Chapter 4

Eggshell Structure of the Narrow-headed Softshell Turtle

Chitra chitra Nutphand, 1986 (Testudines: Trionychidae)

Abstract

Eggs of Chitra chitra were examined for: 1) eggshell thickness by stereo microscope 2) eggshell structure by scanning electron microscopy (SEM) 3) qualitative and quantitative elements of eggshell structure by energy dispersive x-ray analysis (EDX), and 4) eggshell composition by X-ray diffraction analysis. It was found that the eggshell thickness of the outer layer and inner layer detected by stereo microscope were 0.14 $\pm$ 0.02 mm and 0.13 $\pm$ 0.02 mm, respectively. The result of SEM showed that the eggshell had three layers; an outer calcareous sheet (previously unseen), a middle crystalline layer and an inner fibrous layer. The eggshells were composed of oxygen = 52.96$\pm$4.81%, carbon = 35.03$\pm$9.17%, magnesium = 5.55$\pm$0.34%, calcium = 5.37$\pm$7.16%, silica = 2.87$\pm$1.64%, aluminum = 2.30$\pm$1.07%, potassium = 0.17$\pm$0.1%, and sodium = 0.74$\pm$0.3%. The eggshell was the aragonite form of $CaCO_3$.

Key words: Chitra chitra, Softshell turtle, Eggshell Structure, Eggshell Thickness, Eggshell Composition, SEM

Introduction

The narrow-headed softshell turtle Chitra chitra Nutphand, 1986 is one of the largest, and one of the least known turtles in the world. Since its recent recognition as a distinct species (Nutphand, 1986), its population size has almost certainly continued to decline across its limited range in Thailand and elsewhere in southeast Asia (Thirakhupt and van Dijk, 1994; Engstrom et al, 2002; Kitimasak and Thirakhupt, 2002) due to over fishing, pet trade, and habitat alteration and degradation (mainly due to reservoir construction). Its biology and natural history are very poorly known, and the lack of information seriously limits conservation efforts in its behalf. An ongoing study of this species in the field and in captivity is designed to rectify this deficiency. As part of this investigation of the reproductive biology of this species the structure and composition of the eggshell of eggs from a wild caught female were analyzed. The results of the analysis are presented here, and compared with the few other structural analyses of turtle eggshells which have been published (ie. Young, 1950; Solomon and Baird, 1976; Baird and Solomon, 1979; Packard and Packard, 1979; Packard, 1980; Woodall, 1984; Roberts and Sharp, 1985; Packard and Hirsch, 1986; and Wangkulangkul et al., 2000).

Materials and Methods

A female C. chitra (~80 kg) was caught in Srinagarind Reservoir, Kanchanaburi Province, Thailand in March, 2001. Subsequently she laid 3 eggs in a fiber holding tank. All of these eggs were broken. After that synthetic oxytocin was used to induce oviposition. All of the eggs obtained from oxytocin injection (N=32) were incubated in a styrofoam box. Six undeveloped eggs, two weeks old, were separated for study. Specific eggshell characteristics examined and techniques utilized in the analyses are described below: 1) **Eggshell thickness**; the eggshells were randomly cut into 5 pieces/egg of about 1 x 5 mm. They were dehydrated and stained by 0.5% eosin. Thirty thicknesses (5 locations/egg) were measured under a stereo microscope with an ocular micrometer. The thicknesses of outer and inner layers were compared by t-test analysis using SPSS program (Ver. 10). 2) **Scanning Electron Microscopy (SEM);** the dehydrated eggshells were coated with gold and were examined by a J.S.M.-6400 scanning electron microscope, operated at 15 kV. 3) **Energy dispersive x-ray analysis (EDX);** the J.S.M.-6400 electron microscope was used to examine both qualitative and quantitative elements in the dehydrated eggshells. 4) **X-ray Diffraction analysis;** the eggshells were ground down to powder-sized particles to examine their composition. An X-ray Diffractometer model JDX-8030 was used at 45 kV for this procedure.

Results

Eggshell thickness

Eggshells of C. chitra were examined and were separated into 2 distinct layers through eosin staining. The outer layer was unstained but the inner layer had a pink color from the eosin. It was found that the mean thicknesses of the outer layer and inner layer were 0.14 ± 0.02 mm and 0.12 ± 0.02 mm, respectively (Table 4.1). The thicknesses of the outer and inner layers of the eggshells were significantly different ($p < 0.05$).

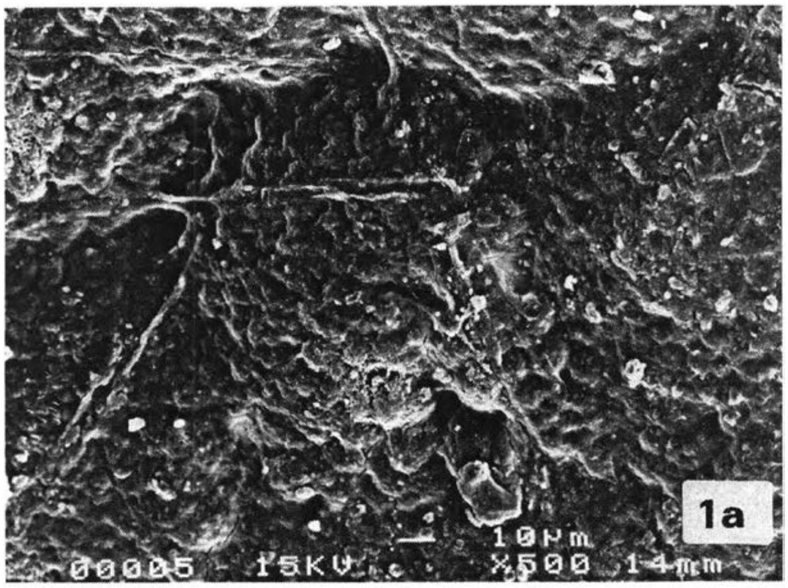
Table 4.1 Outer layer thickness, Inner layer thickness (mm), Energy dispersive x-ray analysis (EDX) (%) and X-ray Diffraction of wild Chitra chitra eggs.

Eggshell Thickness	n	Mean $\pm$ SD
Outer layer thickness (mm)	6	0.14 $\pm$ 0.02
Inner layer thickness (mm)	6	0.12 $\pm$ 0.02
Energy dispersive x-ray analysis (EDX) (%)	9	O = 52.96+4.81 C = 35.03+9.17 Mg = 5.55+0.34 Ca = 5.37+7.16 Si = 2.87+1.64 Al = 2.30+1.07 Na = 0.74+0.3 K = 0.17+0.1
X-ray Diffraction	9	Aragonite

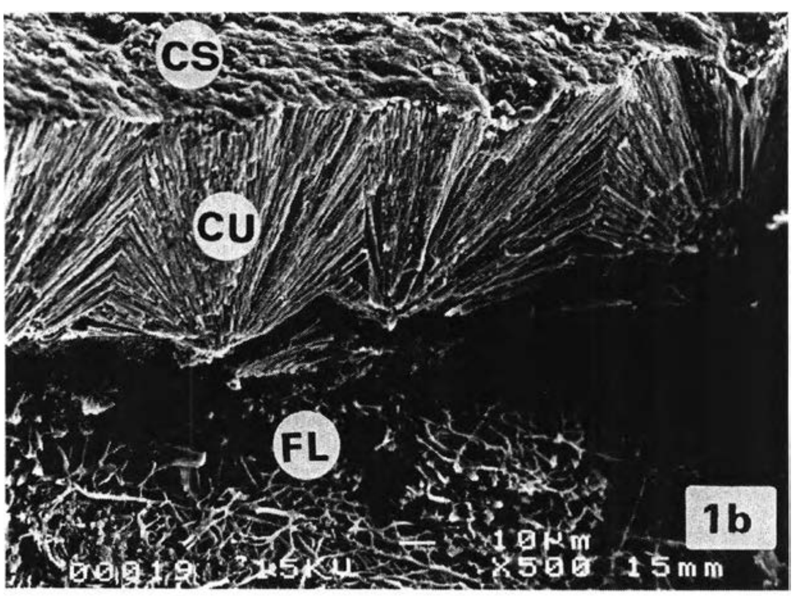
Scanning Electron Microscopy (SEM) Analysis

SEM determined that C. chitra eggshells were divided into three distinct layers – an outer calcareous sheet, a middle crystalline layer, and an inner fibrous layer (Figure 4.1).

The outer surface was composed of a nearly continuous calcareous sheet that covered the whole of the egg (Figure 4.1a). It was a very thin layer that could not be observed under a stereo microscope. The middle layer was composed of fan shaped crystalline units packed together throughout the layer (Figure 4.1b; 4.2). These units displayed the radial fractured symmetry of aragonite projecting from nucleation centers located on the surface of the inner layer. The inner layer of the eggshell was composed of multiple layers of reticular fibers (Figure 4.1b, 4.2, 4.3). Interspersed at intervals along the surface of the calcareous sheet were pore opening which extended downward between the adjacent crystalline units of the middle layer (Figure 4.4).



a



b

Figure 4.1 SEM showing the calcareous sheet (CS) covering the outer layer of eggshell, middle layer crystalline unit (CU) and an inner fibrous layer (FL).

a = top view, b = side view, Bar = 10 µm

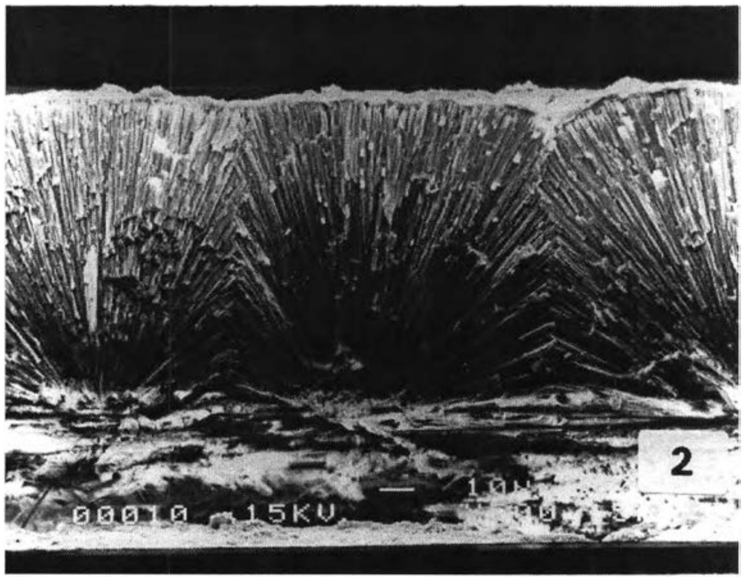
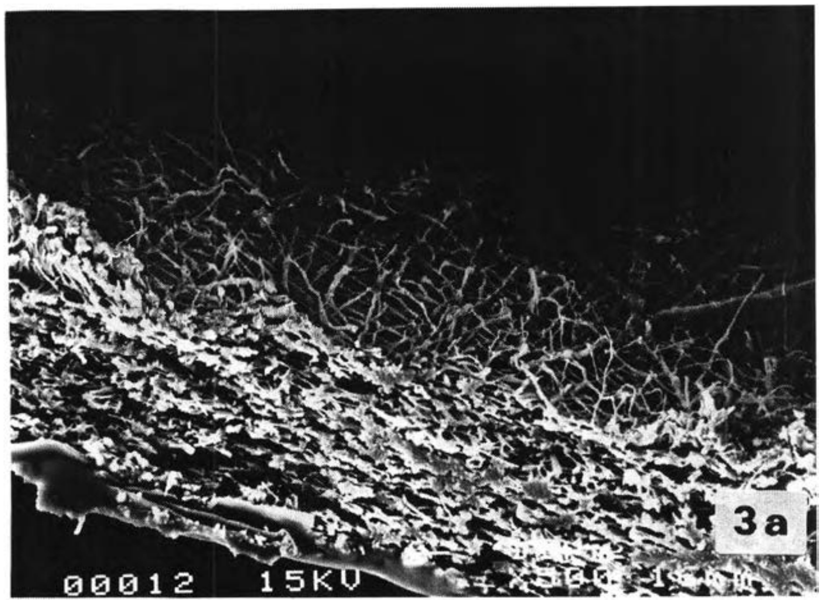
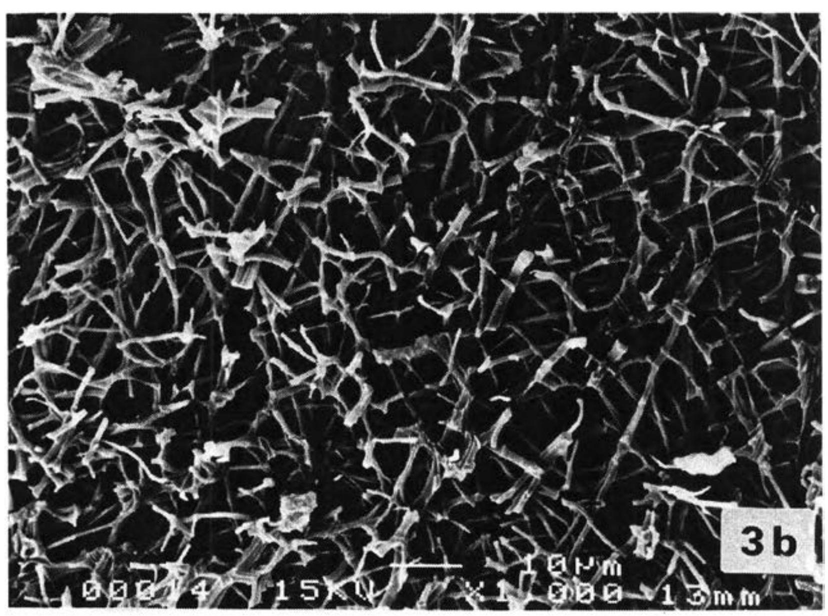


Figure 4.2 SEM showing the radially fractured appearance of crystalline units of the middle layer. Bar = 10 μm



a



b

Figure 4.3 SEM showing the inner layer of the eggshell.

a = side view (Bar = 10 μm), b = top view (Bar = 10 μm)

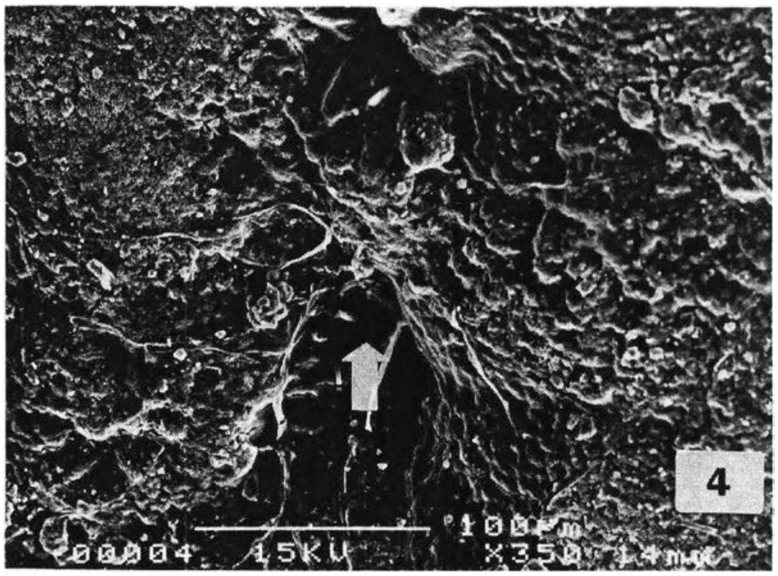


Figure 4.4 Outer surface of C. chitra eggshell showing pore (arrow) at the intersection of crystalline units. (Bar = 100 μm)

Energy Dispersive X-Ray Analysis (EDX)

EDX analysis revealed that C. chitra eggshells were composed of the following elements (ranked in order of relative abundance): oxygen, carbon, magnesium, calcium, silicon, aluminum, sodium and potassium (table 4.1).

X-ray Diffraction Analysis

The powder X-ray photographs indicated that eggshells of C. chitra were composed of the aragonite form of $CaCO_3$.

Discussion

The eggshell of C. chitra displayed only two layers, the middle and inner layers under stereo microscope, but showed 3 layers including an outer calcareous sheet under SEM. Numerous other studies of turtle eggshell structure record the presence of two shell layers. These include broad-shelled river tortoises Chelodina expansa, spiny softshell turtles Apalone spiniferus, olive ridley turtles Lepidochelys olivacea, Euphrates softshells Rafetus euphraticus, European pond turtles Emys orbicularis and radiated tortoises Geochelone radiata (Young, 1950; Packard and Packard, 1979; Woodall, 1984; Wangkulangkul et al., 2000).

Similar to the finding of our study of C. chitra, three layers, were recorded in snapping turtles Chelydra serpentina by Packard (1980). The outer layer of the snapping turtle eggshell was covered by a thick organic calcareous sheet which obscures the tips of the crystalline units of the middle layer. The middle layer was structurally similar to that observed in C. chitra in this study. The outer calcareous sheet may not have been recorded in many turtle eggshells due to the researchers' use of old preserved eggs in their studies. The calcareous sheet may have deteriorated in the preservative over time. The inner layer of C. chitra has multiple layers of fiber similar to those observed in the previous studies of C. serpentina and Trionyx spiniferus (Packard and Packard, 1979; Packard, 1980)

Young (1950) recorded that the eggshell thicknesses of R. euphraticus, Emys orbicularis and G. radiata were 0.23 – 0.29 mm, 0.27 – 0.28 mm and 0.59 – 0.84 mm, respectively. In this study the mean total eggshell thickness of C. chitra was 0.26 mm, falling within the range of eggshell thicknesses of the aquatic turtles Young studied.

Solomon and Baird (1976) analyzed the elements in eggshells of Chelonia mydas. They found the shell composed of ~ 20% Ca, 0.06% Mg, and ~ 1% P. In C. chitra eggshells only ~ 5.37% is composed of Ca but Mg content (5.55%) is substantially higher than that in C. mydas. The reasons for the differences in composition of these species' eggshells are unknown.

The composition of C. chitra eggshells from the wild caught female was $CaCO_3$ in aragonite form. This result was similar to that of other studies of turtle eggshells (Solomon and Baird, 1976; Baird and Solomon, 1979; Packard and Packard;'1979; Packard, 1980; Woodall, 1984; Roberts and Sharp, 1985; and Packard and Hirsch, 1986).

The author is conducting a captive breeding program for C. chitra at Kanchanaburi Inland Fisheries Research and Development Center, Kanchanaburi, Thailand to promote the long-term conservation of this critically endangered species. Knowledge of the structure and elemental composition of the eggshells of C. chitra obtained from wild stock may be useful for comparison with those produced by captive females. The similarity of eggshells of brood stock and wild stock may be a useful indicator to determine nutritional requirements of captives and to increase the likelihood that healthy hatchlings would be produced.

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