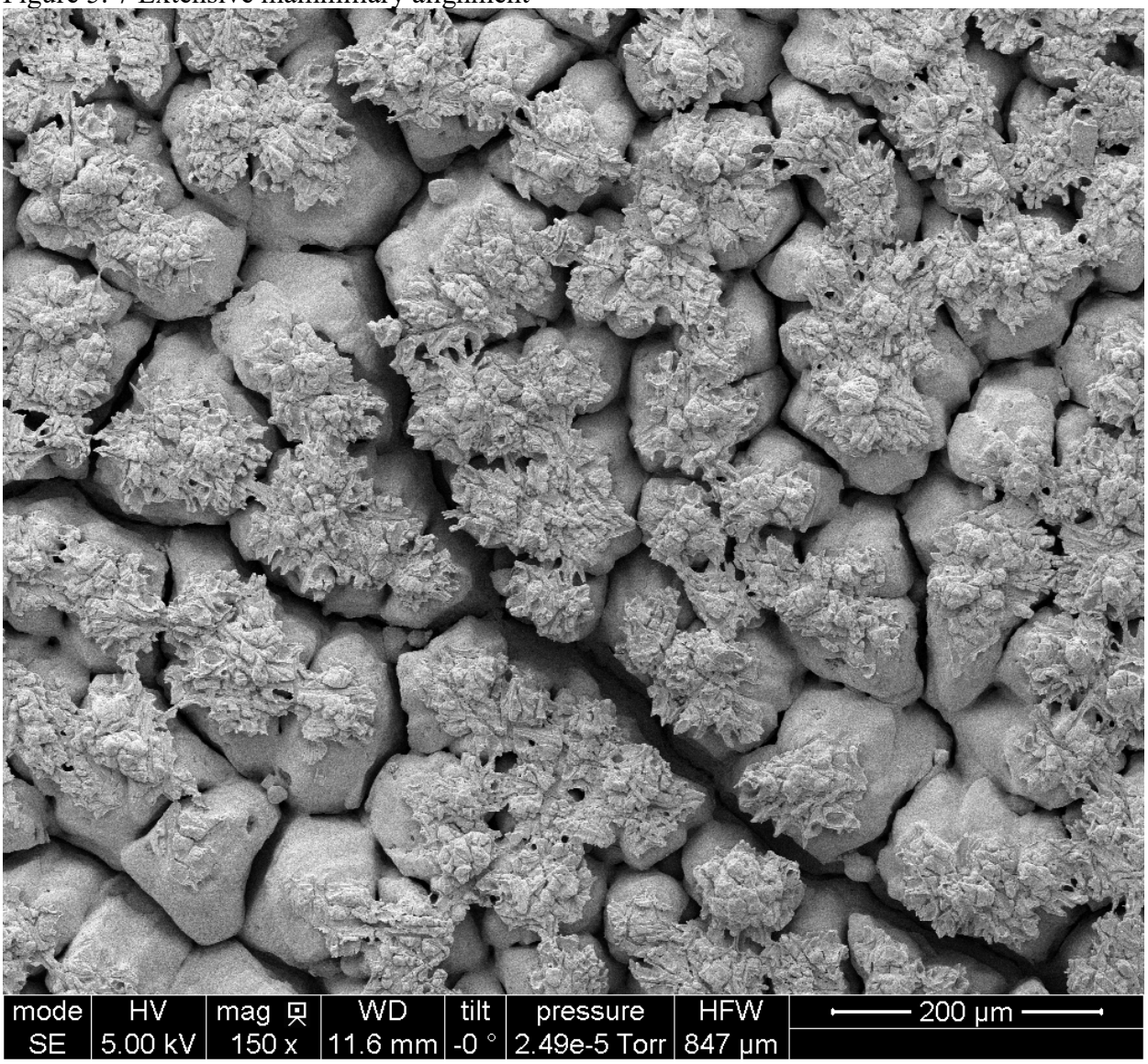
Figure 5. 6 Palisade layer with late fusion



Mammillary Alignment

In a microstructurally strong egg, mammillary bodies should be randomly distributed over the eggshell, in order to stop crack propagation that can occur between mammillary bodies Solomon and Bain (1996). Figure 5.14 demonstrates an eggshell that would be given a score of 7, for extensive mammillary alignment.

Figure 5. 7 Extensive mammillary alignment

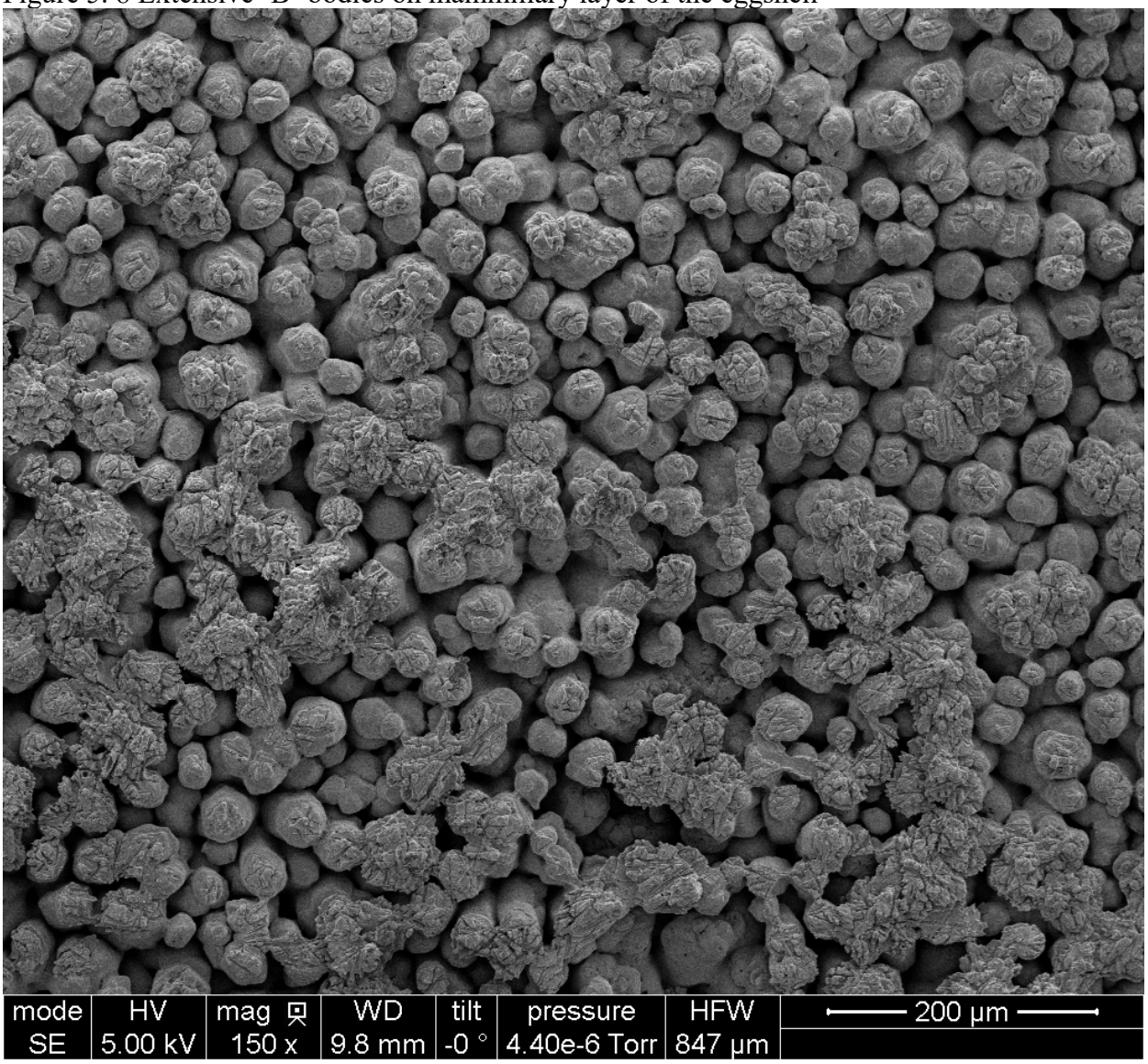


If an outside force were to hit the eggshell at this point on the egg, a crack could occur between the mammillary bodies, along the fault line seen above. This egg was taken from a hen receiving the control diet, diet 1, at 45 week of age, collection date 5, and had a breaking strength of 3.689kgf. Although this egg was considered strong with a breaking strength above 3.000kgf, this was a weak spot in the egg.

Type 'B' bodies

B bodies are round bodies that make minimal contact with membrane fibers, they make no contribution to the "true" thickness of the eggshell (Solomon, 1997). Figure 5.15 demonstrates extensive 'B' bodies.

Figure 5. 8 Extensive ‘B’ bodies on mammillary layer of the eggshell

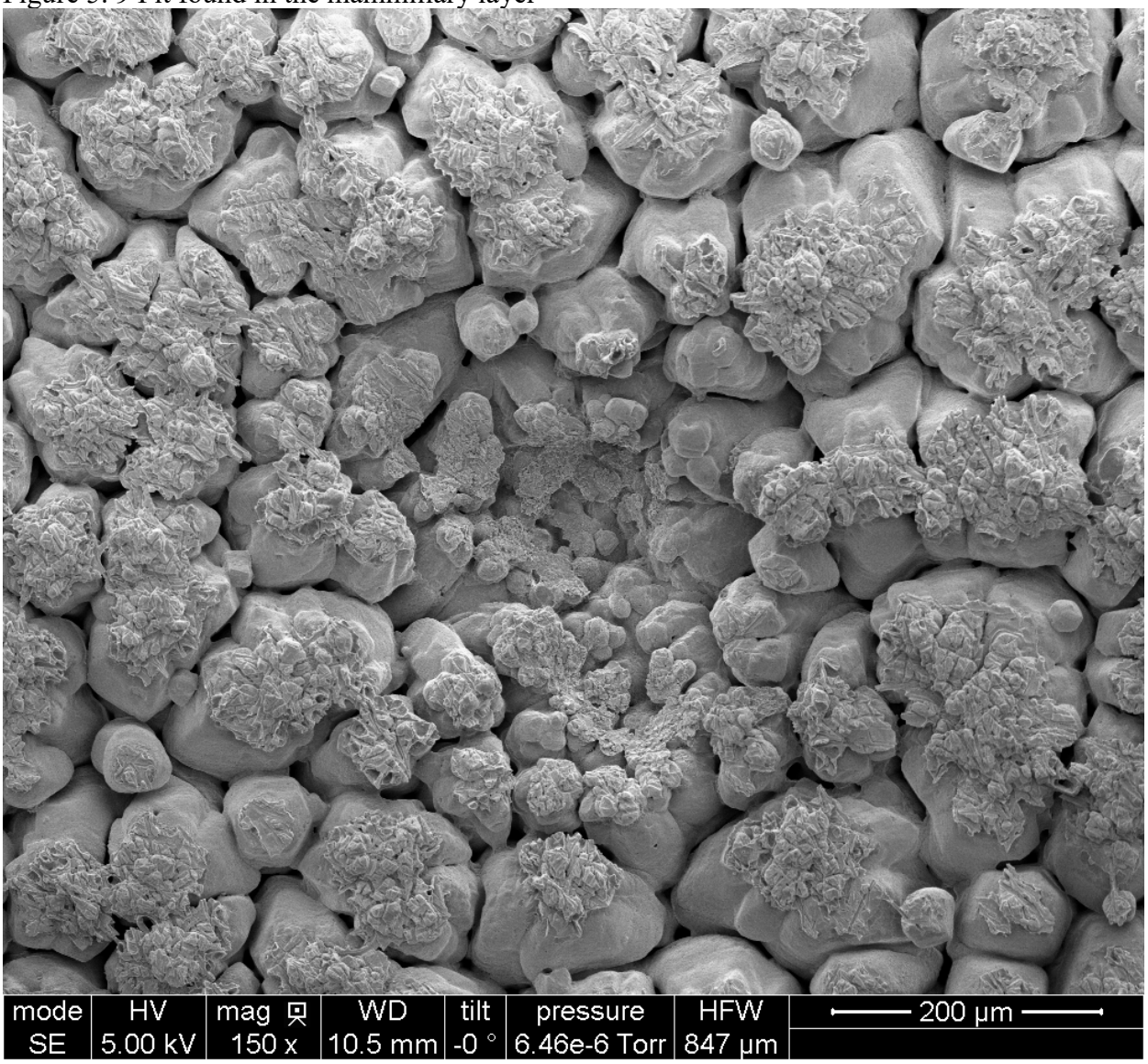


With extensive ‘B’ bodies distributed on the mammillary layer, palisade thickness is minimal, because the ‘B’ bodies are unable to contribute to overall shell thickness. This image was taken from an egg at the end of the experiment, date 10, from a hen receiving the control diet, diet 1, and had a breaking strength of, 2.637kgf.

Pitted

Occasionally, pits of cavities can be seen in the mammillary layer of the eggshell. These pits can obstruct the entire depth of the shell, often disrupting nucleation sites (Solomon, 1997). Figure 5.16 demonstrates a pit found in the mammillary layer.

Figure 5. 9 Pit found in the mammillary layer



This image was taken from an egg at 65 weeks of age, collection date 10, from a hen receiving 30ppm Bioplex® Zn. Additionally, this egg had a breaking strength of 1.987kgf.

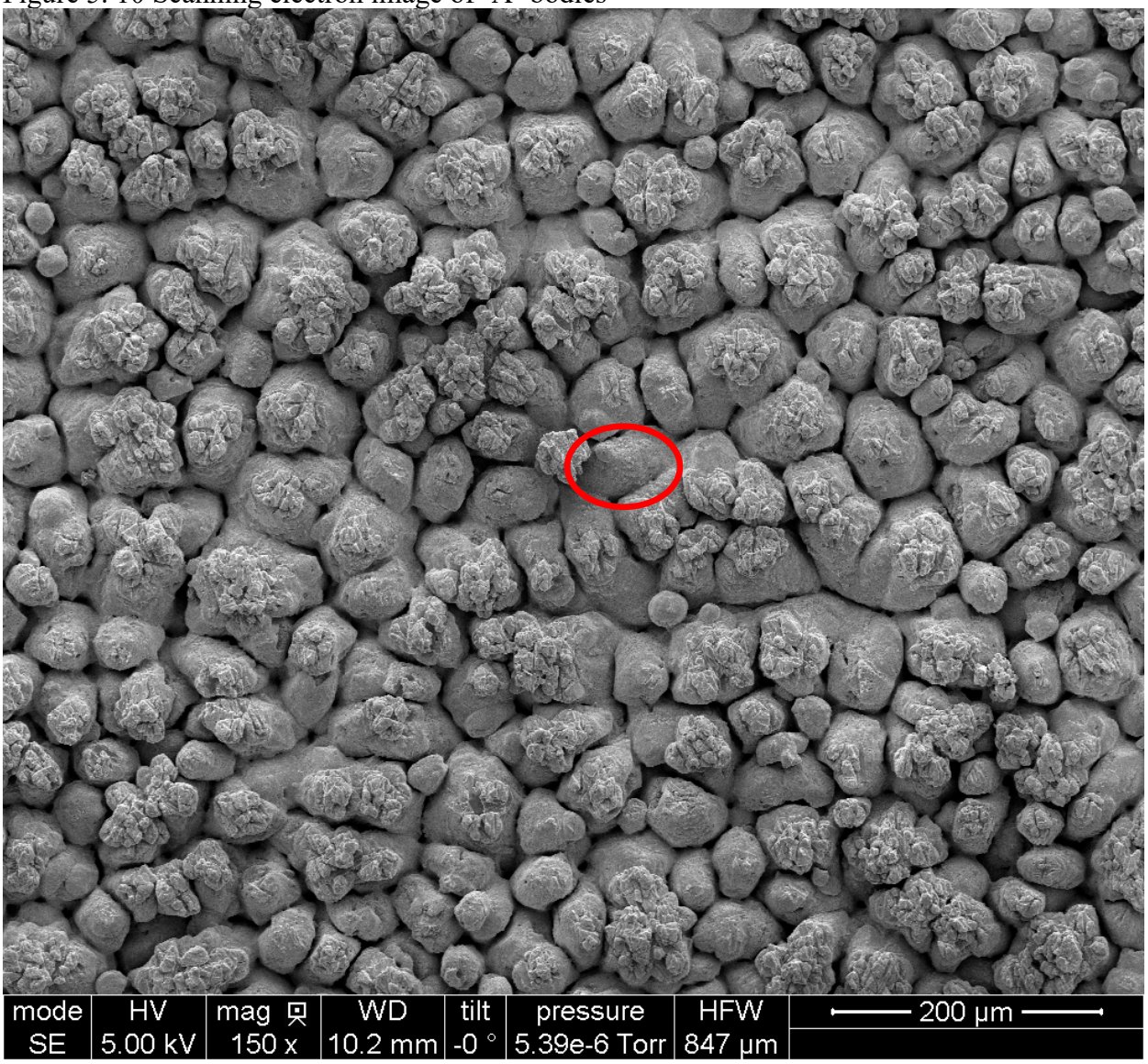
Aragonite

A "normal" shape is primarily composed of calcium carbonate, which is one of three polymorphic variations of calcite (Solomon, 1997). Throughout this experiment, aragonite formations were not seen.

Type 'A' bodies

Type 'A' bodies occur less frequently compared to type 'B' bodies and have similar qualities of 'B' bodies. Similarly, to 'B' bodies, their contact with membrane fibers is minimal, however, 'A' bodies do contribute palisade layer, but rarely contribute to "true" shell thickness (Solomon, 1997). Figure 5.17 is an image of 'A' bodies.

Figure 5. 10 Scanning electron image of ‘A’ bodies



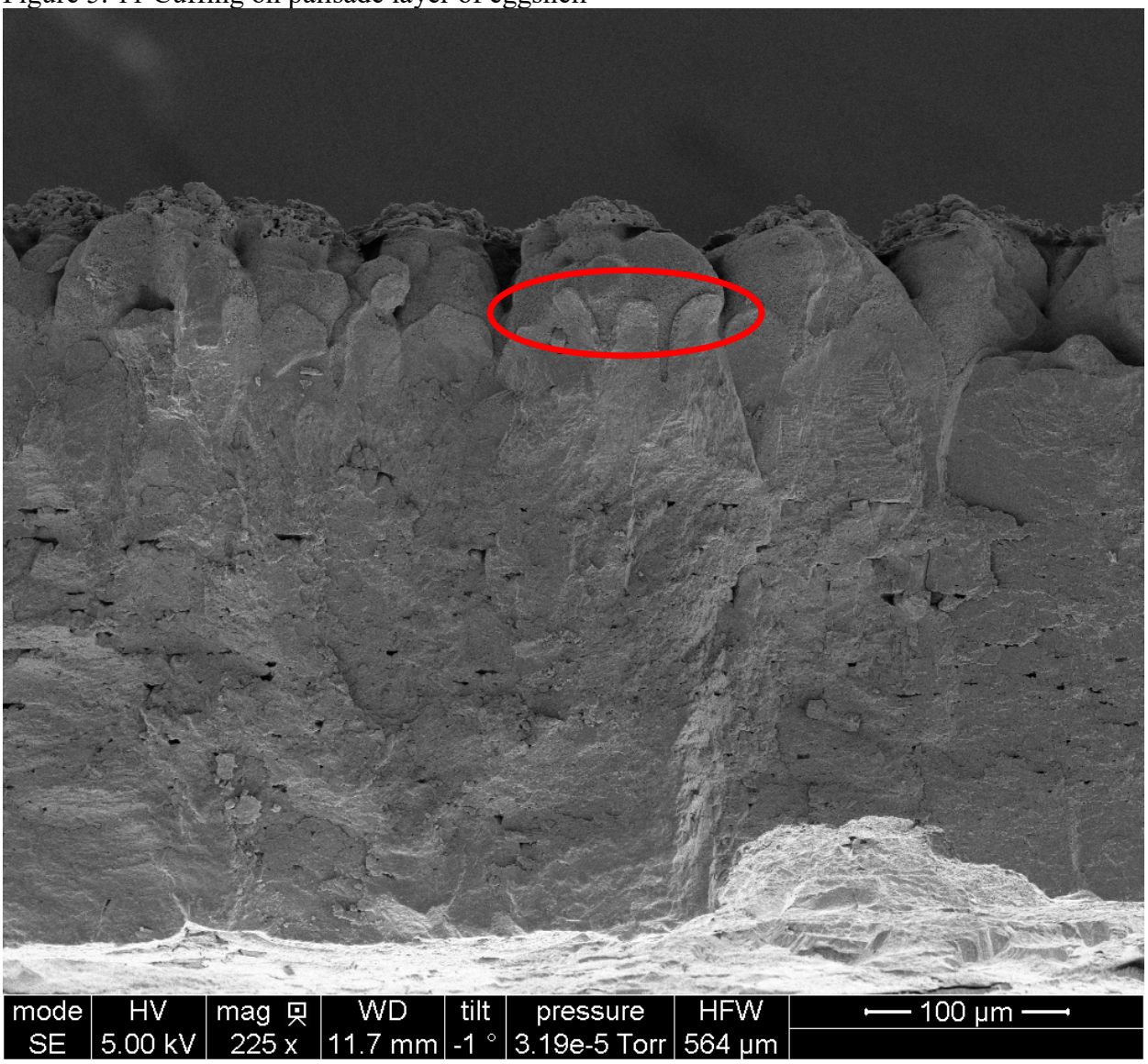
Cubics

Cubic formation occurs when birds experience stress and occupy spaces between mammillary caps (Solomon, 1997). However, cubic formations were not seen throughout this experiment.

Cuffing

Cuffing occurs where the palisade layer and cone meet and assist in the early fusion of palisade columns, increasing strength of the palisade by distributing any outside stress among the shell, decreasing the likelihood of the eggshell breaking (Solomon, 1997). Figure 5.18 demonstrates cuffing.

Figure 5. 11 Cuffing on palisade layer of eggshell



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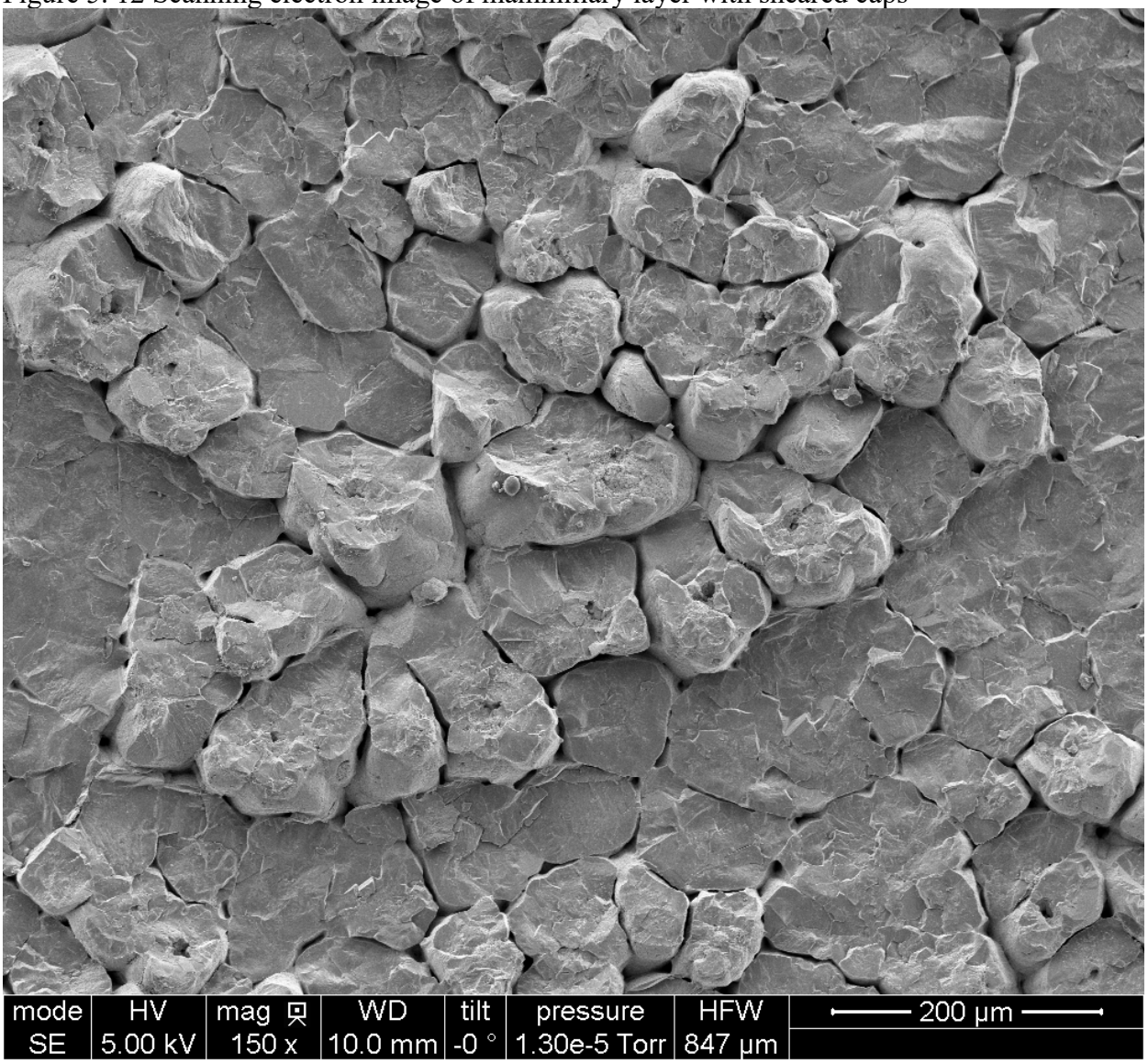
This egg was from a hen receiving diet 4, 80ppm Bioplex®Zn, at date 5, 45 weeks of age. The breaking strength of this egg was 5.616kgf.

Changed Membrane

In the presence of a sulphur-rich environment, weakness between the mammillary and palisade layers is created, causing the caps to readily shear (Solomon, 1997). Figure 5.19 shows a mammillary layer with sheared caps.

Figure 5. 12 Scanning electron image of mammillary layer with sheared caps



This egg was taken from a hen at 65 weeks of age, date 10, receiving diet 3, 30ppm ZnO. This egg had a breaking strength of 4.266kgf, indicating that this was a weak spot in this otherwise strong egg.

5.5 Conclusion

From this data, it can be concluded that Bioplex® zinc doesn't have a consistent effect on eggshell quality. Eggs weights were significantly higher at the end of the trial compared to the start of the trial for both brown and white eggs, regardless of diet. Breaking strengths for white and brown eggshells increased from the start of the trial to the end of the trial for all diets, but, significant differences were not detected between dietary treatments. Additionally, haugh units decreased throughout the 36 week trial for both white and brown eggs, but eggs were still considered AA quality by the end of the experiment. Percent shell was unaffected by diet over time, except for diet 4. This indicated that Bioplex® zinc 80ppm, did have an effect on the percent shell, compared to the other diets. Percent yolk of white eggs was significantly higher for all diets in period 2, compared to period 1, while inconsistent results were seen concerning percent yolk in brown eggs. Shape index was calculated to determine if it could be used as a nondestructive test to predict eggshell quality. From this data, it can be concluded that shape index does not look promising as an indicator of eggshell quality, especially for white and brown eggs. This is likely due to intense genetic selection of these breeds.

Scanning electron images were taken at two different points of the trial, but consistent results were not seen from these images. Although common negative eggshell defects were seen, eggs with higher breaking strengths displayed some of these negative defects. This can be attributed to eggs being broken along the x-axis, where it is known to

be stronger, while eggshell samples to imaged taken from the equator. To better understand the true microstructure of the egg, samples to be imaged should be taken from where the eggshell breaks during quasistatic compression.

CHAPTER 6. KEEL BONE DEFORMITIES IN LAYING HENS FED DIFFERENT ZINC SOURCES

6.1 Introduction

Bone health and egg production go hand in hand. Issues with bone and egg production often arise from deficiencies, imbalance or malabsorption of calcium, phosphorus, and/or Vitamin D_3 (Hy-Line, 2013b). The skeleton of the laying hen is strongly influenced by the level of egg production, diet formulation in relation to feed consumption and disease status. The incidence of broken and weak bones is an increasing problem as hens progress through the lay cycle in the table egg industry (Riczu et al., 2004). The effect of bone fractures on bird welfare is unknown, however bone fractures cause pain and is likely to cause the same effect in birds (Nasr et al., 2012). Pain caused by keel bone fractures may have an effect on egg production and eggshell quality (Nasr et al., 2012). The aim of this study was to determine if diet had an effect on keel bone deformation, and ultimately affecting egg production, as hens progressed throughout the laying cycle.

6.2 Materials and Methods

Two hundred and forty commercial white egg layers and 240 commercial brown egg layers were used during this study. All procedures were conducted under protocols approved by Institutional Animal Care and Use Committee (IACUC). Birds were places on one of five treatments, with 8 replications per diet, for each color of bird. Birds were place on their experimental diet at 29 weeks of age and were remained on the experimental diet for 36 weeks of lay (65 weeks of age). Hens were housed in conventional cages, with 2 birds per pen and supplied *ad* libitum access to feed and

water. Birds were weighed every 4 weeks throughout the experiment and keel bones were scored. The ingredient composition for each treatment can be seen in Table 6.1 and component composition for each treatment in Table 6.2. Premix composition for each diet can be found in the appendix.

Table 6. 1 Ingredient composition of diets

Ingredient (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Corn	56.00	56.00	56.00	56.00	56.00
SBM, dehulled (48% cp)	28.00	28.00	28.00	28.00	28.00
Soy oil	3.75	3.75	3.75	3.75	3.75
Dical (23-18)	1.00	1.00	1.00	1.00	1.00
Limestone	7.20	7.20	7.20	7.20	7.20
Oyster shell	3.00	3.00	3.00	3.00	3.00
Salt, iodized	0.38	0.38	0.38	0.38	0.38
DL-methionine	0.17	0.17	0.17	0.17	0.17
Vitamin premix (No Mineral)	0.25	0.25	0.25	0.25	0.25
Mineral premix 1 (No Zn)	0.25	-	-	-	-
Mineral premix 2 (80 ppm Zn as ZnO)	-	0.25	-	-	-
Mineral premix 3 (30 ppm Zn as ZnO)	-	-	0.25	-	-
Mineral premix 4 (80 ppm Zn as Bioplex)	-	-	-	0.25	-
Mineral premix 5 (30 ppm Zn as Bioplex)	-	-	-	--	0.25
Total	100	100	100	100	100

Table 6. 2 Analyzed diet composition

Component (%)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Crude Protein	16.30	17.72	17.43	17.82	17.05
Crude Fat	5.13	5.47	5.52	5.47	5.46
Crude Fiber	4.67	3.61	2.79	3.09	2.51
Calcium	4.13	5.12	4.73	5.30	5.12
Phosphorus	0.57	0.61	0.52	0.49	0.52
Zinc (ppm)	26.7	107.0	55.9	105.0	54.3

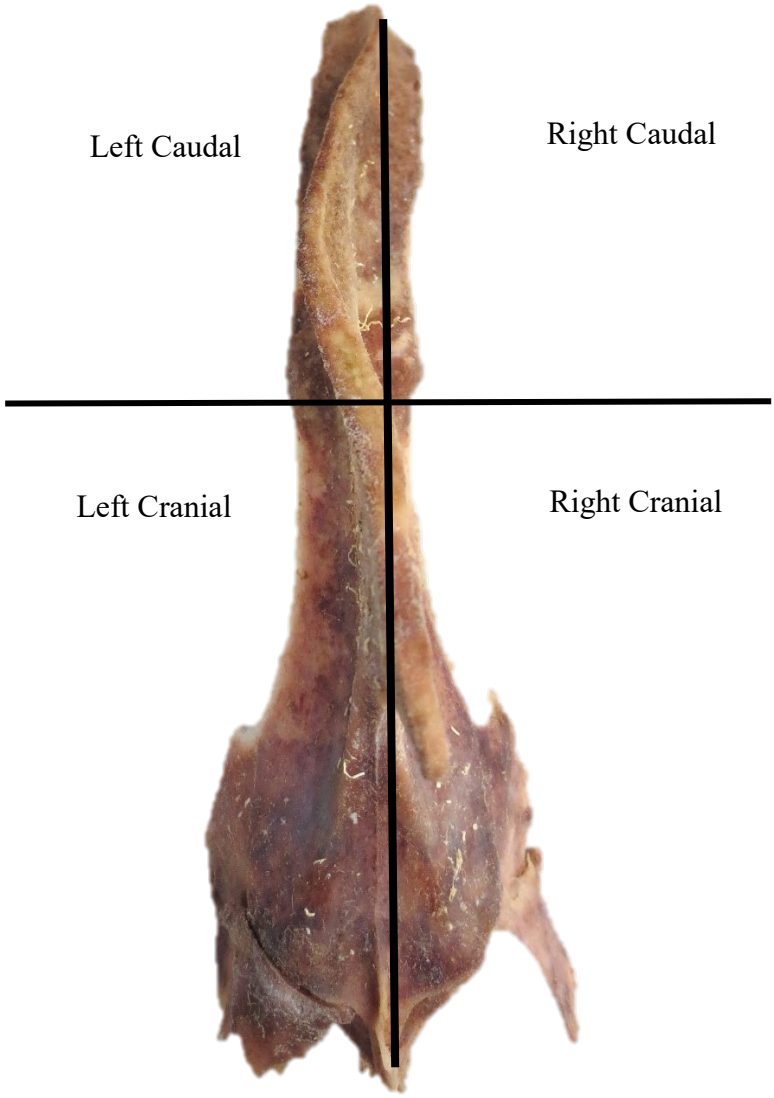
Keel bones were scored every 4 weeks on the live bird, with the first scores being taken 2 weeks after the start of the experiment and the final scores taken two weeks after the last weigh date. Scores were taken on four of the eight replications and one bird was picked at random and scored every 4 weeks. Three different scores were recorded by three different scorers on each date. The same three people scored throughout the 36 week trial. Palpation of the keel bone was used for scoring was based off of scoring methods outlined by Casey-Trott et al. (2015). Two weeks after the last weigh date, keels were scored one final time and all birds from the pens randomly selected for SEM imaging of eggshells were harvest for keel bones. All keel bones shown in table 6.3 were taken for analysis.

Table 6. 3 Pens used for Keel Bone Analysis

	Diet	Pen Number
White Egg Layers	1	14
		29
	2	5
		50
	3	2
		35
	4	30
		33
	5	18
		23
	Diet	Pen Number
Brown Egg Layers	1	24
		34
	2	7
		40
	3	15
		22
	4	10
		36
	5	8
		31

Harvest keel bones were cleaned using *Dermestidae* beetles**.** Bones were places in the beetles for 36-48hrs until cleaned of all meat and cartilage. Digital images were taken of each keel and divided into four quadrants and deviations from a straight line were recorded. Quadrants deviations measured can be seen in figure 6.1.

Figure 6. 1 Keel Bone Quadrants for Deviation Measurements



Deviations that occurred in either the left cranial *and* right caudal or right cranial *and* left caudal were considered an ‘S’ shape. Deviations that occurred on one side either left cranial *and* left caudal or right cranial *and* right caudal or just one or the other; were considered a ‘U’ shape. These shapes can be seen in figure 6.2 and 6.3.

Figure 6. 2 Keel Bone Designated as ‘S’ shape

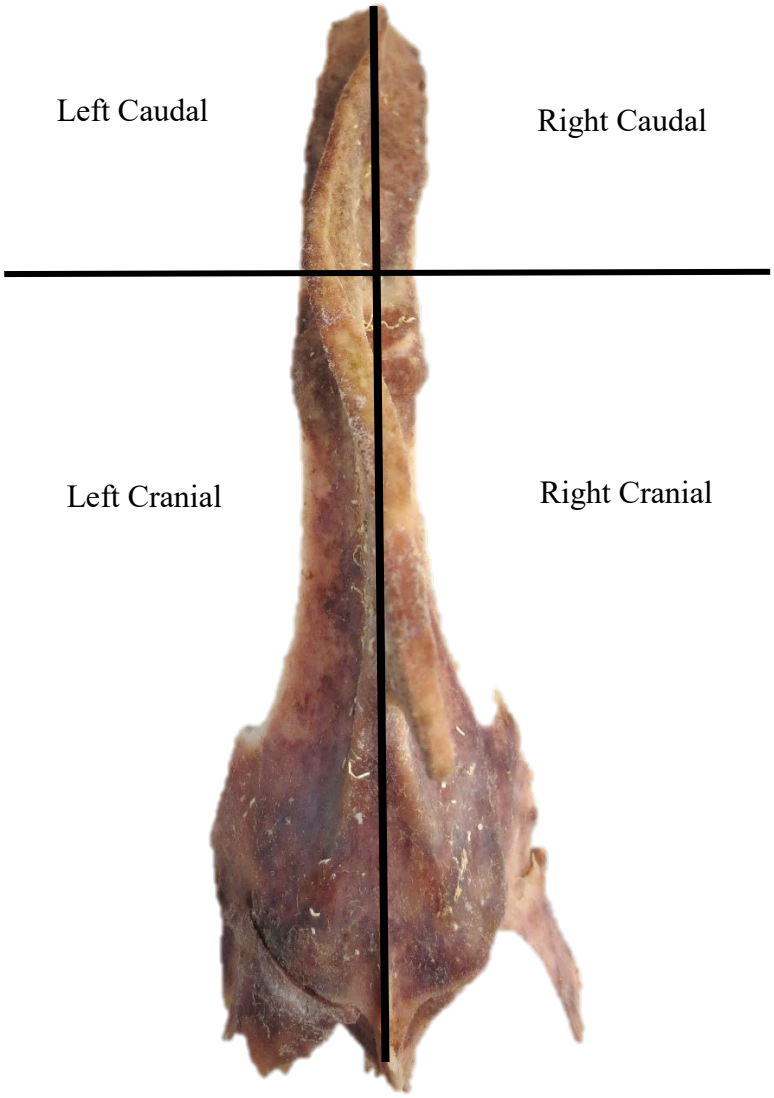
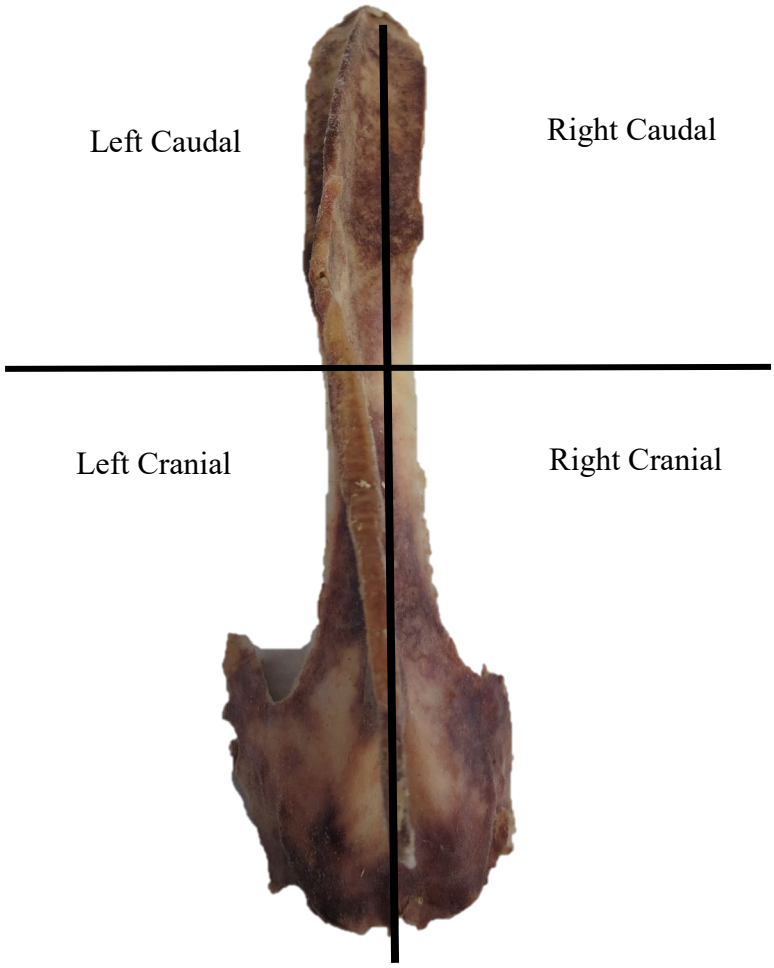


Figure 6. 3 Keel Bone designated as ‘U’ shape.



6.3 Statistical Analysis

Keel bone scores were analyzed using PROC FREQ function in SAS 9.4. Deviation of keel bones were analyzed using PROC GLM. Significant differences were detected at $P \leq 0.05$.

6.4 Results and Discussion

Keel bone scores increased from a score of 1 (straight keel) to a score of 3 (‘S’ shape curve), as laying hens (white and brown) progressed through production. This can be seen in table 6.4 and 6.5.

Table 6. 4 Keel bone scores of live white layers over 36 week period (29 to 65 weeks of age)

	Score		
Date	1	2	3
1	38	22	0
2	13	44	3
3	11	47	2
4	15	50	5
5	14	45	1
6	13	45	2
7	19	28	13
8	16	36	8
9	10	39	11
10	5	33	22

Date : 1= 3/12; 2= 4/9; 3=5/7; 4=6/4; 5=7/2; 6=7/30; 7=8/27; 8=9/24; 9=10/22; 10=11/19

Table 6. 5 Keel bone scores of live brown layers over 36 week period (29 to 36 weeks of age).

	Score		
Date	1	2	3
1	16	42	2
2	20	39	1
3	6	51	3
4	3	57	0
5	14	46	0
6	9	47	4
7	15	39	6
8	5	42	13
9	10	39	11
10	4	40	16

Date : 1= 3/12; 2= 4/9; 3=5/7; 4=6/4; 5=7/2; 6=7/30; 7=8/27; 8=9/24; 9=10/22; 10=11/19

From this data set, it was determined that at the start of the experiment (29 weeks of age) 63% of white egg layers, were given a score of 1 during palpitation, 37% with a score of 2, and 0% of the birds given a score of 3. By the end of the 36 week trial (65 weeks of age) 8% of the white egg layers, were given a score of 1, 55% a score of 2, and 37% of birds given a score of 3. This data agrees with data found by Richards et al. (2012), who sampled 2 different flocks of birds at 25 weeks of age, with 94% and 95% of birds with a score of 0 *(which would be a 1 from our scoring system)*, 6% and 4% of birds given a score of 1 *(related to our 2)*, and 0% and 1% given a 2 *(related to our score of 3)*. When palpating those same birds at 65 weeks of age, 28% and 36% of birds were given a score of 0, 46% and 38% a score of 1, and 26% and 26% of a score of 2 (Richards et al., 2012). Additionally, fewer brown egg layers started with a keel bone score of 1, 27%, and a greater percent started the trial with a keel bone score of 2, 70%. Unlike the white egg layers, 3% of the birds started the trial with a keel bone score of 3. By the end of the 36 week trial, only 6% of brown egg layers had a keel bone score of 1, and 27% of the birds ended the trial with a keel bone score of 3%. About the same amount of birds had a score of 2 throughout the experiment, starting with 70% to 67% by the end.

Keel bone score should correlate with type of deviation. A score of 1 was given to a straight keel, with zero deviation from a straight line. A score of 2 would be anything with a deviation to one side, in one of the four quadrants, or forming a 'U' shape, deviations that occurred on one side either left cranial *and* left caudal or right cranial *and* right caudal or just one or the other. A score of 3 was an 'S' shape, where deviations that occurred in either the left cranial *and* right caudal or right cranial *and* left caudal. In commercial white laying hens, keel bones that formed a 'U' shape had significantly lower

total deviation of 22.55 mm compared to total deviation of keel bones forming an ‘S’ shape of 36.36 mm ($P \leq 0.05$). Similar results were found in the commercial brown laying hens, with total deviation of ‘U’ shape keels being 27.02 mm, compared to 42.40 mm ($P \leq 0.05$) of ‘S’ shape keel bones. From this data, it can also be concluded that commercial brown layers have a greater deviation from straight for both ‘U’ and ‘S’ shape deformations. These results can also be seen in table 6.6 and 6.7.

Table 6. 6 Deviation of keel bones for white laying hens (mm)

Type of Deviation	Deviation
'U' shape	22.55 ± 1.79^{b}
'S' shape	36.36 ± 2.89^{a}

Different letters within a row indicates differences between type of deformation; significance detected at $P \leq 0.05$

Table 6. 7 Deviation of keel bones for brown laying hens (mm)

Type of Deviation	Deviation
'U' shape	27.02 ± 2.14^{b}
'S' shape	42.40 ± 2.45^{a}

Different letters within a row indicates differences between type of deformation; significance detected at $P \leq 0.05$

This data agrees with data found by Fleiming et al. (2004) who found that “normal keels” decreased from “normal” to “twisted” as hens aged. At 15 weeks of age, 99.2% of hens had “normal” keels, to 93.7% normal, at 70 weeks of age. Additionally, total deviation for each diet were analyzed, and results can be seen in table 6.8 and 6.9.

Table 6. 8 Keel bone deviation (mm) of white laying hens by diet on date 10

Diet	Deviation on Date 10
1 (Control)	27.03 ± 3.40[a]
2 (80 ppm ZnO)	27.32 ± 3.42[a]
3 (30 ppm ZnO)	26.38 ± 3.23[a]
4 (80 ppm Bioplex® Zn)	24.04 ± 3.37[a]
5 (30 ppm Bioplex® Zn)	26.72 ± 3.15[a]

Different letters between rows indicate differences between diets; significance detected at $P \leq 0.05$.

Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.

From this data, it can be concluded that no significant differences were detected between diets concerning keel bone deviation. However, only a small number of keel bones were collected at the end of the experiment, and a larger data set could lead to significant differences. Additionally, this small data set shows numerical differences, with keel bones from birds on diet 4 had numerically lower deviations (24.04 mm) compared to the keel bone deviation from birds receiving other diets.

Table 6. 9 Keel deviation (mm) of brown laying hens by diet on date 10

Diet	Deviation on Date 10
1 (Control)	29.92 ± 3.55^{b}
2 (80 ppm ZnO)	29.40 ± 4.10^{b}
3 (30 ppm ZnO)	41.08 ± 3.75^{a}
4 (80 ppm Bioplex® Zn)	29.12 ± 3.88^{b}
5 (30 ppm Bioplex® Zn)	32.36 ± 3.45^{ab}

Different letters between rows indicate differences between diets; significance detected at $P \leq 0.05$.

Diet 1=commercial corn-soy diet with no additional Zn supplementation; 2=corn-soy diet 80 mg Zn/kg diet; 3=corn-soy diet 30mg Zn/kg diet; 4=corn-soy diet 80 mg Zn/kg as Bioplex® Zn, 5= corn-soy diet 30 mg Zn/kg as Bioplex® Zn.

In brown layers, keel bone deviations were significantly less in diet, 1, 2, and 4 compared to diet 3 (41.08 mm, $P \leq 0.05$). Similar to the commercial white layers, this was a small data set and a larger data set could lead to more significant differences. Keel bone deviation for inorganic Zn, ZnO at 30 ppm, may have been significantly higher due to antagonist absorption of minerals at this level of supplementation. Ao and Pearce (2013) describe the negative competition between minerals. Especially copper, manganese and zinc, for binding ligands and uptake sites in gut mucosa. This could explain the extreme deviation of keel bones from birds receiving diet 3, compared to the other diets.

6.5 Conclusions

From this data, as expected, a greater percentage of keel bones from white egg layers were scored as 1 at the start of the 36 week trial, 63%, compared to the end of the experiment, 8%; and 27% to 6% for brown egg layers. Additionally, 0% of the birds had a score of 3 at the start of the experiment, compared to 37% thirty-six weeks later, for the white egg layers; and 3% to 27% of a score of 3 from the start to the end of the trial. Keel bone scores of 2 increased from 37% to 55% for white egg layers throughout the 36 weeks of this trial; brown egg layers stayed pretty consistent throughout the trial, starting at 70% and ending with 67% birds having a keel bone score of 2. For both white and brown egg layers, the 'U' shape deviation had significantly less deviation from a straight line, with 22.55 mm and 27.02 mm respectively, compared to the 'S' shape deviation for white and brown layers, 42.40 mm and 36.36 mm respectively. Diet had different effect on keel bone deviation, depending on the egg layer. Diet did not have a significant effect on keel bone deviation for white egg layers. However, there was a numerical trend, with keel bones from hens being fed diet 4, as 80 ppm Bioplex® Zn, having a numerically

lower deviation of 24.04 mm, compared to the deviation of keel bones from hens on the four diets. On the other hand, keel bones from brown egg layers were significantly affected by the diet they were receiving. Keel bones from hens on diet 3 had significantly higher deviation, 41.08 mm, compared to diets 1, 2, and 4. Keel bones from hens receiving diet 5, had intermediate keel bone deviation of 32.36 mm. This data looks promising for Bioplex® Zn, at 80 ppm, especially brown egg layers. However, more research in this area is needed to determine the amount of Bioplex® Zn required to alleviate issues with bone health, especially keel bone damage.

CHAPTER 7. OVERALL IMPLICATIONS

The layer industry is facing a major shift in housing, so understanding the factors that affect eggshell quality are vital, as the industry progresses through this shift. A series of experiments evaluated shell quality. Two studies were conducted to create extreme differences in eggshell quality, at two different stages of lay. In chapter 3, eggshell quality of eggs from hens at the end stages of their laying cycle, 88 weeks of age, were examined. From this data, it was determined that eggshell breaking strength and percent shell showed consistent results when determining eggshell strength. However, specific gravity was not precise enough to determine differences in eggshell quality. Additionally, when looking at eggshell microstructure, using the scanning electron microscope, total number of 'normal' to 'b' bodies, as a ratio, could be indicative to eggshell quality, especially with hens at the end of their lay cycle. The same diets were used on hens at the start of their lay cycle and similar results were seen concerning eggshell breaking strength and eggshell percent, as a great indicator of eggshell strength and integrity. Additionally, scanning electron microscopy, for eggs from hens during peak lay, showed promise for indicating eggshell integrity. From chapter 4 it was determined that eggshells with higher breaking strengths and greater percent shell had thicker palisade layers, and narrower mammillary caps. On the other hand, eggshells requiring less force to break and a lower percent shell had thinner palisade layers and wider mammillary caps. From this data, it was concluded that scanning electron microscopy, especially palisade thickness can indicate eggshell integrity, along with eggshell breaking strength and eggshell percent.

Organic and inorganic zinc sources, Bioplex® and zinc oxide respectively, at levels of 30 ppm and 80 ppm, were provided to white and brown laying hens for 36 weeks, from 29 to 65 weeks of age. Throughout this study, eggs were collected every 4 weeks to determine eggshell quality and keel bones were assessed for keel bone deformation. From this data it was concluded that egg production was unaffected by diet throughout the 36 weeks of the trial. Egg weights were significantly higher by the end of the trial, for both white and brown egg layers, regardless of treatment. Eggshell breaking strength was significantly higher at the end of the trial for white eggs and brown eggs on diets 1 (control), 2 (80 ppm ZnO), and 3 (30 ppm ZnO). Percent shell was unaffected by period or diet for white shelled eggs, while Bioplex® zinc at 80 ppm had significantly lower percent shell in period 2 compared to period 1. No significant differences were detected for shape index, indicating that this measurement is not a good indicator of shell strength and overall shell integrity. However, in a previous study conducted by Nolan (2017) looking at shape index in heritage breed eggs, significant differences were detected between heritage breeds. This indicates that shape index shows promise in indicating differences in eggshell integrity, especially in laying hens that have not been through intense genetic selection. Concerning scanning electron microscopy, microstructural differences were detected on eggshells, but consistent results between diets were not seen. Minimal work has been done with scanning electron microscopy and eggshell integrity, however work that has been done, has taken samples for microscopy along the equator of the egg. If conventional methods of eggshell quality, especially breaking strength, are being taken, samples for scanning electron microscopy should be taken along the same axis as breaking strength.

From this research, it can be concluded that eggshell breaking strength and integrity is a very complicated matter to discuss. Breaking strength still proves to be the most effective in determining eggshell strength. Unfortunately, breaking strength is a destructive method, losing the egg in the process of determining eggshell strength. Scanning electron microscopy, also a destructive measure, was successful in detecting structural differences between eggshells, however due to sampling location, breaking strength and eggshell imaging results did not correlate. Although scanning electron microscopy was able to detect structural differences on the eggshell, results were inconsistent compared to standard eggshell measurements. In previous research, sampling for scanning electron microscopy was taken along the equator. Samples for these studies was also collected along the equator of the egg. However, breaking strength was taken along the long axis of the egg, which could explain inconsistent results seen between breaking strength and scanning electron imaging. Future research looking at breaking strength and imaging should take breaking strength measurements and sample for microscopy along the same axis. Unfortunately shape index, a non-destructive test, showed unfavorable results due to intense genetic selection of the two strains used in this research. The white and brown strains used during these studies may have reduced the natural variation that would typically be seen within bird strains. Although differences were not seen in this experiment, scanning electron microscopy and breaking strength show promise in determining eggshell integrity, so more research in this area is needed.

APPENDICES

Table 1. Mammillary body count of eggs from hens receiving sufficient Ca

	Hen Number											
	1		2		3		4		5		6	
Date	Normal	b	Normal	b	Normal	b	Normal	b	Normal	b	Normal	b
1	129.8	4.5	.	.	94.67	1.0	51.0	1.0	39.0	2.7	.	.
20	82.5	6.8	.	.	107	1.3	130.0	18.0	49.0	14.0	.	.

Table 2. Mammillary body count of eggs from hens receiving reduced Ca

	Hen Number											
	7		8		9		10		11		12	
Date	Normal	b	Normal	b	Normal	b	Normal	b	Normal	b	Normal	b
1	50.0	7.7	.	.	46.3	2	55.0	5.0	88.8	4.3	82.5	6.75
20	111.1	4.8	.	.	58.9	2.67	88.5	4.3	79.4	9.7	.	.

Table 3. Mammillary width and palisade layer thickness for each egg of one hen on each experimental diet for 20 days

	Hen 4		Hen 10	
Date	Mammillary Cap Width (µm)	Palisade Layer Thickness (µm)	Mammillary Cap Width (µm)	Palisade Layer Thickness (µm)
3/8	-	-	117.70	209.08
3/9	109.06	232.98	98.58	142.08
3/10	84.51	236.98	-	-
3/11	86.96	228.76	-	-
3/12	102.50	226.07	102.00	143.20
3/13	90.56	223.58	153.90	135.63
3/14	94.90	233.53	84.1	142.52
3/15	75.67	197.49	-	-
3/16	66.36	235.00	70.09	113.58
3/17	87.82	241.72	-	-
3/18	98.41	237.93	76.49	152.90
3/19	78.94	218.38	-	-
3/20	76.39	244.48	113.83	168.35
3/21	121.81	254.98	-	-
3/22	-	-	-	-
3/23	107.08	221.08	81.80	170.20
3/24	86.30	220.02	-	-
3/25	-	-	77.75	154.66
3/26	109.76	173.13	118.54	158.86
3/27	79.94	229.60	-	-
3/28	75.58	228.05	85.75	164.18

‘-‘ means no egg was produced by that hen that day

Table 4. Premix composition in diet 1

Premix 1. Inorganic mineral premix (no Zn)			
Mineral	Form	Element Content, %	Minerals for 1 kg premix (g)
Se	Na-Selenite	45.4	0.176
Copper	CuSO4.5H2O	25	16
Iodine	KIO_3	59.31	2.02
Iron	$FeSO_4$-H_2O	30	106.67
Manganese	MnSO4-H2O	29.5	108.47
Total Mins			233.34
Carrier	Limestone		766.66
Total			1000

Table 5. Premix composition in diet 2

Premix 2. Inorganic mineral premix (80 ppm Zn)			
Mineral	Form	Element Content, %	Minerals for 1 kg premix (g)
Se	Na-Selenite	45.4	0.176
Copper	CuSO4.5H2O	25	16
Iodine	KIO_3	59.31	2.02
Iron	$FeSO_4$-H_2O	30	106.67
Manganese	MnSO4-H2O	29.5	108.47
Zinc	Zn oxide	72	44.44
Total Mins			277.61
Carrier	Limestone		722.39
Total			1000

Table 6. Premix composition in diet 3

Premix 3. Low level ZnO mineral premix (30 ppm Zn)			
Mineral	Form	Element Content, %	Minerals for 1 kg premix (g)
Se	Na-Selenite	45.4	0.176
Copper	CuSO4.5H2O	25	16
Iodine	KIO_3	59.31	2.02
Iron	$FeSO_4$-H_2O	30	106.67
Manganese	MnSO4-H2O	29.5	108.47
Zinc	Zn oxide	72	16.67
Total Mins			249.83
Carrier	Limestone		750.17
Total			1000

Table 7. Premix composition in diet 4

Premix 4. High level Bioplex Zn premix (80 ppm)			
Mineral	Form	Element Content, %	Minerals for 1 kg premix (g)
Se	Na-Selenite	45.4	0.176
Copper	CuSO4.5H2O	25	16
Iodine	KIO_3	59.31	2.02
Iron	$FeSO_4$-H_2O	30	106.67
Manganese	MnSO4-H2O	29.5	108.47
Zinc	Bioplex Zn	10	320.00
Total Mins			553.16
Carrier	Limestone		446.84
Total			1000

Table 8. Premix composition in diet 5

Premix 5. Low level Bioplex Zn premix (30 ppm)			
Mineral	Form	Element Content, %	Minerals for 1 kg premix (g)
Se	Na-Selenite	45.4	0.176
Copper	CuSO4.5H2O	25	16
Iodine	KIO_3	59.31	2.02
Iron	$FeSO_4$-H_2O	30	106.67
Manganese	MnSO4-H2O	29.5	108.47
Zinc	Bioplex Zn	10	120.00
Total Mins			353.16
Carrier	Limestone		646.84
Total			1000

REFERENCES

Alltech. 2018. Bioplex. Accessed June 25 2018.

Altuntaş, E., and A. Şekeroğlu. 2008. Effect of egg shape index on mechanical properties of chicken eggs. Journal of Food Engineering 85: 606-612.

Anderson, K., J. Tharrington, P. Curtis, and F. Jones. 2004. Shell characteristics of eggs from historic strains of single comb white leghorn chickens and the relationship of egg shape to shell strength. Int. J. Poult. Sci 3: 17-19.

An, S., D. Kim, and B. An. 2016. Effects of dietary calcium levels on productive performance, eggshell quality and overall calcium status in aged laying hens. Asian-Australasian journal of animal sciences 29: 1477.

Athanasiadou, D. et al. 2018. Nanostructure, osteopontin, and mechanical properties of calcitic avian eggshell. Science Advances 4.

Ao, T., and J. Pierce. "The replacement of inorganic mineral salts with mineral proteinates in poultry diets." *World's Poultry Science Journal* 69.1 (2013): 5-16.

Bain, M. 2005. Recent advances in the assessment of eggshell quality and their future application. World's poultry science Journal 61: 268-277.

Bar, A. 2009. Calcium transport in strongly calcifying laying birds: Mechanisms and regulation. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 152: 447-469.

Belyavin, C., and K. Boorman. 1980. The influence of the cuticle on egg-shell strength. British Poultry Science 21: 295-298.

Casey-Trott, T. 2016. Opportunities for Exercise during Pullet Rearing: Effects on Bone Health and Keel Bone Damage in Laying Hens. Dissertation, The University of Guelph, Ontario, Canada.

Castillo, C., M. Cuca, A. Pro, M. González, and E. Morales. 2004. Biological and economic optimum level of calcium in white Leghorn laying hens. Poultry science 83: 868-872.

Cutts, J. A., G. C. Wilson, and S. Fernández. 2007. Optimum egg quality: a practical approach. 5M Publishing.

Eissa, A. H. A., and A. R. O. Alghannam. 2011. New trends for understanding stability of biological materials from engineering prospective Progress in Molecular and Environmental Bioengineering-From Analysis and Modeling to Technology Applications. InTech.

Fernandes, J. I. M., et al. "Effects of organic mineral dietary supplementation on production performance and egg quality of white layers." *Brazilian Journal of Poultry Science* 10.1 (2008): 59-65.

Fleming, R., H. McCormack, L. McTeir, and C. Whitehead. 1998. Medullary bone and humeral breaking strength in laying hens. Research in veterinary science 64: 63-67.

Guo Y.M., Yang R., Yuan J., Ward T.L., Fakler T.M. (2002): Effect of Availa Zn and ZnSO4 on laying hen performance and egg quality. Poultry Science, 81 (Suppl.), 40.

Gupta, L. 2008. How to Improve Shell Quality http://www.thepoultrysite.com/articles/1004/how-to-improve-shell-quality/ Accessed June 25 2018.

Hamilton, R. 1986. The microstructure of the hen's egg shell-a short review. Food Structure 5: 13.

Hincke, M. et al. 2012. The eggshell: structure, composition and mineralization.

Leeson, S., and L. Caston. 1997. A problem with characteristics of the thin albumen in laying hens. Poultry science 76: 1332-1336.

Hunton, P. 2005. Research on eggshell structure and quality: an historical overview. Revista Brasileira de Ciência Avícola 7: 67-71.

Hy-line. 2013a. The Sceince of Egg Quality. http://www.hyline.com/aspx/resourcelibrary/downloads.aspx Accessed October 6, 2017 2017.

Hy-Line. 2013b. Understanding the Role of the Skeleton in Egg Production. http://www.hyline.com/userdocs/pages/TU_SKELETON_ENG.pdf Accessed October 6, 2017.

Jacob, J. 2015. Raising Chickens for Egg Production. Small and Backyard Flocks https://articles.extension.org/pages/71004/raising-chickens-for-egg-production Accessed Date Accessed.| doi:DOI|

Jiang, S. et al. 2013. Effects of dietary energy and calcium levels on performance, egg shell quality and bone metabolism in hens. The Veterinary Journal 198: 252-258.

Mabe, I., C. Rapp, M. M. Bain, and Y. Nys. 2003. Supplementation of a corn-soybean meal diet with manganese, copper, and zinc from organic or inorganic sources improves eggshell quality in aged laying hens. Poultry Science 82: 1903-1913.

Macleod, N., M. M. Bain, and J. W. Hancock. 2006. The mechanics and mechanisms of failure of hens' eggs. International journal of fracture 142: 29-41.

Martin, K. M. 2016. The effects of zinc supplementation from two sources on egg quality and bone health in laying hens.

Methods, M. I. 2013. SEM: Scanning Electron Microscopy. file:///C:/Users/lnwood4/Downloads/33%20(2).pdf Accessed August 22, 2018 2018.

Mishra, S., K. Sethi, P. Swain, A. Das, and S. Kanunugo. 2014. Importance of zinc supplementation in layer bird nutrition.

Nakano, T., N. I. Ikawa, and L. Ozimek. 2003. Chemical composition of chicken eggshell and shell membranes. Poultry Science 82: 510-514.

Nasr, M., J. Murrell, L. Wilkins, and C. Nicol. 2012. The effect of keel fractures on egg-production parameters, mobility and behaviour in individual laying hens. Animal Welfare-The UFAW Journal 21: 127.

Nedomová, Š., J. Trnka, P. Dvořáková, J. Buchar, and L. Severa. 2009. Hen's eggshell strength under impact loading. Journal of Food Engineering 94: 350-357.

Nolan, Lauren W., et al. "Use of shape indext to determine differences of eggs from hertiage breeds vs. commercial white egg strains." International Poultry Scientific Forum, Atlanta.

Nys, Y., J. Gautron, J. M. Garcia-Ruiz, and M. T. Hincke. 2004. Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. Comptes Rendus Palevol 3: 549-562.

Nys, Y., J. Gautron, M. McKee, J. Garcia-Ruiz, and M. Hincke. 2001. Biochemical and functional characterisation of eggshell matrix proteins in hens. World's Poultry Science Journal 57: 401-413.

Nys, Y., M. Hincke, J. Arias, J. Garcia-Ruiz, and S. Solomon. 1999. Avian eggshell mineralization. Poultry and Avian Biology Reviews 10: 143-166.

Plaimast, Hatairat, et al. "Effect of supplementary zinc from organic and inorganic sources on laying performance and zinc deposition in eggs." *The Thai Journal of Veterinary Medicine*38.3 (2008): 47-53.

Parsons, A. H. 1982. Structure of the Eggshell1. Poultry Science 61: 2013-2021.

Riczu, C., J. Saunders-Blades, Hu. Yngvesson, F. Robinson, and D. Korver. 2004. End-of-cycle bone quality in white-and brown-egg laying hens. Poultry science 83: 375-383.

Roberts, J. R. 2004. Factors affecting egg internal quality and egg shell quality in laying hens. The Journal of Poultry Science 41: 161-177.

Rodriguez-Navarro, A., O. Kalin, Y. Nys, and J. Garcia-Ruiz. 2002. Influence of the microstructure on the shell strength of eggs laid by hens of different ages. British poultry science 43: 395-403.

Roland, S. D. A., D. R. Sloan, and R. H. Harms. 1975. The Ability of Hens to Maintain Calcium Deposition in the Egg Shell and Egg Yolk as the Hen Ages1. Poultry Science 54: 1720-1723.

Solomon, S. E., and M. M. Bain. "Structural and physical changes in the hen's eggshell in response to the inclusion of dietary organic minerals." *British poultry science* 53.3 (2012): 343-350.

Solomon, Sally E., and M. Bain. 1996. "The normal eggshell." *Proceedings of the national breeders roundtable*: 42-53.

Solomon, S. E. 1997. Egg & eggshell quality. Iowa State University Press.

Sciences, E. M. 2018. Critcial Point Drying Principles. https://www.emsdiasum.com/microscopy/technical/datasheet/critical_drying.aspx Accessed August 22, 2018 2018.

Simkiss, K. 1968. The structure and formation of the shell and shell membranes. Egg quality: A study of the hen's egg: 3-25.

Simons, P. C. M. 1971. Ultrastructure of the hen eggshell and its physiological interpretation. Centre for Agricultural Publishing and Documentation Wageningen.

Stadelman, W. J. 1990. Quality Identification of Shell Eggs. In: W. J. S. a. O. J. Cotterill (ed.) Egg Science and Technology. p 37-61. Food Products Press, Binghamton, New York.

Swain, P. 2014. Importance of zinc supplementation in layer bird nutrition.

Swiatkiewicz, S., and J. Koreleski. 2008. The effect of zinc and manganese source in the diet for laying hens on eggshell and bones quality. Veterinarni Medicina 53: 555-563.

Trindade Neto, M. A., et al. "Dietary effects of chelated zinc supplementation and lysine levels in ISA Brown laying hens on early and late performance, and egg quality." *Poultry science* 90.12 (2011): 2837-2844.

Tyler, C. 1961. Shell strength: Its measurement and its relationship to other factors. British Poultry Science 2: 3-19.

Van Toledo, B., A. Parsons, and G. Combs Jr. 1982. Role of ultrastructure in determining eggshell strength. Poultry Science 61: 569-572.

Whitehead, C., and R. Fleming. 2000. Osteoporosis in cage layers. Poultry Science 79: 1033-1041.

Wilson, S., and B. Thorp. 1998. Estrogen and cancellous bone loss in the fowl. Calcified tissue international 62: 506-511.

Wistedt, A. 2013. Shell formation and bone strength in laying hens.

Yenice, E., C. Mızrak, M. Gültekin, Z. Atik, and M. Tunca. 2015. Effects of organic and inorganic forms of manganese, zinc, copper, and chromium on bioavailability of these minerals and calcium in late-phase laying hens. Biological trace element research 167: 300-307.

Zamani, A., H. Rahmani, and J. Pourreza. 2005. Supplementation of a corn-soybean meal diet with manganese and zinc improves eggshell quality in laying hens. Pak. J. Biol. Sci 8: 1311-1317.

Zhang, Y. N. et al. 2017. Effect of dietary supplementation of organic or inorganic zinc on carbonic anhydrase activity in eggshell formation and quality of aged laying hens. Poultry Science 96: 2176-2183.

VITA

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