

Eggshell Structure of the Siamese Narrow-headed Softshell Turtle *Chitra chitra* Nutphand, 1986 (Testudines: Trionychidae)

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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 ± 0.02 mm and 0.12 ± 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=53.0±4.8%, carbon=35.0±9.2%, magnesium=5.55±0.34%, calcium=5.4±7.2%, silica=2.9±1.6%, aluminum=2.3±1.1%, potassium=0.17±0.1%, and sodium=0.74±0.3%. The eggshell was the aragonite form of CaCO₃.

KEYWORDS: *Chitra chitra*, softshell turtle, eggshell structure, eggshell composition, SEM.

INTRODUCTION

The Siamese narrow-headed softshell turtle *Chitra chitra* Nutphand, 1986 is one of the largest and the least known turtles in the world. Since its recent recognition as a distinct species¹, its population size has almost certainly continued to decline across its limited range in Thailand and elsewhere in Southeast Asia²⁻⁴ due to over fishing, pet trade, population 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 by the authors is designed to rectify this deficiency. As part of our 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 our analysis are presented here, and compared with the few other structural analyses of turtle eggshells which have been published.⁵⁻¹³

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 JSM-6400 scanning electron microscope, operated at 15 kV. 3) **Energy dispersive x-ray analysis (EDX)**; the JSM-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.

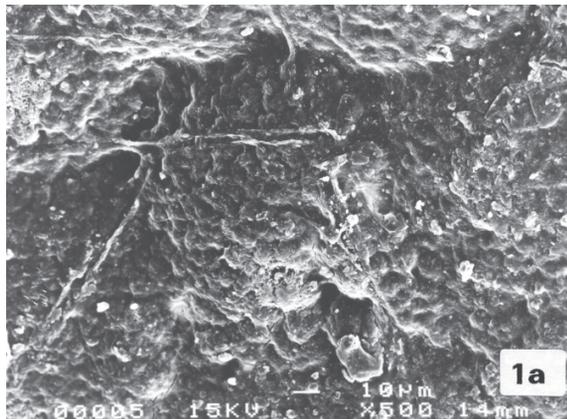
RESULTS

Eggshell thickness

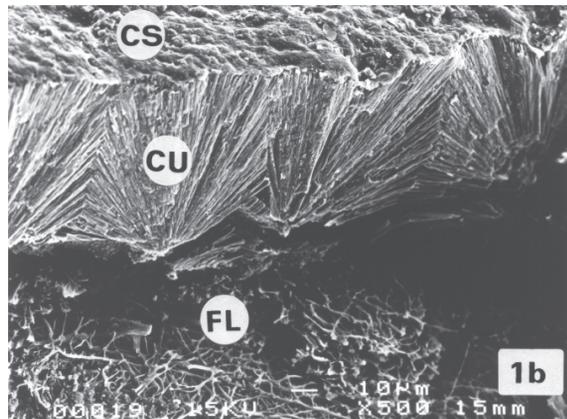
Eggshells of *C. chitra* were examined and were separated into 2 distinct layers through eosin staining. The outer layer was unstained but the inner layer had a pink color from the eosin. It was found that the mean thicknesses of the outer layer and inner layer were 0.14 ± 0.02 mm and 0.12 ± 0.02 mm, respectively (Table 1). The thicknesses of the outer and inner layers of the eggshells were significantly different ($p < 0.05$).

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 (Fig 1). The outer surface was composed of a nearly continuous calcareous sheet that covered the whole egg (Fig 1a). It was a very thin layer that could not be



a



b

Fig 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

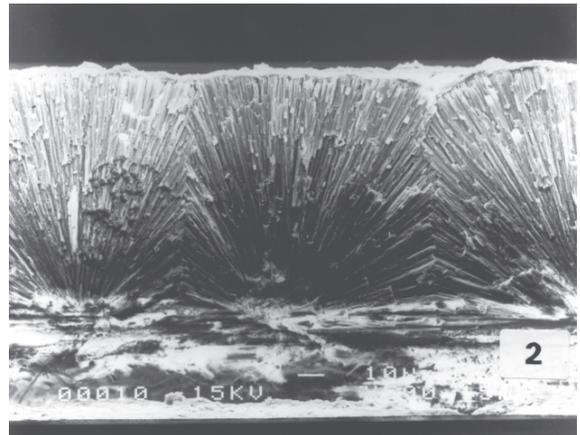
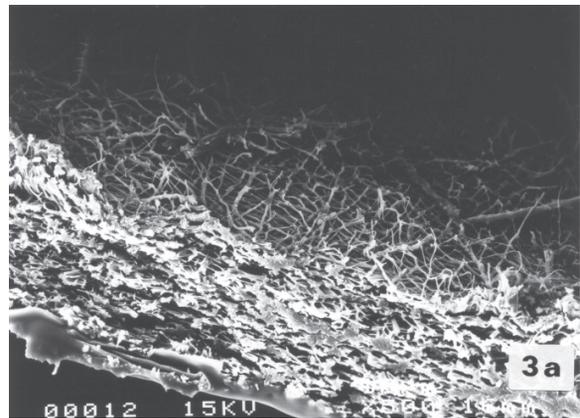
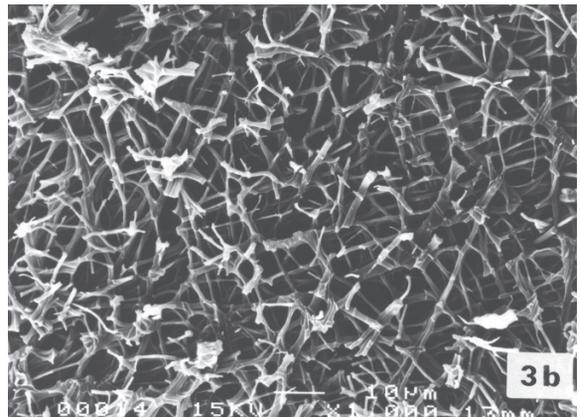


Fig 2. SEM showing the radially fractured appearance of crystalline units of the middle layer. Bar = 10 µm



a



b

Fig 3. SEM showing the inner layer of the eggshell.
a = side view (Bar = 10 µm), b = top view (Bar = 10 µm)

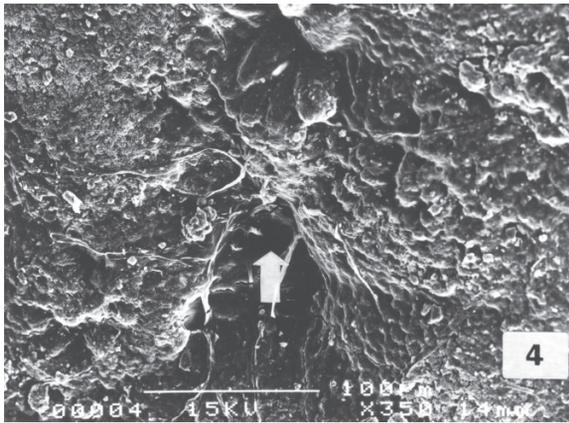


Fig 4. Outer surface of *C. chitra* eggshell showing a pore (arrow) at the intersection of crystalline units. (Bar = 100 μ m)

observed under a stereo-microscope. The middle layer was composed of fan-shaped crystalline units packed together throughout the layer (Fig 1b; 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 (Fig1b; 2, 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 (Fig 4).

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 1).

X-ray Diffraction Analysis

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

Table 1. Outer layer thickness, Inner layer thickness (mm), Energy dispersive x-ray analysis (EDX) (%) and X-ray Diffraction of wild *Chitra chitra* eggs.

| Analysis Method | 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 \pm 4.81 C = 35.03 \pm 9.17 Mg = 5.55 \pm 0.34 Ca = 5.37 \pm 7.16 Si = 2.87 \pm 1.64 Al = 2.30 \pm 1.07 Na = 0.74 \pm 0.3 K = 0.17 \pm 0.1 |
| X-ray Diffraction | 9 | Aragonite |

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*¹⁰, spiny softshell turtles *Apalone spiniferus*⁸, olive ridley turtles *Lepidochelys olivacea*¹³, Euphrates softshells *Rafetus euphraticus*⁵, European pond turtles *Emys orbicularis*⁵, and radiated tortoises *Geochelone radiata*.⁵

Similar to the finding of our study of *C. chitra*, three layers were recorded in snapping turtles *Chelydra serpentina*.⁹ 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*⁹ and *Trionyx spiniferus*.⁸

Young (1950)⁵ 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.⁵

Solomon and Baird (1976)⁶ analyzed the elements in eggshells of *Chelonia mydas* and found that the shell was 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.⁶⁻¹²

The authors are conducting a captive breeding program for *C. chitra* at Kanchanaburi Inland Fisheries 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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REFERENCES

1. Nutphand W (1986) "Man Lai," the world's biggest softshell turtle. *Thai Zoological Magazine* **1(4)**, 64-70. (in Thai)
2. Thirakhupt K and van Dijk PP (1994) Species diversity and conservation of the turtles of Western Thailand. *Nat Hist Bull Siam Soc* **42**, 209-59.
3. Engstrom TN, Shaffer HB and McCord WP (2002) Phylogenetic diversity of endangered and critically endangered Southeast Asian softshell turtles (Trionychidae: Chitra). *Biol Conserv* **104(2)**, 173-9.
4. Kitimasak W and Thirakhupt K (2002) New record of *Chitra chitra* Nutphand, 1986 in Mae Ping River, Thailand. *Nat Hist J Chulalongkorn Univ* **2(1)**, 62-3.
5. Young JD (1950) The structure and some physical properties of the testudinian eggshell. *P Zool Soc Lond* **120**, 455-69.
6. Solomon SE and Baird T (1976) Studies on the egg shell (oviducal and oviposited) of *Chelonia mydas* L. *J Exp Mar Biol Ecol* **22**, 145-60.
7. Baird T and Solomon SE (1979) Calcite and aragonite in the egg shell of *Chelonia mydas* L. *J Exp Mar Biol Ecol* **36**, 295-303.
8. Packard MJ and Packard GC (1979) Structure of the shell and tertiary membranes of eggs of softshell turtles (*Trionyx spiniferus*). *J Morphol* **159**, 131-44.
9. Packard MJ (1980) Ultrastructural Morphology of the shell and shell membrane of eggs of common snapping turtles (*Chelydra serpentina*). *J Morphol* **165**, 187-204.
10. Woodall PF (1984) The structure and some functional aspects of the eggshell of the broad-shelled river tortoise *Chelodina expansa* (Testudinata: Chelidae). *Aust J Zool* **32**, 7-14.
11. Roberts HS and Sharp RM (1985) Preferred orientation of calcite and aragonite in the reptilian eggshells. *P Roy Soc Lond B Bio* **255**, 445-55.
12. Packard MJ and Hirsch KF (1986) Scanning electron microscopy of eggshells of contemporary reptiles. *Scan Electron Micros* **4**, 1581-90.
13. Wangkulangkul S, Thirakhupt K and Chantrapornsyl S (2000) Comparative study of eggshell morphology in wild and captive Olive ridley turtles *Lepidochelys olivacea* at Phuket, Thailand. In: *Sea turtles of the Indo-Pacific: Research, Management and Conservation* (Edited by Pilcher N and Ismail G), pp 208-217. Asean Academic Press, London.