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The Ultrastructure of *Chrysemys picta* Eggshell and Shell Membrane

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Abstract: The structure of eggshells plays a multifunctional role in successful embryogenesis. The avian eggshells have been extensively studied while there are few studies on reptilian eggshells. Painted turtle eggs exhibit pliable shells and have received attention by researchers because of its availability but general morphological characteristics of their eggshells have not been previously reported. The objective of the study is to describe the ultrastructure of the eggshell and shell membranes of preincubated and post incubated eggs of painted turtles (*Chrysemys picta*) using a Scanning Electron Microscope (SEM). Painted turtles lay oval-shaped, pliable-shelled eggs, eggshell is composed of outer calcareous layer and inner shell membrane. Outer mineral layer composed of loosely arranged shell units separated with pores traversing through the calciferous layer to the membrane. The size of shell units and pores increase with incubation suggests that the developing embryo derives calcium and water from the eggshell. The outer mineral layer is connected to the multilayered shell membrane at the basal knob. The outer shell membrane is composed of randomly arranged fibers organized into rough, filamentous meshwork and the inner membrane is in contact with the albumin consist of smooth, parallel arranged, featureless f ibers. In conclusion, eggs of painted turtle showed some similarities in morphology to other species of turtles that lay pliable-shelled eggs, the description on structural characteristics of an eggshell is essential to understand the chemical composition of the eggshell and its development.

Keywords: Painted Turtle, Eggshell, Shell Membrane, Scanning Electron Microscope, Shell Units, Shell Membrane

1. Introduction

Vertebrates such as reptiles, birds and mammals lay land eggs and were classified as amniotes. In evolution development of amniotic egg was an important landmark that allowed amniotes to completely adapt to terrestrial environment [1]. Reproduction in reptiles and birds is unique in producing eggs with an eggshell [2-3]. The eggshell surrounds a series of membranes that encapsulate the albumen, yolk sac, and the embryo itself [2, 3]. The eggshell and shell membranes are secreted around the developing egg in the oviduct and provides a self-contained life support system for the developing embryo. The eggshell serves various function for the developing embryo such as mechanical stability, calcium reserve, as well as controlled transfer of heat, moisture and respiratory gasses [2-3]. Additionally, since the eggshell is formed from protein and mineral reserves of the female, understanding the structural properties of an eggshell can provide insight into parental investment specific to the reptile egg.

All extant turtle species are oviparous and based on the physical properties, turtle eggs are classified into three categories, soft, flexible/pliable and rigid [4-5]. Morphological characteristics and composition of eggshells of different turtle species are reported for snapping turtle, *Chelydra serpentina*; soft-shelled turtle, *Trionyx spiniferus*; loggerhead turtle, *Caretta caretta*; leatherback turtle, *Dermochelys coriacea*; olive ridley turtle, *Lepidochelys olivacea*; green turtle, *Chelonia mydas*; different species of Kinosternid turtles; pond turtle, *Emys orbicularis* [2, 5-11]. Comparatively the structural attributes of eggshell and shell membrane of *Chrysemys picta* have been partially neglected despite being one of the more common species in the scientific literature.

The painted turtle, *Chrysemys picta* belong to the order Testudines and family Emydidae [12-13]. Currently painted turtles are widely distributed through North America and are seen in shallow water bodies like ponds, marshes, lakes, and creeks. Females nest from mid-May until mid-July in sandy or loamy soil. Nests are relatively small compared to other turtle species and can contain approximately 1-20 eggs depending on the age of the female. Other than the studies on the effects of anoxia or temperature, painted turtle was the first turtle species to have its genome sequenced [14-16]. Painted turtles are used as a model system for developmental biology, evolutionary and ecological genomics studies due to genomic resources available [17].

Knowledge of structural properties of an eggshell is important to understand development because of the parental investment specific to reptile eggs. The primary investment of energy is towards the development of the embryo inside the egg, and secondly, the energy is used in enhancing hatchling quality [18]. The function of eggshell in protecting the embryo can be compared to the function performed by the carapace and plastron during post-embryonic development, thus the eggshell is a type of maternal investment to ensure hatchling survival. Like the yolk, the shell is formed from the protein and mineral reserves of the female, plays a significant role in the embryo development, and is not seen at later stages.

Recently we published optimal sample preparation protocols for imaging eggshells using SEM [19]. Painted turtle eggshell was used to elucidate the efficiency of the preparation methodologies based on acceptable image quality obtained [19]. In the present study, the ultrastructure of the eggshell and shell membranes of painted turtles is described using the Scanning Electron Microscope (SEM) to compare the structural morphology of preincubated (fresh) and post incubated (spent) eggs. The increase in sizes of the eggshell units and pores were elucidated during development. This is to examine if pore size increases in size with incubation as observed in snapping and sea turtles, or remains unchanged. Further, the width of nodules was analyzed to compare the differences between developmental stages. Lastly, the shell membrane layers were examined for any changes achieved in their size during development. Morphological differences between fresh and spent eggshells were examined with a focus on the role of the eggshell in incubation and the changes it undergoes in fulfilling that role. The qualitative analysis provided agrees with known changes in the morphology of eggshell due to embryonic use of shell resources.

2. Materials and Methods

2.1. Collection and Incubation of Eggs

Collection, incubation, fixing of eggs and processing of eggshells were done according to the methods described previously [19]. Six clutches of *Chrysemys picta picta* (eastern painted turtle) eggs were collected during the 2016-breeding season (mid-May- mid-July) from natural nests at Rice Creek Field Station in Oswego, NY. This collection of eggs was in accordance and performed with permission gained from the New York State Department of Environmental Conservation (DEC). As well, all animal work including collection, incubation, fixing, and processing of turtle eggs was approved under SUNY Oswego Institutional Animal Care and Use Committee (IACUC) ethics protocol. The incubation and staging were done according to a complete developmental table published earlier [20]. The bedding medium consisted of half and half vermiculite and peat moss. Eggs collected were post incubated with half of their surface in direct contact with the moistened bedding medium at 30°C.

2.2. Preparing Egg Shells and Shell Membranes

All the shell fragments included for the examination presented here are from six clutches collected. Shells of all post incubated eggs were saved after embryo fixation and used for the study. Also, eggshells from preincubated/unhatched eggs were obtained, some of these shells were gathered from the vicinity of nests opened by predators the next day after laying. To note the location of all the nests were marked immediately after laying, only 35 eggs were collected for the study and the other nests were left undisturbed. All shells from post incubated or preincubated eggs were rinsed with distilled water to remove dirt and egg components from outside and inside of the shell respectively. Cleaned shells were air-dried for at least 12 hours and stored at room temperature in a clean specimen bottle labeled by number and stage [19].

2.3. Preparation of Sample for SEM

Dried shell fragments were mounted on stubs with double-sided carbon tape. The shells were then subjected to gold plating for 60 seconds at 35mA sputter followed by imaging using a Scanning Electron Microscope (JEOL, USA) at an accelerating voltage of 10kV and emission current 10µA. Analyses were carried out on the outer calcareous shell layer; inner shell layer, shell membranes and innermost surface of the shell in all possible orientations and magnifications, mounting of shell fragments were done according to desired orientation needed [19]. To expose the underlying inner surface and shell membrane, fragments of shells were soaked in water to regain flexibility, then the upper calciferous layer was mechanically stripped and peeled using fine forceps.

2.4. Statistical Analysis

For both preincubated and post incubated eggshells, observations were made from biological replicates of 6-8 eggs for each developmental stage (preincubated, stage 9, 12, 17 and 20). Three fragments from the middle region of each egg were subject to a random analysis of 50 separate pore/nodule sizes for each fragment, which were used to determine an average pore/nodule size for each stage. The nodules or the pores are not perfect circles, maximum width was measured across their perimeter for estimating their sizes. Measurements of different regions of shell and shell membrane were done using the measuring tool option in the scanning electron microscope image processing software, measured parameters include the thickness of the calcareous layer, inner layer with shell membrane and space between these two layers. One-Way ANOVA, Tukey simultaneous tests were used to analyze the differences of means for pore and nodule sizes between the developmental stages.

3. Results

3.1. Painted Turtle Egg and Eggshell

Painted turtle eggs are white, elliptical, and flexible, the size of the eggs range between 3-3.5 cm in length (Figure 1A). Eggs of painted turtle have shell structure that relatively easy to analyze with the following components from outer to inner region in a sequence: outer calcareous surface layer, inner surface, shell membrane and basal boundary like other testudines species. Radial view along the edges of the shell fragments revealed inner surface layer and basal boundary are a thin flimsy covering attached to the ventral side of the calciferous layer and shell membrane respectively. The average thickness of the calcareous layer with an inner surface and shell membrane with basal boundary was measured to be 27.5µm and 64 µm respectively.

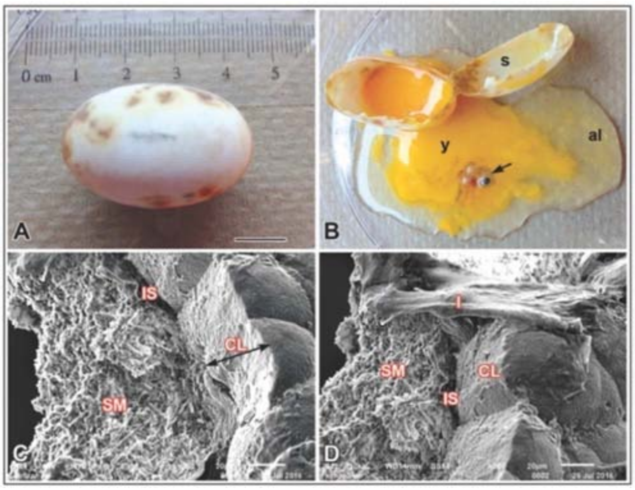

Figure 1. *A) External morphology of painted turtle egg. Scale bar 400 µm B) Inside of incubated egg cut open during embryo fixation, arrow marks the developing embryo, y: yolk, al: albumin, s: flexible eggshell. (C-D) Cross-section of a shell fragment, inner and outer surface towards the left and right respectively. Note the calcareous layer (CL) outside, inner surface (IS) and fibrous shell membrane (SM) inside. The thickness of the calcareous layer and shell membrane was measured to be 27.5µm and 64 µm respectively. The space between the two layers measured to be 8.5µm thick and is held together by interconnection bridge (I) at regular intervals.*

Shell fragments (n=50) from preincubated and post incubated eggs were used for analyses and we observed no difference in thickness of these regions between preincubated and post incubated eggs (data not shown). The outer calcareous layer and shell membrane are not continuing with each other instead a narrow space of 8.5µm thickness is present. A smooth interconnection bridge holds the outer layer and shell membrane together at regular intervals (Figure 1D).

3.2. Surface View of Outer Calcareous Layer

The outer calcareous layer is being organized into individual units called nodules or shell units. The nodules are globular, discernible units compactly arranged forming an irregular polygonal pattern (Figure 2A). Spicules are not seen on the surface but coated by a slightly granulated smooth covering that appears to be brittle (Figures 1C, 2A-B). This might be the reason for observing a few fine cracks running across some nodules (Figure 2A). These fine cracks are not an artifact due to drying, we observed these cracks even on the surface of washed shell fragments before drying.

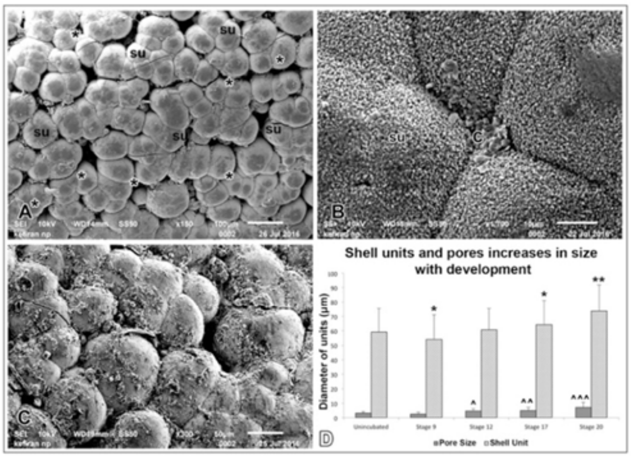

Figure 2. *A-C) The calcareous layer consists of individual globular units, the nodules or shell units (su). Note the pores (asterisks) occurring between adjacent shell units. A) Surface view from a preincubated egg. B) High magnification of a shell unit, note the connection/center point (c) between the units and each varying in their size. C) Close to hatching the calcareous layer exfoliate forming fragments and the coarse powder due to calcium removal by the embryo. D) Size of the shell unit and pores increases with incubation. One asterisk (*) showing a significance level of p<0.10, and a double asterisk describes a significance level of p<0.0.*

The adjacent shell units or nodules interlock, and there are relatively few spaces traversing the thickness of the eggshell. We termed the observed free spaces of varying sizes between adjacent nodules as pores. The average measurement of both pores and nodules (n=50 measurements for each 3 fragments of each 6-8 eggs) showed increased size with development from stage 9 to 20 (Table 1). While both the parameters measured vary between experimental replicates of each egg, greater variation is seen with nodules size compared to pore size.

Table 1. *The average size of pores and nodules in the calcareous layer from eggshells of painted turtles.*

Developmental Stage	Pore size			Nodule size		
	Range	Average	SD	Range	Average	SD
Pre-incubated	0.74 – 5.39 µm	3.09 µm	1.22	30.66 – 140.95 µm	59.30 µm	16.15
Stage 9	0.89 – 6.45 µm	2.60 µm	1.19	20.61– 125.50 µm	54.19 µm	16.85
Stage 12	2.72 – 9 µm	4.60 µm	1.60	30.51 – 100.73 µm	60.64 µm	14.87

Developmental Stage	Pore size			Nodule size		
	Range	Average	SD	Range	Average	SD
Stage 17	1.81 – 15.87 µm	4.91 µm	2.18	34.13 – 117.03 µm	64.24 µm	16.74
Stage 20	2 – 18.43 µm	7.05 µm	3.61	22.5 – 128.39 µm	73.84 µm	18.07

Note: All the measurements were made from biological replicates of 6-8 eggs for each developmental stage. Three fragments from each egg were subject to a random analysis of 50 separate pore/nodule sizes.

Table 2. *One-Way ANOVA, Tukey simultaneous tests for differences of means for pore and nodule sizes between the developmental sizes.*

Difference of Levels	Pore size		Nodule size	
	T- value	Adjusted P- value	T- value	Adjusted P- value
Stage 9- Preincubated	-1.13	0.79	-2.67	0.05
Stage 12- Preincubated	3.51	0.004	0.70	0.95
Stage 17- Preincubated	4.22	0.0002	2.58	0.07
Stage 20- Preincubated	9.23	<0.0001	7.60	<0.0001
Stage 12-Stage 9	4.64	<0.0001	3.37	0.006
Stage 17-Stage 9	5.35	<0.0001	5.25	<0.0001
Stage 20-Stage 9	10.35	<0.0001	10.27	<0.0001
Stage 17-Stage 12	0.71	0.95	1.88	0.32
Stage 20-Stage 12	5.72	<0.0001	6.90	<0.0001
Stage 20-Stage 17	5.01	<0.0001	5.02	<0.0001

An increase in pore sizes with development was found to be significant (p>0.1) for all stages examined except for the difference observed between preincubated and stage 9 eggshells (Table 2). Differences in nodule sizes between all the incubation stages were found to be significant (p>0.1) except for preincubated and stage 12 eggs (Table 2). The point of meeting between each discrete unit consisting of 4-5 nodules, we termed this connection junction as center point (Figure 2B). The calcareous layer is in close association with the underlying membranous layer in eggs that were preincubated and post incubated for 9 days (stage 9-10). Flaking of the mineral layer is seen from stage 12-13 (days 12-15), maximum exfoliation is observed as powdered calcareous fragments (Figure 2C) and the mineral layer is completely separated from the shell membrane by stage 20 (day 32-33).

3.3. Inner Eggshell Layer

Manual stripping of the shell units allowed us to visualize and examine the inner shell layer. The inner shell layer consists of the basal knob which are the broken remnants of the shell units (Figure 3A-C). These remnants show the original organization of the intact eggshell units. The shell nodules seem to radiate from the central plaques from which the mass of crystals grows towards the surface. The center of the basal knobs is open and consists of fibers from the underlying shell membrane making the connection points where an intact shell nodule is attached to the shell membrane.

3.4. Shell Membrane

The membranous region below the shell layer consists of layers of fibers that can be separated into two distinct regions, outer and inner shell membranes. Magnified view of the shell membrane could identify at least five layers of randomly organized fibers (Figure 3D) but cannot eliminate the possibility of additional layers attached to each other. The outer shell membrane consists of randomly arranged rough fibers whereas the inner shell membrane is appearing smooth in comparison (Figure 3E). The inner membrane is in contact with the extraembryonic membranes of the developing embryo and albumin.

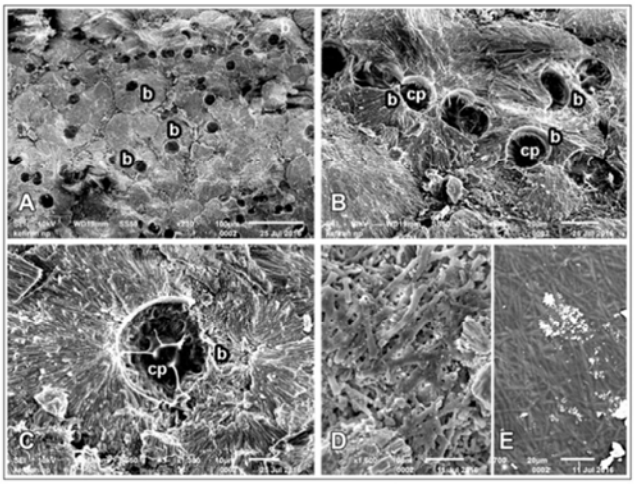

Figure 3. *A-C) Low to high magnification view of the inner eggshell layer. Manual stripping left the remnants of the shell units, the basal knobs (b). The central plaques (cp) of the basal knobs show radially symmetrical fiber tracks left by the broken tips on the facing surface of the shell membrane. D) Multiple layers of fiber network randomly oriented to one another. E) The ventral surface of the shell membrane close to the albumin appears smooth in comparison to the outer shell membrane.*

4. Discussion

The structure of eggshell observed in painted turtles showed several similarities and differences to the descriptions provided in studies previously published. Like the eggs of other turtles and tortoises, the eggshell of painted turtle consists of a calcareous layer and fibrous shell membrane. The outer surface of painted turtle eggshells is whorled, and the outline of individual shell units is apparent along with the pores traversing the eggshell. Based on the observation on the painted turtle eggshell and comparing with the classical criteria used to classify egg-shells from the viewpoint of shell hardness [21], eggs of the painted turtle may be categorized to have a pliable shell. The pliable nature of the eggshell allows flexing without exerting pressure on the calcium layer. The surface of the painted turtle eggshell is covered with an external matrix material of unknown nature and no defined cuticle covering is seen in both preincubated and post incubated eggs, this is comparable to most turtles except for the members belonging to the families Kinosternidae and Geoemydidae [6-8, 10, 22-24]. The calcareous layer of eggshells of all species of turtles examined to data is composed of calcium carbonate, which occurs as aragonite [4-7, 25-26]. The similar composition is expected to be present in the mineral layer of painted turtle eggs, although the chemical nature of painted turtle eggshell is the subject of future investigation.

Calcification of the eggshell evolved as a defensive measure to overcome microbial and invertebrate infections [6]. Also, the level of calcification is directly related to water absorption by the egg during post-incubation [6]. Eggs with pliable calcified shells like in the painted turtles will require more uptake of water for development, thus will take up water facultative, enhancing the quality of the hatchling. The shell units or nodules are compactly arranged and possess a demarcated boundary that does not traverse to the adjacent units. This might serve as an advantage for high conductance to water vapor as seen in snapping turtles [6-7]. An increase in pore and nodule sizes observed in post incubated eggs supports this interpretation with the expansion of the outer calcareous layer due to intake of water. The swelling of eggs due to water absorption was reported previously in snapping turtle eggs [6]. By comparing the structure of shells from preincubated and post incubated eggs, the other most obvious difference is seen with the attachment of the shell membrane with the calcareous layer. The outer surface of the eggs incubated close to hatching (day 23) showed coarse powder of calcareous fragments due to complete separation of the shell membrane from the calcareous layer while separation and flaking are initiated around the mid-point of incubation around day 12-15. This might be due to the developing embryos of painted turtles remove and use calcium from the eggshell in development. A similar withdraw of calcium from the eggshell is seen in other turtles, snakes, crocodilians and birds [5-6, 26-28]. Although exfoliation from the surface of shell units is seen with post incubated eggs, no disruption of shell unit alignment is observed in eggs of any post-incubation stages. Individual boundaries of nodules remain intact even with the mineral layer flaking as calcium powder and no broken edges of the units were observed as in snapping turtle, softshell turtle and avian eggs [6-7, 27-28]. Unlike in snapping and softshell turtles, flaking of calcium layer was seen through the egg surface while in former species of turtles flaking and distortion of the nodules were seen in random patches along the outer surface [6-7].

The inner shell layer exposed by manual stripping showed the basal knobs which are the basal tips of the shell units connected to the shell membrane. Shell units of the painted turtle are mostly uniform in size between the distal and proximal tips like softshell turtles whereas in snapping turtle there is a distinct variation between the proximal tip and the rest of the shell unit. The basal knob itself consists of the central plaque and fiber connections were seen arranged within the grooves which might be attachment point between the nodules and the membrane. A similar structure is observed in softshell turtle eggshell whereas in snapping turtle and sea turtle eggs, the central plaque is covered with a membranous structure. The basal knobs are believed to function for nucleation of the calciferous mineral layer during shell formation as seen in avian and trionychid turtle eggs [7, 10, 30]. The size and arrangement of the basal knobs of painted turtle showed no variation between preincubated and post incubated eggs.

The thickness of the shell membrane varies greatly within turtle species due to differences in the number of layers present within the membrane [6-7, 31]. The shell membrane of painted turtle egg is organized into an outer shell membrane connected to the calcareous layer and inner shell membrane in contact with the albumin and extra-embryonic membranes. As seen in snapping turtle and avian eggs, no air space is seen in the eggs of painted turtles between the outer and inner shell membranes or between the albumen and the inner surface of the shell membrane (data not shown). The shell membrane of painted turtle eggs is identified to be a multilayered intact structure and does not undergo any change with incubation. Although both the outer and the inner shell membranes are made up of fibrous tissue, inner membrane consist of parallel placed smooth fibers while the outer membrane layers consist of rough fibers with complicated processes as seen in softshell turtles [7]. Arrangement of fibrils within the shell membrane is linked to the water pliability and determines the selection of nesting sites in turtles and tortoises [7]. The inner membrane in avian eggs is not perforated whereas in the Chinese softshell turtle several canals are present in between the inner membrane fibers indicating high water passage through the membrane [32-34]. In painted turtle eggs no canals are seen between the intertwining fibers that are closely bound and the spaces in the meshwork were small, this might play a role in retaining water.

5. Conclusion

This study provides a detailed description of the ultrastructure of painted turtle eggshell and shell membrane using SEM. The shells of eggs of painted turtles showed some similarity in gross morphology to other species of turtles that lay pliable-shelled eggs [7, 14, 29-31]. Eggshells of painted turtle consist of a thick firm calcareous layer and a multilayered shell membrane. Both these layers are connected by an interconnection bridge at regular intervals. The size of the nodules and pores of the calcareous layer increase with incubation due to water and calcium absorption by the embryo during development. There is negligible variability in morphology among the eggs in different clutches, within a clutch, and within the regions on a single eggshell except for the thickness of the eggshell. General morphological characteristics of the painted turtle eggshell reported will be used to elucidate the chemical composition of the eggshell in the future.

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