THE EGGSHELL STRUCTURES OF RINGED TURTLE - DOVES (*STREPTOPELIA RISORIA*)

by

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ABSTRACT

THE EGGSHELL STRUCTURES OF

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The avian egg provides nutrients and protects the growing embryo from physical and microbial harm. Structures fundamental to the protection are the membrane layers, which are tightly meshed networks of collagen fibers, and the eggshell, which is composed of calcite crystals, ($CaC0_3$). The eggs of Ringed Turtle-Dove and commercially bred chickens were selected for the study. Eggs of commercially bred chickens are twice in length and width of the dove eggs. Features analyzed are components of quality and strength of eggshells included shell thickness, membrane thickness, and concentration and size of vesicles. A comparative study of the bird eggs was designed to examine these structures. Scanning electron microscopy was

used to examine and compare the eggs from two species of birds. The hypothesis was that the relationship of the ultrastructures seen in chicken eggs would be twice the measurement of those found in the dove eggs.

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CHAPTER 1

BACKGROUND ON BIRDS ANDTHIER EGGS

1.1 Birds and Eggs in research

The egg is important economically. The formation of the calcified shell provides an interesting model for biomineralization. Scientific technology advances improve the ability to study and identify the proteins in the egg. The observations and data from this study could be applied to study the eggs of birds found in the wild. In order to appreciate the structure and ultrastructure of the dove egg, the comparison to the most studied avian egg was made. Although it is possible to find papers written on other species, such as Japanese quail eggs, the chicken, specifically a White Leghorn (*Gallus gallus)*, is the bird of choice in egg studies. Commercially purchased chicken eggs were used as the comparison due to the vast information available in published research studies. The domesticated dove was selected because of its relationship to birds found in the wild. The birds that laid the eggs were raised in captivity, which allowed easy collection of eggs and elimination of variables of diet and environment.

1.1.1 Phylogeny and Classification of Birds

The study of the evolutionary development of a genus or species and classification aids in understanding relationships of animals. Names of birds vary with locations, therefore the need of a standard naming and classification. Francis Willoughby and John Ray published the first formal classification of birds in 1676. The

model system of classification, taxonomy was developed by Carl Linnaeus, a Swedish botanist in 1758. Linnaeus' system designates two Latinized names to each species. Name combinations are unique to a specific animal. The first part, genus, refers to a group of an evolutionary similar species; the second indicates the species. Evolutionary relationships are more broadly grouped by families, orders and classes. (Gill, 1990) Birds are in the class Aves. The Ringed Turtle - Dove belongs to the order Columbiformes, which include pigeons and doves. The number of taxa included in Columbiformes is 1 Family, 42 Genera, and 303 Species. The Chicken is classified in the order Galliformes, which includes grouse, quails, pheasants, and guinea fowl. Taxa distribution for Galliformes is 4 Families, 78 Genera and 268 Species.

Table 1.1 Classification of Species of Birds in study

Taxon	Ringed Turtle-Dove	**Chicken**
Class	Aves	Aves
Order	Columbiformes	Galliformes
Family	Columbidae	Phasianidae
Genus	*Streptopelia*	*Gallus*
Species	*risoria*	*gallus*

1.1.2 Ringed Turtle- Dove (*Streptopelia risoria*)

The Ringed Turtle- Dove belongs to the Dove Family Columbidae. *Streptopelia risoria* is a domesticated species of the African Ring Dove or African Collared Dove *Streptopelia roseogrisea*. The Ringed Turtle- Dove is twelve to fourteen inches in long. Ringed Turtle-Doves, social birds that live in colonies, are raised in captivity. Eggs are laid in clutches of two and are incubated by both male and female for 14 days. Their diet consists of a seed mix and water. The type of seeds eaten by doves largely depends

on the species. The most common seeds given to medium sized doves, such as the Ringed Turtle-Dove, are milo, wheat a mixture of millets, cracked com, and hulled oats. Doves do not husk the seeds before eating them; they swallow the seeds whole. Crushed eggs or oyster shells are included as a calcium source, which is essential to bone and egg development. Charcoal, to help digestion, and sand, to help grind food, may be provided for caged doves. (Oliver, 2005) Because the female prepares her body year round for breeding, the dietary needs do not change. The Ringed Turtle-Doves that produced the eggs used in this study live in captivity; therefore, the diet and environment of these animals are known. (Figure B.1). The doves are raised in clean wire cages; climate conditions are dry with a temperature range of 20°—25° C. Eggs were collected at different stages of incubation with limited disturbance to the birds.

1.1.3 Chickens bred for commercial use

The domestic chicken, *Gallus domesticus,* has been documented as existing in human populations in Indo–Pakistan and Egypt as early as 3250 B.C. and 2000 B.C., respectively. It is believed to have originated from one of several jungle fowl in Southeast Asia. The bird most commonly used in research is the White Leghorn chicken. This species, *Gallus gallus*, or crosses that resemble it, are also used in the commercial poultry industry because they lay pure white eggs. These developed crosses and the specifics to obtain desired qualities in broilers and layers are considered a trade secret in the industry. (Coronado, 2003)

1.2 The Avian egg

The egg is single cell, though very complex in design and function. Eggs of oviparous animals provide an environment for developing the embryo external and separate from the female. The avian egg has characteristics that distinguish it from eggs of reptiles, mammals, and insects. The eggs are cleidoic (containing calcium carbonate), amniotic, and, in addition to the shells, have three membranes around the yolk sac. (Gill, 1990) The avian eggshell protects the embryo from parasite and invertebrate predation and helps prevent desiccation. The shell must be strong enough to support the weight of the parent during incubation yet permitting the emerging chick to break out. Pores in the shell permit the exchange of water vapor, ions, and gases necessary for life of the growing bird. Protection from bacteria comes from the membranes and features that prevent the pores' being completely exposed to the environment. Amniotic eggs have fluid that surrounds the fetus during development. Membranes allow improved gas exchange and structural support. The protective membranes include the amnion, which surrounds the embryo; the chorion, surrounding all embryonic structures; and the allantoic membrane, which creates the area in the egg used during gas exchange and provides a storage place for waste products. (Gill, 1990) Inside the egg, yolk provides nutrition and albumen, is a source of water, buffers temperature variations, and provides protective cushioning for the embryo. The membranes and shell provide protection in several ways. Evolution has a role in the formation of larger eggs. The advantage of size increase is an improvement in thermal stability within the egg. (Gill, 1990) Studies advancing understanding of the avian egg have commercial and environmental

implications. Shell integrity is relevant commercially whether the end consumer product is eggs or meat from animal. Environmental consequences include population decline and unsuccessful propagation of species, which can lead to extinction.

1.2.1 Shell Composition and Quality

The commercial egg industry is concerned with the production of the strongest possible eggshells to maximize the number of eggs that reach the market. Many factors affect the relationship of shell breakage and quality: flock health and nutrition, breeding, and environmental conditions. Dried shells of commercially laid chicken eggs have a 95% composition of calcium, 0.3% phosphorous, 0.3% magnesium, and traces of sodium, potassium, zinc, manganese, iron, and copper. Thicker eggshells are not automatically stronger than thinner ones. The shape of the shell and the arrangement of inorganic and organic components are factors considered that contribute to strength. (Gautron, et al, 2001)

1.2.1.1 Calcium; a necessity for development

The diet of the bird is partially responsible for the calcium for the eggshell. Hens' diets generally contain 3 grams of calcium per day. Calcium stores in the medullary bone of the adult hen provide up to 40% of mineral for shell production. Higher blood calcium levels are found in laying bird versus non-laying birds. Estrogen influences growth of the medullary bone. During laying periods, hens utilize a considerable amount of calcium from their skeleton. As the eggshell forms, the medullary cavity of the pigeon is broken down; some break down of the medullary bone is also associated with the chicken during egg production. (Burley and Vadehra, 1989)

1.3 Egg production as represented by the chicken

Egg production takes place in the avian oviduct, a long tubular organ that makes eggs by the secretion of materials that envelop the yolk. The six regions of the oviduct are the infundibulum, magnum, white isthmus, red isthmus, uterus, and the vagina. Passage and the resultant egg production take approximately twenty-four hours. (Figure B.2) The ovaries in an adult hen hold five to six developing egg yolks. The ovum enters the oviduct through the infundibulum and travels via peristaltic contractions of the elastic walls of its smooth muscle layers. After twenty minutes in the infundibulum, the yolk moves into the magnum. The rate of movement is 2.3 mm per minute in the magnum. (Gill, 1990) The yolk rotates in the magnum for three to four hours while four distinct layers of albumen form around it. These layers are of different viscosities and composition. The chalazae, one such composition and composed of twisted strands of albumen, is a structure that stabilizes the yolk position and stabilizes embryo orientation. The rate of passage of the yolk and albumen slows to 1.4mm/minute in the white isthmus. In an hour, the inner and outer membranes form to enclose the yolk. (Gill, 1990) The inner membrane encloses the albumen. The outer membrane is later attached to the shell. Initiation of calcification takes place in the distal red isthmus. During the first five hours of time in the uterus (also called the shell gland), albumen plumping, a process that adds more albumen, is said to give final form and shape to the egg. Pore formation is thought occur during this process. (Gill, 1990; Burley and Vadehra, 1989) Shell calcification and the addition of pigment and the cuticle, occurs in the uterus during the last twenty hours. Within thirty minutes to an hour of

completion of production, mineralization is stopped, and the cuticle and pigments are deposited. After approximately twenty-four hours, production is completed and the egg then passes into the vagina and exits the body through the cloaca. The cloaca, which opens to the external body, is a chamber that is shared with the digestive and urinary tracts. Oviposition is complex; its timing is determined by the effects of the last ovulation. (Burley and Vadehra, 1989) Formation of the eggshell occurs around 41° C. The structure stabilizes around 24° C, room temperature. (Nys, et al., 2004)

1.4 The Structures of Eggshell Membranes

Deposited after entering the white isthmus, three collagen types are secreted by the isthmus tubular gland cells. (Fernandez et al., 1997) Collagen was identified in the inner and outer membranes of chicken eggs due to the presence of hydroxylysine and the digestion of eggshell membranes by collagenase. Its presence was confirmed by immunochemistry using antibodies against type-I, -V and -X collagens. (Nys, et al 2004) Collagen is the most abundant glycoprotein in the extracellular matrix of animal cells and in animal connective tissue; its molecules contain three polypeptide α chains which wrap into a triple helix. The fibrous proteins of the three helical polypeptides are super coiled and form a ropelike structure. The strong collagen fibers are made up of bundles of fibrils of microfibrils, which are composed of coiled, helical collagen molecules. (Campbell, 2002) Classic fibril forming collagen molecules are synthesized as larger procollagen molecules that are cut by proteinases as the cell secretes the molecules. The molecules band into fibrils and are stabilized by covalent cross-links.

Collagen types can vary in length, carbohydrate content, procollagen processing, helical interruptions, and cross-linking. (Kuhn, 1987)

1.4.1 Collagen Types in membranes

Collagen types are classified and grouped by the networks formed. Type I and V, seen in the shell membranes are grouped with fibril-forming collagens that include type II, III and IX. (Figure 3) These five collagen types are long, 300nm, uninterrupted triple helical molecules that form banded fibrils, which have been found in skeletal tissue. Collagen fibrils in most connective tissues contain more than one type of collagen. Bone is made up of 90% type I and 10% type V collagen. (Schmid et al., 1987) Electrophoresis on SDS-polyacrylamide gels was used to identify collagen types I and V in chicken shell membranes. Differential staining of the gels established a ratio of collagen types I: V as about 100:1. The inner and outer membranes contain both types of collagen coated with glycoproteins. Large 2.5 micron fibers of each membrane contained type I material. Type V, with a narrow fiber of 0.6 microns, tends to be associated with the inner membrane. Interstitial type collagen synthesis and epithelial secretions in the hen oviduct are responsible for the deposition of these fiber membranes. (Wong et al, 1984)

1.4.1.1 Collagen Type I

Collagen type I, a fiber forming collagen is found in skin, tendons, and bones. Type one collagen builds thick fibrils. The molecular arrangement is triple helical, chains α1(I), α2(I), with a molecular of structure 300 nm length and a super molecular structure 67 nm banded collagen fibrils.

1.4.1.2 Collagen Type V

The four chains of this collagen are α1(V), α1' (V, α2(V), and α3(V) with a molecular structure of 300 nm in length. Collagen type V occurs in a wide variety of tissues such as basement membranes, tendons, and corneal tissue. The relationship and location to basement membranes suggests that type V can act as a linker and contribute to fiber structure. This collagen has been said to promote cell attachment and movement. (Fessler et al, 1987)

1.4.1.3 Collagen Type X

Type X collagen is distinguished from other collagens by distinctive features. The short chain non-fibrillar molecule is 138 nm in length. The molecules are three identical α1(X) chains encoded by a condensed gene. (Jacenko, 1998) Collagen type X has a greater thermal ability than type I collagen. Calcium ions have been shown to stimulate the synthesis of type X collagen (Schmid et al., 1987). Type X collagen has been identified in the inner uncalcified layers of the chicken eggshell (Arias et al., 1991). A net of collagen type X and osteopontin fibers provide a scaffold for mammillae in which the calcite crystals are the randomly deposited. (Fernandez et al., 2003) Shell membranes are necessary for shell calcification; these membranes are also a barrier to inward mineralization. Chemical removal of collagen type X induces in vivo calcium crystal formation on shell membranes, which indicates the barrier relationship. (Nys et al., 2004)

1.5 Matrix components and mineralization

Matrix components have a functional role in control of crystal growth. The organic matrix is also thought to control spacing of crystal nucleation sites, growth, and orientation with its mechanical properties. Half of the matrix becomes soluble after demineralization with EDTA, which allows further isolation and characterization of constituents. The complex mixture of proteins, glycoproteins, and proteoglycans can be divided into three groups. The first group includes osteopontin, a bone protein, and clusterin, an extracellular chaperone cluster. The second group is made up of elements that are also major components of albumen: ovalbumin, lysozyme, and ovotransferrin. The third group consists of molecules specific to the eggshell that are produced and secreted by cells in the uterus. Proteins specific to the uterus and eggshell are ovocleidins and ovocalyxins; ovocleidin-17 ovocalyxin-32 and ovocleidin-116/ovoglycan. (Nimtza et al., 2004)

Of the major proteins found in the eggshell, ovalbumin was the first albumen protein expressed in the shells. (Gautron, et al, 2001) Osteopontin is found in mammalian bone and is thought to inhibit the formation of hydroxyapatite and calcite crystals or to vary the rate of calcium carbonate precipitation from the uterine fluid. Clusterin is found in the palisade and mammillary layers and is secreted as a chaperone. Clusterin is involved in stabilization, prevention of aggregation, and shell precipitation prior to composition on the scaffold used in mineralization. (Nys et al, 2004)

1.5.1 Uterine fluid

Uterine fluid is secreted in the distal oviduct. The acellular fluid is saturated with calcium and bicarbonate. Changes in uterine fluid occur during the different stages of calcification. (Nys et al, 2004) Mineralization of the eggshell is regulated by proteins and side chains of proteoglycans. Osteopontin is an extracellular protein that has been identified in the oviduct and the eggshell. Fernandez et al (2003) designed a study using anti-osteopontin antibody (OPN 1) to determine the location in the regions of the oviduct. Their results in the eggshell found the protein in the core of the shell membrane fibers and in the base of the mammillae. In the oviduct the presence of osteopontin was related to the presence of the egg. Found in ciliated epithelial cells of the uterus, osteopontin plays a regulatory role in other mineralized systems. Its presence in the nonmineralized structures and outer section of the shell suggests a role in the regulation of calcification. (Fernandez, et al, 2003)

1.5.2 Lysozymes

Lysozymes are enzymes that lyse the cell walls of gram-positive bacteria. Lysozymes, which form crystals, are found in the uterine fluid during the initial, rapid calcification and terminal phases of shell formation. Hinke, et al studied the effect of egg white lysozyme on calcium carbonate by examining the influence of purified lysozyme on calcite crystallization. They noted that crystals devoid of lysozyme formed rhombohedra in 24 hours. Crystal size was reduced, but the number of crystals increased with lysozymes present. They investigated the degree that the composition of uterine fluid affects eggshell properties. Calcite crystal modifications caused by

lysozymes are due to interaction of positively charged lysozyme with specific crystal faces as a result of carbon density differences. Their experiments showed a heterogeneous distribution of lysozymes throughout the membranes and shell. These, along with other molecules in uterine fluid, influence mineralization. (Hinke, et al., 2000)

1.5.3 Initiation of mineralization: Red isthmus

Isthmus, red isthmus, and shell gland are involved in the mineralization responsible for eggshell formation. The interior surfaces of the regions of the oviduct are covered by an epithelium containing ciliated and non-ciliated cells. (Fernandez et al., 2003) Rapid mineralization and calcifications processes occur as a result of precipitation on the eggshell membrane while the egg passes through the oviduct. Initiation of mineralization takes place in the red isthmus, while the majority of mineralization occurs in the uterus. The hen egg is in this 4 cm long region for 15 to 30 minutes. The mammillary cores are the initial centers of calcification. (Burley and Vadehra, 1989)

1.5.4 Mammillary layer and Mammillary cones

Mammillae, or calcium reserve bodies, contain mammalian which is a highly sulfated proteoglycan. The mammillary layer is comprised of mammillae, also called calcium reserve bodies. Mammillae are distinct collections of organic material where the nucleation of calcium crystals occurs. (Fernandez, et al, 2003) Mammillae are small concentrations of organic material found at the end of the end of the mammillary cones. They are formed by covalent and noncovalent interaction of the membrane fibers with

the mammillary layer matrix. Decalcification associated with chick development begins with removal of calcium from this layer. (Burley and Vadehra, 1989) The outer membranes adhere to the calcified layer at the tips of the mammillary cones, called mammillary cores.

1.5.5 Mineralization: Shell Gland (Uterus)

Calcite crystal growth continues outward from the inner mammillary to the outward palisade layer. The first two to three hours of the process have a slow rate of growth. The rate of mineralization increases to 300 mg per hour. The resultant three calcified shell layers are the vertical crystal layer, the palisade layer, and the mammillary knob layer. (Davis et al., 2002) The primary compositon of the inorganic matrix layers is calcite, the trigonal phase of calcium carbonate, $CaCO_3$. Calcite crystal growth continues outward from the inner mammillary to the outward palisade layer. The innermost layer, the mammillary layer, is about 100 μm thick in chicken eggs. The mammillary layer grows on the outer membrane, creating the base for approxiamately 200 μm thick palisade layer. The top layer, the vertical crystal layer, ranges from 5-8 μm and is covered by the organic cuticle. A thin protein film covers the egg to protect the interior from bacteria and microorganisms. The Cuticle is the outermost layer and covers the entire eggshell. This layer contains two-thirds of the eggshell pigments. The glycoprotein layer is 90% protein with small amounts of carbohydrates and lipids. (Davis et al., 2002) (Figure B.5)

1.5.6 Vesicles

"Spherically" shaped structures found throughout the calcified layer of the shell are called vesicles. High concentrations are located in the palisade layer and crown region of the mammillary layer. (Figure 6) The lumpy appearance on the outer surface of the shell has been partially attributed to vesicles found in the cuticle. Vesicle concentration is a factor in quality of the avian eggshell. One theory, with respect to concentration and species differences, predicts fewer vesicles in hard, brittle shells while a greater number of vesicles are found in more flexible shells. Although possible functions considered are roles in gas exchange and thermal insulation, the precise function of vesicles is not known. (Burley and Vadehra, 1989)

Dennis et al used several techniques to identify vesicles in the different parts of the calcified shell from the inner Calcium Reserve Assembly (CRA) to the outermost cuticle. They noted compositional differences in that Crown vesicles were filled with a substance and palisade, appearing empty in SEM examination, thought to have contained oviduct fluid.

Numerous electron-dense spherical vesicles of 300 - 400 nm in diameter were seen in the Calcium Reserve Body (CRB). The interconnected vesicles of the CRB contain granular material. Smaller vesicles of the CRB sac (260nm ± 55nm) are interconnected with a threadlike material and surrounded by a membrane like structure. Crown region structures are similar with a diameter of 320 nm ± 80 nm. The random arrangement of structures of the vesicle rich palisade region, with diameters of 450 nm ± 75 nm, is described as having hollow vesicles with crescent-shaped, electron-dense,

fringe. The vertical crystal layer was reported as being devoid of vesicles. The cuticle layer was identified as being made entirely of spherical vesicles, further described as electron dense at the circumference with needle like projections in a hollow core. (Dennis et al, 1996)

1.5.7 Shell Pores

Shell pores are located on the shell surface and extend through the calcified regions. Pores vary in size and concentration. Pores provide a means for release of water vapors and the exchange of gases: oxygen diffused in and carbon dioxide waste from fetal respiration is diffused out. The exchange of gases and water is essential to the development of the chick. Air inside the shell increases as water vapor escapes from the egg as the chick develops. The egg of a hen has up to 7, 500 pores. A large percentage of pores are located at the blunt end of the egg. (Ramel, 2005)

1.5.8 The cuticle and pigments

The pigments are contained in the cuticle. Shell colors are added in two steps, the first's occurring when pigments are added during shell formation. Most shell pigments are porphyrins, which come from the hematin of old blood cells degraded in the liver and changed into bile pigments. (Gill, 1990) Color and pigments in egg shells are the result of pigment deposits during shell formation. Genetic background of the bird is the key determining factor, rather than diet. The base color is laid as the egg enters the oviduct. Patterns such as dots, lines and swirls are added right before laying. These patterns are controlled by the degree of turning and speed that the egg travels. Explanations for a reason for colors and patterns include camouflage and parental

recognition. The eggs of the White Leghorn used commercially in the United States are always white. Doves lay eggs that vary from creamy, buff to chalk white. Brown pigments are ooporphyrin, a product from hemoglobin. Cyan pigmentation comes from a by-product of bile, oocyanin. Other pigments in hemoglobin are biliverdin for blue and zinc chelate for green. (Burley and Vadehra, 1989)

CHAPTER 2

METHODS AND MATERIALS

2.1 Egg measurements and treatment

Twenty-two eggs of each species were used in the study. Dove eggs were collected in three periods. The first period contained eleven eggs; subsequent periods had collections of five and six eggs. (Figure B.7) Commercially produced chicken eggs were obtained to coincide with the collection of the dove eggs. (Figure B.8)

Measurements and treatment observations were recorded for the dove (Table A.1) and chicken eggs (Table A.2). Measurements recorded for the third series include weight of whole egg, albumen and yolk, and dried shells. Volume of albumen and yolk was also recorded for the third series. (Table A.3) Dove eggs were also identified by fertility and incubation status.

Dove eggs were cut open using a razor blade blunt end to pointed end; chicken eggs cracked open at the midsection. Contents of the first two sets of eggs were discarded. Eggshells were rinsed in tap water and air dried in labeled containers. Shell sample one from each type was treated using water only. Other shells were treated as follows: Household bleach, Clorox brand which contains 6% Sodium hypochlorite by volume, a 3% Sodium hypochlorite solution, and distilled white vinegar, Safeway brand, which has 5% acetic acid content and deionized water.

Sodium hypochlorite, 6% solution and 3% solution, was used to remove the two membrane layers from the shell. Acetic acid, 5% solution, was used to examine effects on calcite. Water was used on samples for minimal structural change, with careful rinsing to remove albumen residue. Samples were treated for one, two, and five minutes in the various treatments. Shells were rinsed carefully under running water and returned to containers to dry.

2.2 Preparation for scanning electron microscopy

Aluminum stubs were labeled and placed in stub boxes. Random sections of the shells were cut, using a razor blade and secured to a specimen stub. Bleached shells were very brittle and required careful handling. The substances used to secure the specimens were tacky wax, Bond 527 glue (Bond Adhesive Company), double-sided stick tape, and Duco cement. A microwavable soy based wax was also used, but discontinued because the shells popped off of the wax. Glued specimens were permitted to cure for 24 hours to prevent adverse effects of the glue in the vacuum chamber of the sputter coater. Most of the specimens were secured satisfactorily to the stub using a double-sided tape product that was designed for use with the stubs. Specimens used for cross sections studies were secured using Duco cement. The best method was as follows: metal stub placed in cork holder, a dime-sized portion of glue was placed on a glass slide, and the top of the stub was lowered into the glue and rotated, leaving a thick and even layer on the surface. After the smooth layer of glue was obtained it was permitted to set up for 15 seconds. Eggshell specimens were placed

in the cement as the cement began to cure. Glued specimens were cured overnight to prevent reactions in the vacuum chamber of the sputter coater.

Once specimens were secured to the stub, they were placed in the sputter coater with a gold and palladium target. Shell specimens coated for 4 ½ minutes displayed charging problems when examined in the JEOL 35 Scanning Electron Microscope (SEM). Charging from secondary electrons was especially noted when viewing the outer membrane surface of the dove eggs**.** Two-step coating was used on subsequent samples. The first step was 4 ½ minutes, followed by placing the specimens at an angle on the stage in the chamber and repeating the coating. Samples were treated for totals of 6 ½ and 9 minutes to decrease incidence of charging.

Standard procedures were used to set up the JOEL 35 SEM; essential parts of this set up include the correct filament saturation and proper stigmation. Contrast and brightness were monitored. Images were obtained using Vital Scan Software. Images were scanned at 1024 X 1024, with a dwell time of 196 for high image resolution. Images were saved as grayscale TIFF files.

2.3 Image preparation for quantitative analysis

SEM images of shell cross sections did not require further image modifications prior to analysis with Image Pro Plus analysis. However, the wide range of tonal values found in vesicle structure made it difficult to count and measure them. In order to study the structures, an outline mask was created using layers using Adobe Photoshop 7.01. For each 3000X image of vesicles, the following procedure was employed. The grayscale TIFF file was opened in Photoshop 7.01. The mode of the file was changed

from grayscale to RGB. A new layer was made. Traces of random vesicle perimeters were made using a Wacom Intous$_3$ professional pen and tablet. The pixel size required to trace vesicles ranged from 3 to 5 pixels. After the tracing was completed, the paint bucket tool was used to fill in the area of the perimeter traces. The scale bar was copied from the layer of the SEM and added to the new vesicle image. The layer containing the vesicle tracings was copied into an image file for analysis using Image Pro Plus. (Figure B.9)

2.4 Image Pro Plus Analysis

The initial step of analysis was establishing a master calibration using the Spatial Calibration tool. A master calibration was made using the 10 μm bar in the 3000X vesicle image and of the 200 μm bar for the cross section images. Prior to collecting measurements from the image, a verification measurement was taken of the scale bar of the each image.

Cross-section images of dove and chicken eggshells were measured for thickness using the length tool. Three measurements were made for each cross section area; the combined eggshell and membrane, the eggshell only, and the membrane only. Data were collected and exported to Excel. Measurements of the vesicles were made from the traced area images. (Figure B.10) The software was selected to record; area perimeter, roundness, length and width for each vesicle. The measurements and statistics were exported to Excel spreadsheet files.

CHAPTER 3

RESULTS

3.1 Results of Structure measurements

Dove eggs measured half the size of the chicken eggs for length and width. Ultrastructure measurements were not directly related the gross dimensions of the egg. Measurements made on the structures studied indicate that the dove eggshells were thinner than chicken eggshells; membranes of the dove eggs are thinner than the chicken ratio of shell to membrane is thicker membrane to shell thickness. Chicken eggs had a higher concentration of vesicles in the calcified layers; vesicles in dove eggs, measured by area, were larger than the chicken. (Table A.4)

Cross-section thickness measurements that included the entire shell and membrane structures were made. The variable range for these measurements is large due to variation of integrity of the membranes. The minimum and maximum values for combined shell and membrane thickness were 165.79 μm - 292.7171 μm for doves and 425.30 μm - 582.99 μm for chickens. Several factors affected the range seen in these measurements. While some of the eggs showed outer and inner membranes intact, many of the samples outer membranes separated from either the mammillary cones or the inner layer. During incubation membrane layers shrink causing the outer membrane to pull away from the mammillary cones. Incubation of eleven days in dove eggs, although not fertile, did show signs of outer membrane separation, which was probably

related to incubation and specimen handling. Membranes are also known to separate from the shell where the air cell is formed. (Burley & Vadehra, 1989). Membranes of the chicken eggs did not remain intact on all of the samples examined. Chicken eggs were not fertilized; therefore, membrane separation may have been a result of proximity to the air cell or specimen preparation. Only a small number of samples had outer and inner membranes that remained intact. The ratio of membrane to shell in these cross sections revealed a thick outer and inner membrane on the dove eggs. The sizes of the inner membranes were 54.35% of the size of the dove shells; inner membranes were only 18% of the size of the chicken eggs. The average of inner membrane thickness in dove eggs was 60.20 μm and 76.81 μm for chicken eggs. The range of inner membrane thickness in dove eggs was 34.86 μm - 73.99 μm and 49.56 μm - 109.52 μm for chicken inner membranes. The size differences of the eggs of the two species lead to an expected shell thickness difference.

The dove eggshells were thin enough to cut open with a razor blade, while the chicken eggshell was only scratched. The average of shell thickness in dove eggs was 110.76 μm and 414.75 μm for chicken eggs. The range of shell thickness in dove eggs was 88.46 μm – 128.51 μm and was 323.58 μm – 498. 97 μm for chicken shells. The chicken egg has a thicker shell. Factors that affect the dry weight of the shells may be noted that the chicken shell dry weight was 6.38 g and the dove was only 0.4 g. The thinner shell of the dove is not the only factor to consider in the weight of the shell; the larger vesicle size leaving voids in the calcified layers contribute to the low weight of the shell.

3.1.1 Mammillary cones

Mammillary cones were revealed after the removal of the inner and outer membranes. Solid arrays of the tips of the mammillary cones of varying sizes were seen during examination of the chicken shells. The structures in chicken eggs have a smooth, rounded surface. The structures in the dove eggs have an appearance of stacked layers of crystals. The dove eggshells do not have a solid regular pattern of mammillary cones. Areas devoid of the formations or truncated affect the overall thickness of the eggshell. A size variation in both species of egg is seen. Irregular pattern of the dove shell affects the overall shell structure. A comparison of dove and chicken eggs showed a higher concentration of the cones in chicken eggs. The range of the size was also greater in the chicken eggs. Calcium is taken from these structures to aid in bone development of embryos. Defects in the dove eggs were not due to calcium uptake. The incubation period for doves is fourteen days. Although some of the eggs had been marked “fertilized”, there was no evidence of embryonic development.

3.1.2 Vesicles

Vesicles were seen throughout the calcified layers of both shells. Each vesicle appears to be more than a hole in the layer. Two-dimensional observations only briefly point out that each vesicle goes through many of the crystal layers. (Figure B.13) A higher concentration was found in the palisade region in both dove and chicken shells. The concentration of vesicles was higher in the shells of the chicken. Qualitatively, the vesicles in the dove egg appeared larger, but the concentration in the chicken eggshells seemed to be greater. The structures were not visible in SEM images of 100x, which

was used as the standard for cross section measurements. The structures are recognizable in the 500x images of the dove eggs, but shape is hard to define until magnifications of 2000x are reached. The smaller vesicles of the chicken egg could be seen at 1000x, but required a larger magnification to define the perimeter for the tracing patterns needed for analysis. Counts were made from 3000x magnifications because the size and shape in the chicken shells were too small for measurements and shape determination. The generalized shape of the vesicle is oval to elliptical. Each image examined represents the position and shape of the vesicle in one plane. The forms studied represent the vesicle at the various levels in the planes.

Stereo pairs were made for further observations. The effect of the stereo image pairs is begins in the imaging aspect. Two images are made of the same area, but are shot with a 7° angle difference. Careful alignment of printed images under a stereoscopic viewer created the stereo image. The images can also be created in Photoshop by placing one image into the Red channel layer and the other into the Green and Blue channel layers. The images can be viewed with glasses designed with a red film over the left eye and a blue film over the right eye. Evaluation of the stereo pairs verified depth in the fractured layers that could not been seen from viewing the single images. The three-dimensional view created by the stereo pairs gave a better idea of the relationship of vesicles in the fractured cross sections by giving depth to all of the structures.

Previous studies by Dennis et, al. used diameter as a measure of the size of vesicles. Area measurements of vesicles were made from the SEM images for a better

evaluation of the size of the structures. The size, measured by area, was larger in the dove shells. Quantitative studies of the vesicles were designed around the information that was obtained from two-dimensional images. The small number of studies that described vesicles used diameter as the descriptive measure. In both chicken and dove the vesicles varied from elliptical and were more accurately represented by area measurements. The range of vesicles randomly selected for measurement in the dove eggs was 0.274 μm^2 - 0.570 μm^2, with and 0.063 μm^2 - 0.179 μm^2 for chicken eggs. The average area of vesicles seen in the dove was 0.386 μm^2 and 0.106 μm^2 seen in the chicken. Measurements are given in microns for comparison with other structures. The average size of the vesicles in the dove eggshells was 2.81 times larger than those measured in chicken eggshells.

3.2 Data Analysis

Features of the avian egg that contribute to eggshell quality include shell thickness, membrane attachment strength, density and distribution of pores, matrix properties, presence of pigment, and concentration of vesicles. This study examined eggshell quality in a comparison of two species of birds. The Ringed Turtle- Dove was the animal of primary interest. In order to appreciate the structures in the Ringed Turtle-Dove eggshell, the highly studied chicken is used as a comparison. Quantitative analysis was made using SEM images of the structure. Data were analyzed for correlation to answer questions determining the relationship between the features.

Data used for analysis were averages for each individual sample. Data were confirmed for normality using Systat 8.0 as visual examinations of histograms. Lines in scatter plots fit with regressions. The overall goal was to determine if relationships exist between the size of the two species of eggs and egg length, egg width, concentration of vesicles, vesicle size (area), shell thickness, membrane thickness, and a combined thick of shell and membrane. The Pearson Correlation Matrix revealed that all data were highly correlated, with vesicle area highly negative correlation. Log_{10} transformation of the data was performed on all variables, except the vesicle count to correct for allometric differences. A Principle Component Analysis (PCA) was used. The PCA multivariate analyses of the seven variables showed the data results were highly correlated. Component loadings; two variables accounted for correlation. A scatter plot of the factor scores was set up to show the orthogonal relationships. (Figure B.14) General Linear models and Regression analysis were used to address statistical questions. The relationship of dove egg length (dependant variable) to vesicle area, shell thickness, membrane thickness and combined membrane and shell thickness was significant ($F_{4, 19} = 3.325$ $P = 0{,}035$), Testing the relationship of dove egg width, as the dependant variable was also significant ($F_{4, 19} = 3.857$ $P = 0.022$), Dove egg length relationship to vesicle area when controlling for thickness of membrane and shell combined was significant ($F_{2, 19} = 4.539$ $P = 0.022$). Dove membrane thickness and shell thickness were also significant ($F_{1,20} = 3.857$ $P = 0.050$). Data supported the difference of dove and chicken vesicle sizes (dependant variable) controlling for length and species. ($F_{2, 19} = 10.710$ P= 0.002). (Figure B.15)

CHAPTER 4

DISCUSSION

The comparative study of the chicken and dove eggs was used to learn more about the eggs of the dove. Factors considered in avian eggshell strength include shell thickness, membrane attachment strength, density and distribution of pores, matrix properties, presence of pigment, and concentration of vesicles. This study examined features qualitatively and quantitatively. Shell thickness, membrane thickness and size and concentration of vesicles were compared both ways.

Statistical analysis of the data supported the biological relationships of the structures within each egg. Although the data indicated the relationships of shell structures and membranes were highly correlated, it also showed that the relationships of the ultrastructures between the two species were not consistent with a 2:1 proportion of chicken to dove. The larger chicken egg had a higher concentration of vesicles, but the size of the vesicles reported above was larger in the dove eggs. The smaller dove egg had a thinner shell, yet had membranes that were a larger percentage of the combined structures. It has been reported that the thicker membrane layer has been seen in eggs of birds in birds in tropical climates. (Burley and Vadehra, 1989) A better understanding of the relationships of the ultrastructures in the data that were significant, as well as those that were not can help direct future studies.

Membranes and their attachment to the mammillary cones were qualitatively studied. A one minute treatment with 6% treatment of sodium hypochlorite was not long enough to remove the inner and outer membranes from either the chicken or the dove samples. The layer cracked in both species. The remnants of the outer membrane layer of the chicken egg had the appearance of the single fibers embedded in the layer, whereas the shapes of the mammillae could be seen in the dove shells. (Figure B.14) Samples that were presoaked in water prior to sodium hypochlorite treatments gave an unexpected result. The membranes in both chicken and dove were removed down to a row of single collagen fibers. These showed the best examples of the connection of the membrane fibers to the calcified shell. (Figure B.15) The time for complete removal of the collagens was a two minute treatment followed by careful copious water rinsing. Extended five minute treatments damaged the delicately detailed tips to the mammillary cones. (Figure B.16) Eggs which were kept refrigerated and treated soon after opening showed the rapid removal of membranes. Samples where eggs had been opened, rinsed, dried and required presoak treatments and longer bleaching times to remove the membranes.

Biomineralization is only one topic of interest in egg studies. Aspects such as dietary deletions or chemical factors affecting the process are still areas of interest; advances in the field of genetics will expand understanding. Research is not limited to advances due to the economic significance of the chicken egg; biological processes and the components that drive them are also investigated. Progress in the past decade has shown that membranes of the eggshell were not composed of keratin fibers, but instead

are three types of collagen. Current research focuses on the proteins that make up the 3 – 4 % of the calcified matrix.

4.1 Texas birds in the Family Columbidae

The domesticated dove was selected because of its relationship to birds found in the wild. The birds that laid the eggs were a captive population, which allowed easy collection of eggs and control of variables of diet and environment. The observations and data from this study could be compared with the eggs of birds existing in nature. Visual examination of Eurasian Collared Dove shows a very similar morphology to the Ringed Turtle- Dove, *S. risoria*; length, 12.5 inches and weight, 5.4 ounces. The Eurasian collared dove, *S. decaocto*, is a native of Eurasia, introduced to the Bahamas in 1974. Wild populations of Ringed Turtle - Doves have been known to merge with populations of Collared Doves as a result of escapes from captivity. Populations of this nonmigratory bird were found in Florida in the late 1970's in areas associated in town parks and gardens. Currently, this species is becoming more common from the Gulf Coast of Texas to North Carolina's Atlantic coast and in Tennessee, Oklahoma and Pennsylvania. Like other birds in the Family Columbidae, *S. risoria* and *S. decaocto* are monogamous breeders where both males and females take part in the 14-18 day incubation of eggs. Each clutch generally produces two eggs of around 29 mm long. The Family Columbidae includes nine birds found in Texas. Of these nine birds five different genera are represented: *Columba* (4), *Streptopelia* (1), *Zenaida (*2), *Columbina* (3) and Leptotila (1). The size of these birds ranges from 6 inches up to 15 inches. All are monogamous breeders and both sexes care for and incubate eggs. (Alsop, 2002)

4.2 Future Studies

The comparative study of the Ringed Turtle-Dove and the chicken highlight species differences in structure sizes in eggshells. Further studies of dove eggs may identify chemical composition variances between species. The present study provides an opportunity for further study, collection and comparisons of the domesticated dove to wild doves and thus shows similarities and differences of birds of the same family, Columbidae. Egg integrity and quality are an essential element in the species survival. The dove eggs were able to withstand body weight during the various incubation periods; however, the eggs were easily damaged and dented. Doves in the study were not exposed to temperature variation during egg laying periods or issues related to dietary needs. A study of eggshells of the same family of birds would answer questions related to the thin-shelled eggs and the importance of membrane thickness when supporting fetal development.

The structure which is presently limited by information on function and formation, vesicles presents an area for advancement in the understanding of the avian egg. Vesicles are currently known as structures that contain air when examined after the calcification. A more complete examination of vesicles in eggs of birds with varied habits may give a better understanding of the functions; possibly affecting total egg weight, calcium conservation or a component of gas exchange. Techniques could be designed to learn if the vesicle contents during eggshell formation have a regulatory effect, or if the space holds the organic components prior to oviposition. The key as in

any study to gain knowledge on a topic that can continue to advance with growing scientific technology.

APPENDIX A

TABLES

Table A.1 Dove egg samples with respect to incubation and treatment

Dove Egg	Fertile	Length of Incubation	Length of water pre-treatment	Treatment	Water Rinse	Observations: Shell & membrane
1	Not indicated	11 days	0	0	0	Membranes intact
2	Not indicated	11 days	0	2 minutes Sodium hypochlorite 6%	30 Sec.	dissolved rapidly Shell brittle
3 - 6	Not indicated	11 days	0	2 minutes Sodium hypochlorite 6%	5 min.	Inner and Outer membranes dissolved rapidly Shell brittle
7	no	0 days	30 min.	1 minute Sodium hypochlorite 6%	5 min.	Inner membrane Removed Outer membrane partial removal
8	yes	0 days	30 min.	5 minutes Sodium hypochlorite 6%	5 min.	Moderate reaction Shell brittle, yellow
9	yes	3 days	30 min.	2 min. 5% acetic acid	5 min.	Bubbling on membrane and shell
10	no	3 days	30 min.	5 min. 5% acetic acid	5 min.	Bubbling on membrane and shell
11	no	11 days	30 min.	water	0	Air dry; glossy interior
12 - 16	no	3 days	0	0	0	Air dry; glossy interior
17	no	0 days	0	0	0	Air dry; glossy interior
18-22	no	11 days	0	0	0	Air dry; glossy interior

Table A.2 Chicken egg treatments

Chicken Egg	Water Pre-treatment	Observations: H_2O only inner membrane	Treatment	Water Rinse	Observations: Shell & membrane
1	0	remained intact	0	0	Membranes intact
2	0	remained intact	2 minutes Sodium hypochlorite 6%	30 Sec.	dissolved rapidly Shell brittle
3 - 6	0	remained intact	2 minutes Sodium hypochlorite 6%	5 min.	Inner and Outer membranes dissolved rapidly Shell brittle
7	30 min.	remained intact	1 minute Sodium hypochlorite 6%	5 min.	Inner membrane Removed Outer membrane partial removal
8	30 min.	remained intact	5 minutes Sodium hypochlorite 6%	5 min.	Moderate reaction Shell brittle, yellow
9	30 min.	remained intact	2 min. 5% acetic acid	5 min.	Bubbling on membrane and shell
10	30 min.	remained intact	5 min. 5% acetic acid	5 min.	Bubbling on membrane and shell
11	30 min.	remained intact	water	0	Air dry; glossy interior
12 - 22	0	remained intact	0	0	No bleach used Air dry; glossy interior

Table A.3. Mean measurements for dove and chicken eggs samples 17 through 22.

Means: Measurements, Eggs 17 -22	Chic (n=6)	Dove (n=6)
Length, mm	59.97	28.4
Width, mm	44.92	22.2
Mass: whole egg, grams	70.03	7.22
Mass: dried eggshell, grams	6.38	0.40
Mass: Albumen & yolk, grams	58.95	5.16
Volume: Albumen & yolk, ml.	60.00	4.20

Table A.4 Averages of measurements of the 22 eggs of each species egg evaluated.

Feature	**Dove** (n=22)	**Chicken** (n=22)
Egg Length (mm)	28.65 ± 1.38	59.81 ± 0.97
Egg Width (mm)	22.05 ± 0.56	44.93 ± 0.73
Shell & membranes (μm)	232.53 ± 31.17	519.98 ± 46.41
Shell (μm)	110.76 ± 10.71	425.75 ± 43.71
Membranes (μm)	60.20 ± 9.92	76.81 ± 13.42
Number Vesicles/ 3000x image	208.27 ± 80.67	428.91 ± 118.12
Vesicle Size; area (μm^2)	0.386 ± 0.087	0.106 ± 0.031

APPENDIX B

FIGURES

Figure B.1. Ringed Turtle – Doves. The domesticated doves of the study, *Streptopelia risoria,* that produced the eggs used in the study were raised in captivity. Their environment and diet are controlled.

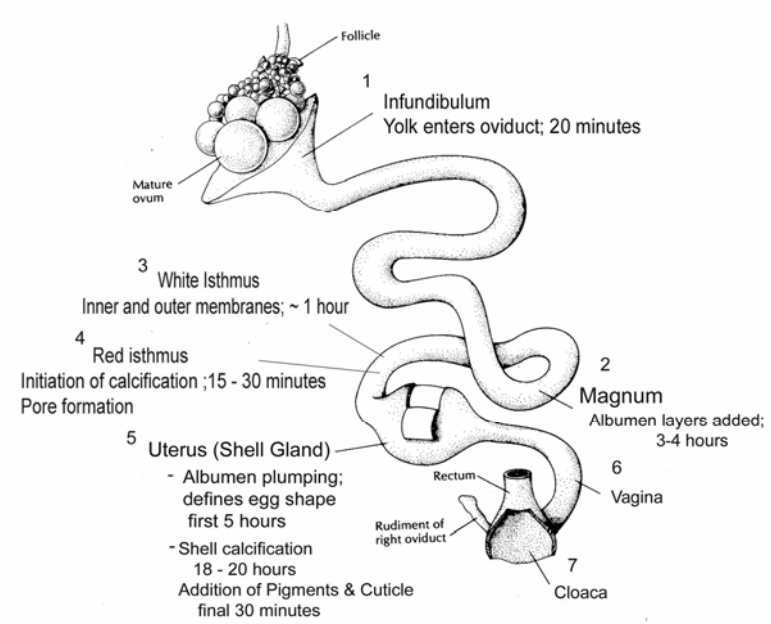


Figure B.2. Avian reproduction as seen in a chicken (Adapted from Gill, 1990)

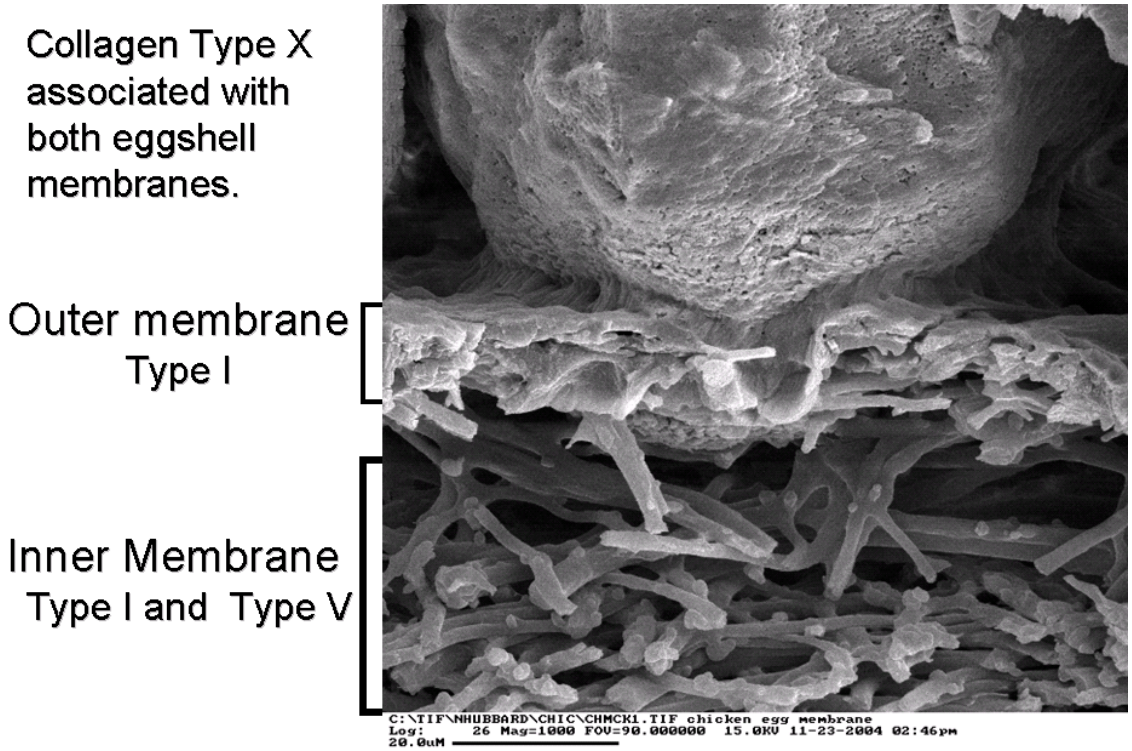


Figure B.3. Collagen found in membranes. Three types of collagen have been identified in the membranes of chicken eggs. Type I and Type X are found in the fibers of the inner and outer membrane. Type V collagen is associated with the outer membranes.

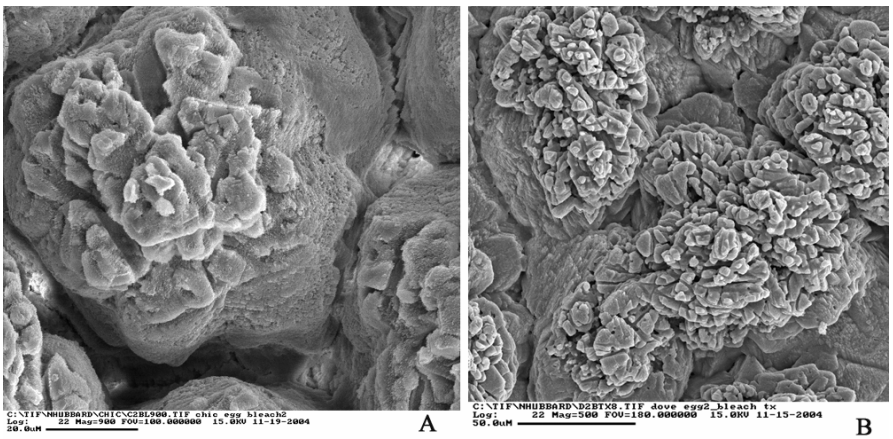


Figure B.4. Mammillary cones. The mammillary cones of avian eggs supply calcium to the developing embryo after the nutrient supply of the yolk is depleted. The tips are the sites of attachment to the outer membrane. A. Chicken B. Dove

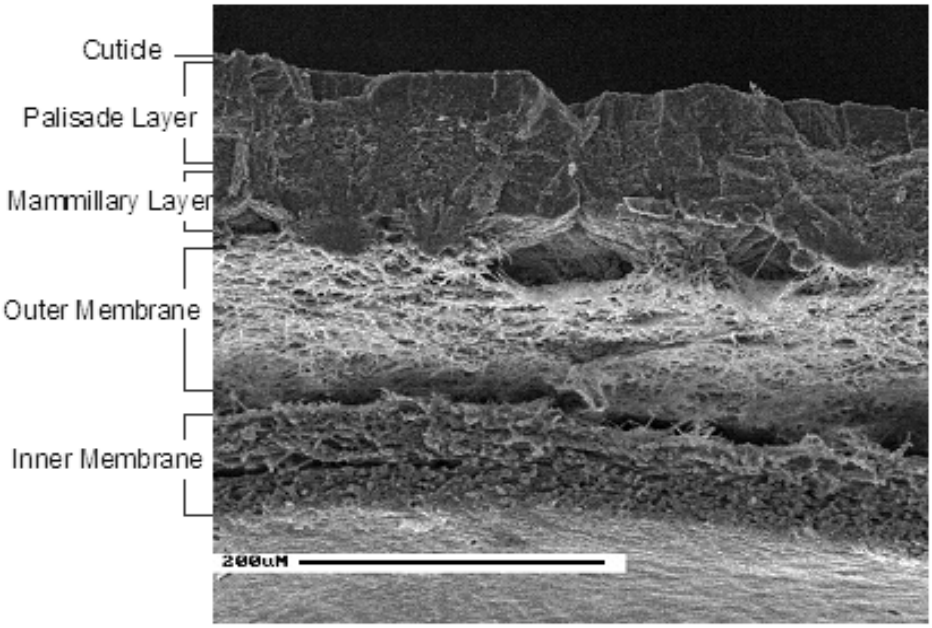


Figure B.5. Cross section of a dove eggshell and membranes. The organic matrix includes the inners and outer membranes and the inorganic matrix the calcified layers, as seen in a dove egg.

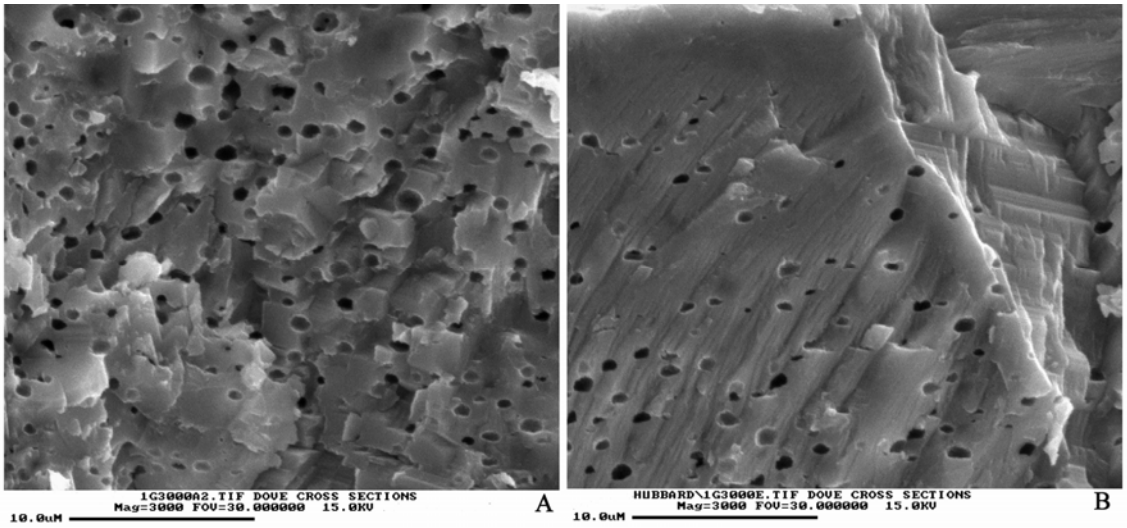


Figure B.6. Spherical vesicles. Random distribution of these small, empty structures is seen throughout the inorganic matrix of the eggshell. Examples of SEM images taken at 3000 x A. Dove egg, Palisade layer B. Dove egg, the outer edge of the Palisade layer and the Vertical Crystal layer.

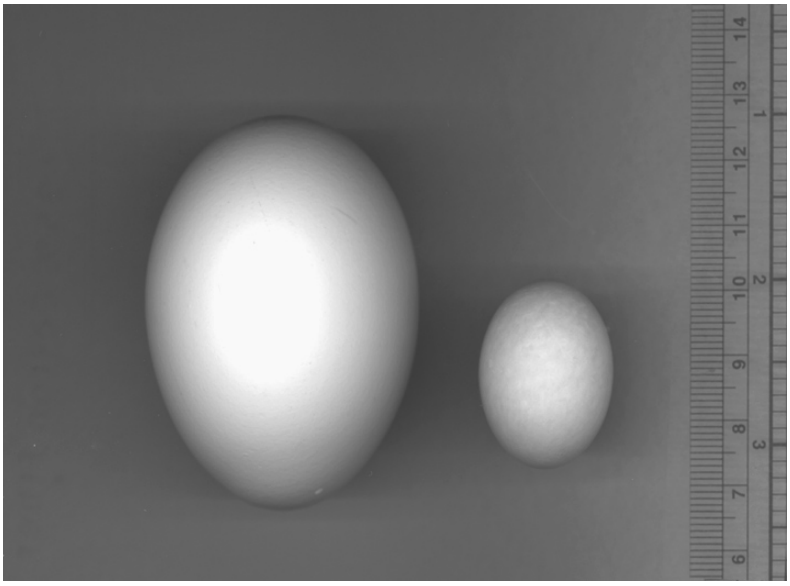


Figure B.7. Chicken and Dove egg from studied eggs. An example of one of the chicken and dove eggs used in the study showing size relationship.

Figure B.8. Four dove eggs collected for the study. Although same eggs were marked with "F" for fertile, due to the possibility, there were no embryos in any of the eggs used.

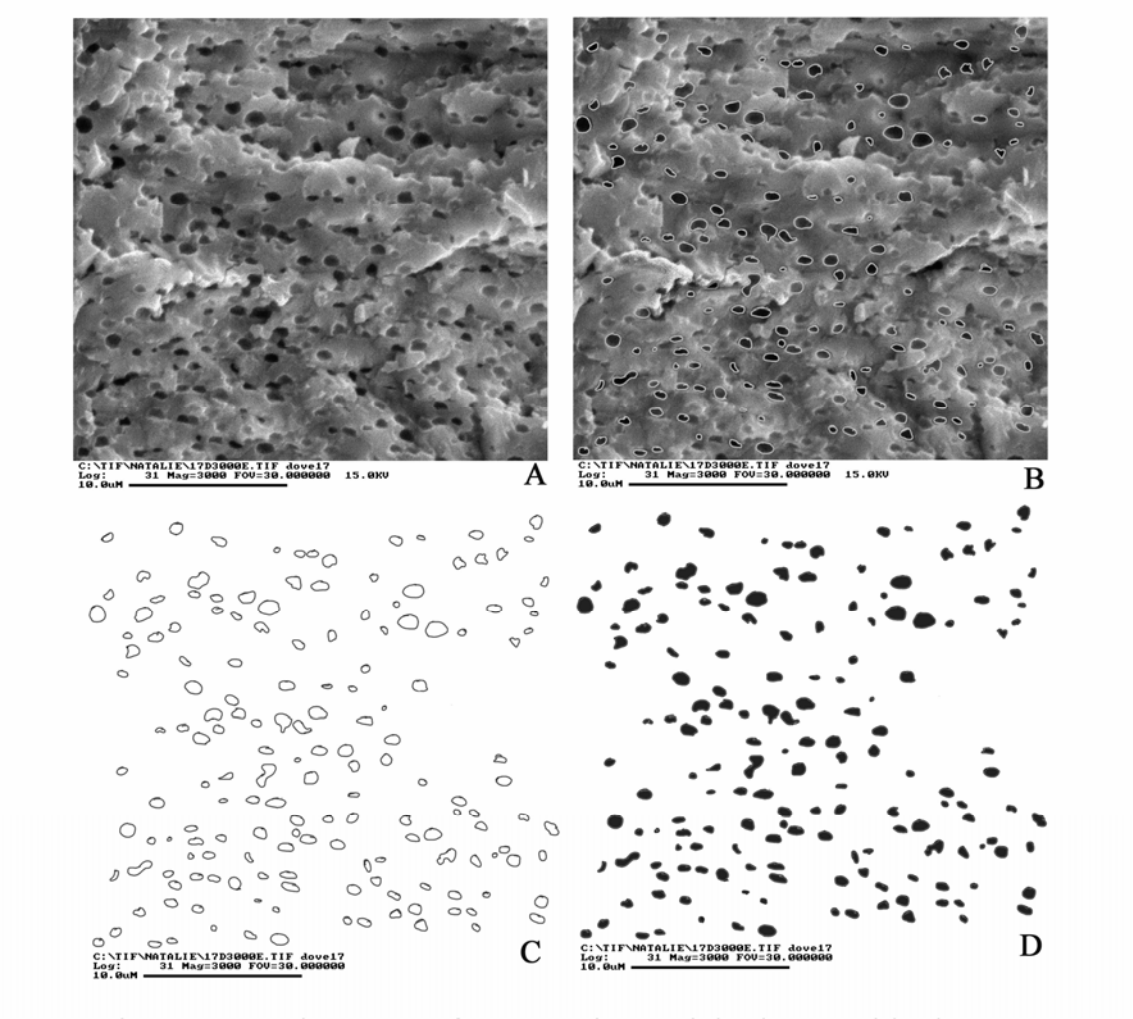


Figure B.9. Image preparations of vesicle images. The process for measuring vesicles began with three steps in Photoshop A. Original TIFF from SEM and Vital Scan. B. Randomly selected vesicles are traced in a new layer using and professional pen and tablet tool, which allowed fine tracing with the 3 pixel size of the paint brush tool. C. A copy was made of the image information, including the micron bar. The traced layer was merged with the copied micron bar and a new image was created. D. Trace images were filled in using the paint bucket tool in Photoshop.

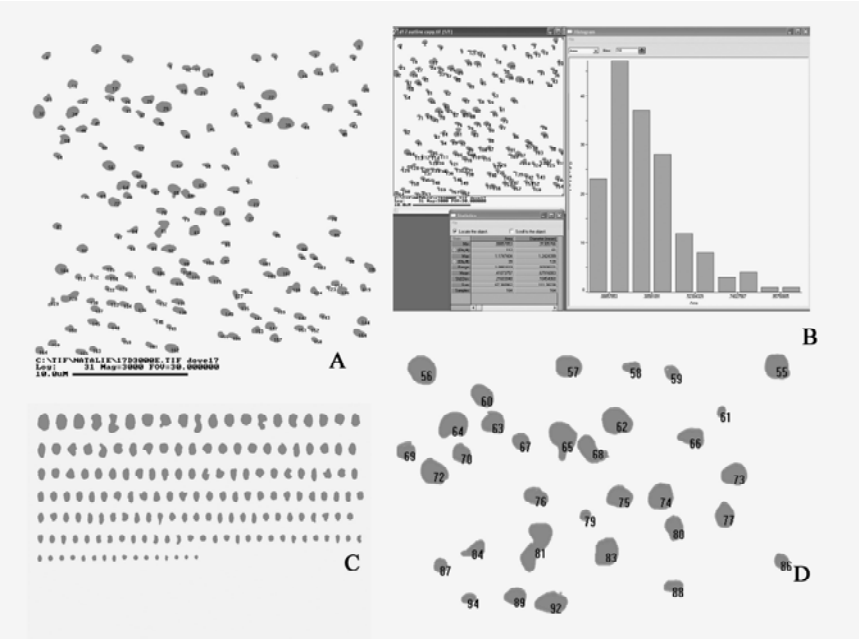


Figure B.10. Image Pro Plus Analysis. Quantitative analysis of structures sized less than a micron was performed on images. A. Counts of vesicles and measurements. B. Data from measurements was downloaded to Excel. C. Special function creates separates vesicles and ranks them by size D. Close up view of count and measure image.

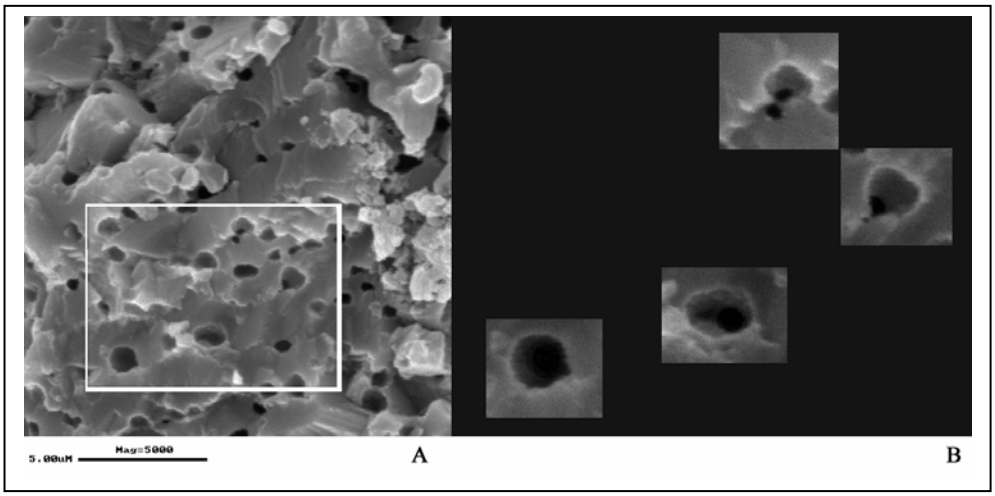
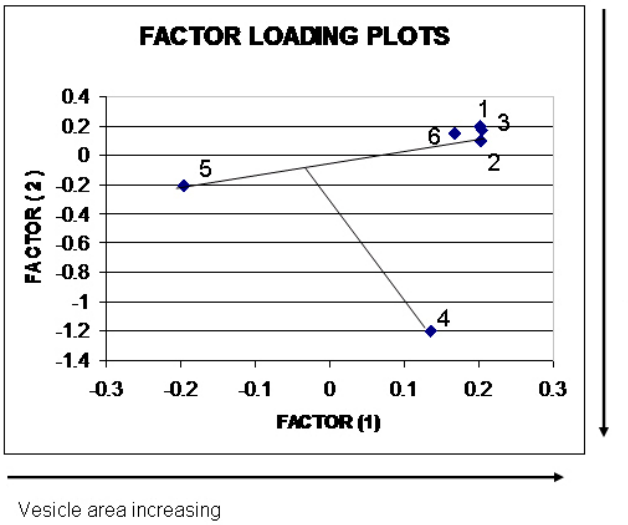


Figure B.11. Vesicle depth and shape variations. A. Vesicles in the region between the palisade and vertical crystal layers of a dove shell. B. The highlighted area from A is enlarged, revealing the shape and depth of the structures through the calcite layers.



Coefficients for Standardized Factor Scores

	1	2
1. L10 EGG LENGTH	0.202	0.195
2. L10 EGG WIDTH	0.203	0.175
3. L10 Shell thickness	0.202	0.097
4. L10 Membrane Thickness	0.135	-1.196
5. L10 Vesicle Area	-0.195	-0.211
6. L10 Vesicle Count	0.167	0.150

Figure B.12 Scatter plot of log transformed data. The PCA multivariate analyses of the seven variables showed stat results were highly correlated, with vesicle area highly negative correlation. Component loadings; two variables accounted for correlation. A scatter plot of the factor scores was set up to show the relationships.

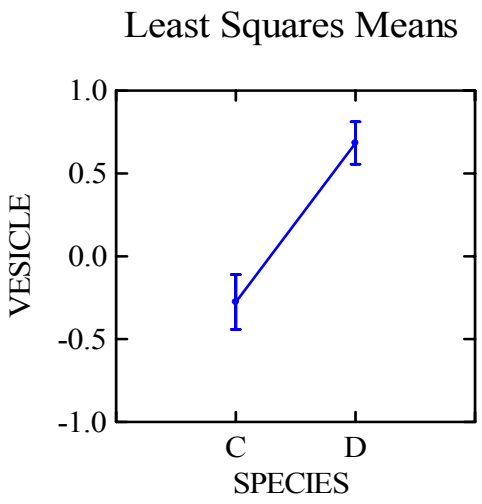


Figure B.13. Species differences of vesicle area size. Difference in Chicken and dove vesicle sizes, Controlling for egg length and species.

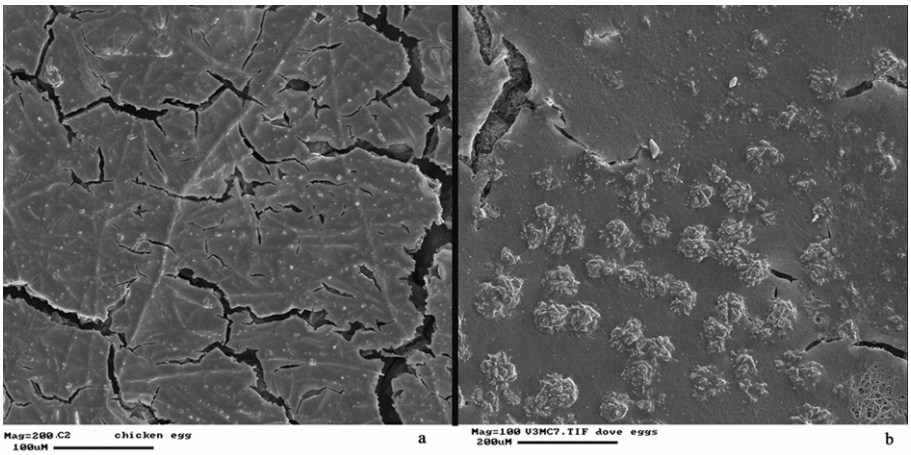


Figure B.14 One minute treatments. A 6% treatment of sodium hypochlorite was not long enough to remove the inner and outer membranes from either the a) chicken shells or the b) dove shells

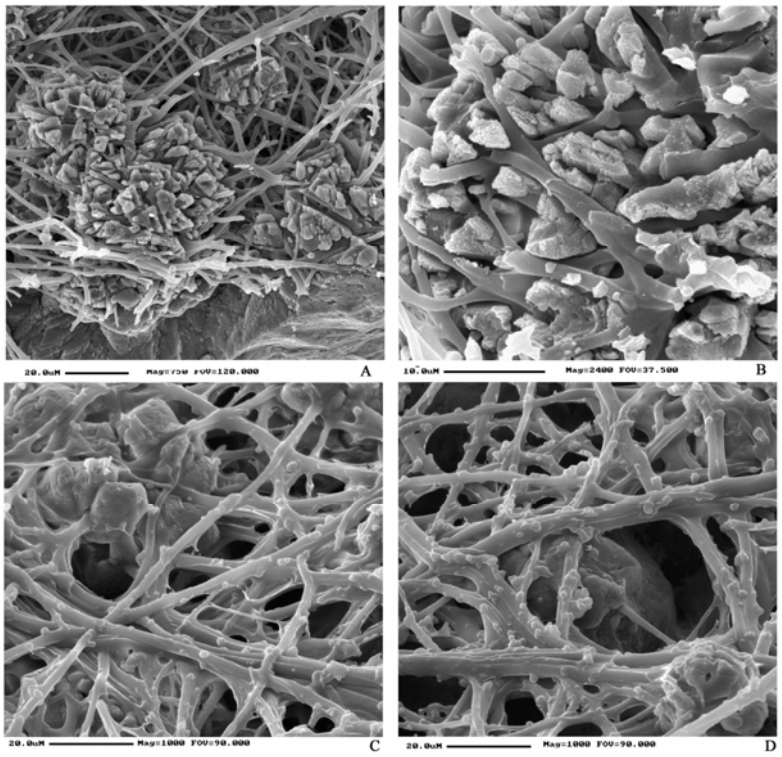


Figure B.15 Outer membrane attachment to mammillary cones. The removal of all but one layer of thick membrane mesh was removed from the mammillary cones, revealing the point of membrane attachment. A. Dove egg B. Dove egg; membrane has a flat band appearance as compared to the membrane seen in the chicken. C. Chicken showing mammillary cone with embedded membrane D. Rounded structure of tip of a mammillary cone is seen through the mesh in this example from a chicken egg.

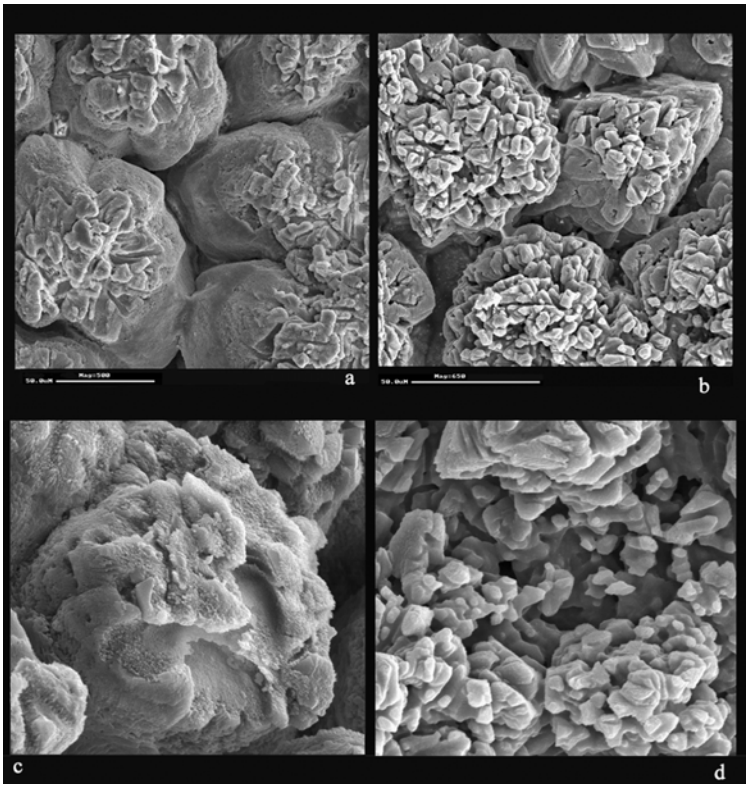


Figure B.16. Treatments exposing mammillary cone tips. Different treatments were used to remove membranes from calcified layers. Eggshells were treated with a 6% solution of sodium hypochlorite to remove the membrane layers. Two minutes was the time that left the intricate structures of the mammillary cones intact. a) Chicken, 2 minutes b) Dove, 2 minutes c) Chicken, 5 minutes d) Dove, 5minutes

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BIOGRAPHICAL INFORMATION

Natalie Hubbard is a former biomedical photographer who returned to college to bridge her knowledge of photography with science. After completing her Bachelor in Science at the University of Texas at Arlington, she decided to further her studies in biology. Whether macro or microscopic subjects are seen, her interest in science and nature is reflected in her photography and when enjoying all of the beauty that is our earth.