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Diversity in the Matrix Structure of Eggshells in the Testudines (Reptilia)

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The eggshells of 56 chelonians were examined by electron microscopy and X-ray diffractometry. They were classified into six types in terms of the matrix structure of their calcareous layer; type I was composed of a thin calcareous layer with minerals in an amorphous structure; type II with shell units composed of mammillary cores calcified with aragonite crystals; type III with shell units composed of mammillary cores, plus a single palisade layer also calcified with aragonite crystals, and with each shell unit separated; type IV with shell units the same as type III, but tightly packed together; type V with shell units composed of mammillary cores plus two palisade layers; and type VI with a cuticle layer calcified with calcite crystals over the same structure as that of type V. X-ray diffraction analyses at the outer surface of eggshells showed a gradual change in crystal disposition from the random disposition of type II to the single direction-oriented disposition of type V. The shell height was approximately parallel to the development of the palisade-layer matrix. The limiting membrane of all eggshell types was perforated with canals and that of type I was partially missing. Type I had a parchment shell, types II and III had a pliable shell (some were rigid) and types IV to VI had rigid shells. The present study showed that the hardness of eggshells can be determined by the composition of the shell matrices, as shell matrices are the framework for mineralization.

Key words: Testudines, diversity, eggshell, electron microscopy, X-ray diffractometry

INTRODUCTION

It is thought that reptilian ancestors underwent many trial-and-error mutations to develop innovative body structures after adopting terrestrial habitats. Innovation also occurred in egg integuments to keep water inside and protect the egg from deformation, while allowing the minimal passage of gases (Colbert and Morales, 2001; Benton, 2005). The majority of reptiles, including all turtles, are oviparous, and viviparity has evolved only in squamates (Piank and Vitt, 2003). Eggs of oviparous extant reptiles can be assigned to one of three categories on the basis of eggshell structure and egg sensitivity to variations in the hydric environment; the parchment-shelled eggs of lizards and snakes, the pliable-shelled eggs of some turtles, and the rigid-shelled eggs of all crocodylians, some chelonians, and a few lizards (dibamid and gekkonid lizards) (Packard et al., 1982). It may be possible to clarify how this innovation occurred by comparing the structure of eggshells of extant

reptiles.

In the eggs of turtles and tortoises, the eggshell consists of two parts: a fibrous shell membrane and a calcareous layer attached to the outer surface of the membrane. As far as we know, the calcareous component of chelonian eggshells is calcium carbonate, which occurs as aragonite (Packard and Packard, 1979; Packard, 1980; Silyn-Roberts and Sharp, 1985, 1986). Studies of chelonian eggshells have focused on the calcareous layer observed with scanning electron microscopy in some representative species such as the sea turtle *Lepidochelys kempi* (Cheloniidae), snapping turtle *Chelydra serpentina* (Chelydridae), soft-shelled turtle *Trionyx spiniferus* (Trionychidae) and kinosternid turtles *Sternotherus minor*, *Kinosternon flavescens*, *K. bauri*, *K. hirtipes*, and *K. alamosae* (Packard and Packard, 1979, 1980; Packard, 1980; Packard et al., 1984). Although not studied so much, the limiting membrane which demarcates the shell membrane at the interface with the egg white may also be a key structure in the selection of reproduction sites in turtles and tortoises, since the membranes of the Chinese soft-shelled turtle *Pelodiscus sinensis* (Trionychidae) eggs are perforated by many canals (Yoshizaki et al., 2004), whereas the canals are not present in the limiting membrane of avian eggshells (Yoshizaki and Saito, 2002; Mao et al.,

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2006), indicating high water passage through the shell membrane, the main body of which is made up of roughly parallel, intertwining fibers, in chelonian eggs.

Oviparity and incubation in terrestrial nests are the typical modes of reproduction in all turtles and tortoises (Ewert, 1979, 1985; Kuchling, 1999; Dinkelacker and Miller, 2008). There are, however, differences in the nest environments; the nests of sea turtles are 'pear-shaped' on beaches and located above the mean water line, those of most other turtles are 'flask-shaped' in substrates ranging from sand to clay or even rotting vegetation, while some turtles laying rigid-shelled eggs prefer to lay their eggs on the surface of the ground with nothing or only a thin layer of substrate covering them (Ewert, 1979; Ehrenfeld, 1979; Kuchling, 1999; Ackerman and Lott, 2004; Dinkelacker and Miller, 2008).

Parataxonomic classification of fossil eggs and general descriptions of their eggshell structures in amniotic vertebrates, including recent eggs, have been attempted (Hirsch, 1996; Mikhailov, 1997), although only for the hard shell components. Thin calcareous layers and non-calcified shell membranes of parchment and pliable eggshell seem to be more easily affected by weathering, which may alter or replace the original structure in fossil remains (Hirsch, 1996). In the present study, we investigated the eggshells of extant turtles and tortoises using electron microscopy and X-ray diffractometry. In particular, we observed the composition of the decalcified eggshell matrix by transmission electron microscopy, and showed that the structure of the calcareous layer is determined by the conformation of its matrix. This study may contribute to the understanding of the fossil records of parchment- and pliable-shelled eggs.

MATERIALS AND METHODS

Eggshells

Shells from the eggs of 56 chelonian species of two families of Pleurodira and nine families of Cryptodira were collected for this study, and were studied using electron microscopy and X-ray diffractometry. These eggshells were from eggs naturally produced in captivity and from unfertilized eggs, eggs stopped in mid-development, and post-hatched eggs. The spawning maternal individuals were in apparently-normal body condition; however, it was difficult to evaluate whether the individuals were in good health and nutritional condition.

Transfer and usage of the eggshells of International Endangered Species of Wild Fauna and Flora designated in the Law for the Conservation of Endangered Species of Wild Fauna and Flora by the Ministry of the Environment, Japan, were done with compliance with the law. All shells were rinsed, air-dried, and stored at room temperature for varying periods prior to study.

Shell thickness was determined on eggshell fragments using an eggshell thickness gauge (Fujihira Co., Tokyo, Japan). Measurements included the calcareous layer and shell membrane. The thickness of the calcareous layer alone was measured on the scanning electron microscopic images. In elongated eggs, the measurement was done on fragments from the egg equator.

Electron microscopy

For scanning electron microscopy, eggshells were coated with a layer of osmium in a Neoc-ST ion sputter (Meiwa, Tokyo, Japan), and examined using an S-4300 scanning electron microscope (Hitachi, Tokyo, Japan). To demonstrate the elements in the eggshells, the specimens were examined with an energy dispersive X-ray micro analyzer, the EMAX EX-220 (Horiba, Tokyo, Japan), attached to the microscope.

For transmission electron microscopy, eggshells were fixed and decalcified in 2.5% glutaraldehyde (vol/vol) in 0.1 M cacodylate buffer (pH 7.2) containing 0.1 M EDTA for several days at room temperature, rinsed in cacodylate buffer without EDTA, postfixed with 1% osmium tetroxide (wt/vol) in the same buffer, and dehydrated in acetone. Specimens were then embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using an S-7000 transmission electron microscope (Hitachi).

X-ray diffraction

To identify their crystalline phases, the eggshells were subjected to X-ray diffractometry in an X-ray diffractometer, the Rad-2R (Rigaku, Tokyo, Japan). Crystal planes were identified by referring to 75-2230 for aragonite and 85-1108 for calcite in the 2009 JCPDS-International Centre for Diffraction Data.

The X-ray diffraction analyses were conducted separately at the inner or outer surface of the eggshells. In some experiments, analyses at the outer surface were done after cuticle materials were removed with a scraper. Removal of the cuticle materials was confirmed by scanning electron microscopy.

RESULTS

Morphological observations

Chelonian eggshells were classified into six types by characteristics obtained from scanning electron microscopy (SEM) and transmission electron microscopy (TEM), as described below. A summary of eggshells used is shown in Table 1.

Type I: Eggshells of the East African serrated mud turtle *Pelusios sinuatus* (Pelomedusidae) were 77 μm in thickness in the cross section and consisted of a shell membrane and a thin crust (2.9 mm in thickness) of calcareous matter on the surface of the shell membrane (Fig. 1A, B). This crust possessed phosphorous in the same distribution pattern as that of calcium (Fig. 1B, C). The outer surface view by SEM of the crust showed a thin sheet, with many holes, covering a meshwork of shell membrane fibers (Fig. 1D). The inner surface view of the limiting membrane of the shell membrane showed two distinct regions, one was a smooth surface region and the other a region in which the membrane was missing and the shell membrane meshwork was exposed (Fig. 1E).

In the cross section by TEM, each shell membrane fiber consisted of a core in which tiny tubes accumulated, surrounded by cortex (Fig. 1G). The fibers of all chelonian eggshells had the same features. The difference was how each fiber bound to its neighbors. In the shell membrane of *P. sinuatus*, they bound very closely, and thus the space in the meshwork was small, as seen in Fig. 1F. The TEM view of the outer crust of the shell showed a layer consisting of small vesicles and particles (Fig. 1G). The TEM view at the limiting membrane showed many wide canals perforating through the smooth region of the membrane (Fig. 1H).

Type II: Eggshells of the loggerhead turtle *Caretta caretta* (Cheloniidae) were 154 mm in thickness in cross section, and had a calcareous shell in which the shell units (84 μm in height) were randomly and discretely arranged (Fig. 2A, B). In each shell unit, crystals of calcium carbonate were radially arranged (Fig. 2C). The SEM view of the outer surface of the eggshell showed an irregular surface with many pores (Fig. 2D), whereas the view of the inner surface of the shell membrane showed a smooth surface (Fig. 2E).

Table 1. The thickness of the calcareous layer and whole eggshell and classification of eggshell type in chelonian eggs.

Sub-order	Family*	Species	Calcareous layer thickness (μm)** mean \pm SD	Eggshell thickness (μm)** mean \pm SD	Eggshell type	
Pleurodira	Pelomedusidae	<i>Pelusios sinuatus</i>	2.9 \pm 0.5	77 \pm 10	I	
		<i>Pelusios castaneus</i>	75 \pm 3	146 \pm 14	II	
	Chelidae	<i>Acanthochelys radiolata</i>	334 \pm 12	370 \pm 16	V	
		<i>Acanthochelys spixii</i>	353 \pm 17	447 \pm 22	V	
		<i>Chelodina mccordi</i>	301 \pm 4	424 \pm 17	V	
		<i>Chelodina rugosa</i>	313 \pm 12	436 \pm 23	V	
		<i>Elseya branderhorsti</i>	458 \pm 7	632 \pm 46	V	
		<i>Emydura subglobosa</i>	166 \pm 6	354 \pm 36	IV	
		<i>Phrynops geoffroanus</i>	388 \pm 8	452 \pm 29	V	
		<i>Phrynops hilarii</i>	353 \pm 14	468 \pm 33	V	
Cryptodira	Chelydridae	<i>Chelydra serpentina</i>	161 \pm 6	268 \pm 16	III	
	Cheloniidae	<i>Caretta caretta</i>	84 \pm 7	154 \pm 29	II	
		<i>Eretmochelys imbricata</i>	75 \pm 5	204 \pm 11	II	
	Trionychidae	<i>Pelodiscus sinensis</i>	173 \pm 6	230 \pm 26	IV	
	Carettochelyidae	<i>Carettochelys insculpta</i>	458 \pm 6	508 \pm 15	V	
	Kinosternidae	<i>Claudius angustatus</i>	157 \pm 9	270 \pm 23	IV	
		<i>Kinosternon baurii</i>	258 \pm 13	317 \pm 18	V	
		<i>Kinosternon cruentatum</i>	269 \pm 38	358 \pm 59	IV	
		<i>Kinosternon flavescens</i>	155 \pm 7	310 \pm 12	IV	
		<i>Kinosternon leucostomum leucostomum</i>	275 \pm 17	385 \pm 5	IV	
		<i>Kinosternon subrubrum</i>	266 \pm 12	408 \pm 31	V	
		<i>Sternotherus carinatus</i>	265 \pm 5	312 \pm 61	V	
		<i>Sternotherus minor minor</i>	148 \pm 4	238 \pm 23	IV	
		<i>Sternotherus minor peltifer</i>	154 \pm 11	233 \pm 15	IV	
		<i>Staurotypus triporcatus</i>	213 \pm 8	378 \pm 64	IV	
		Emydidae	<i>Emys orbicularis</i>	165 \pm 6	220 \pm 10	IV
			<i>Graptemys flavimaculata</i>	97 \pm 17	171 \pm 17	III
			<i>Terrapene carolina bauri</i>	100 \pm 11	162 \pm 40	III
	<i>Trachemys callirostris callirostris</i>		130 \pm 13	168 \pm 33	III	
	<i>Trachemys scripta elegans</i>		108 \pm 6	146 \pm 11	III	
	Platysternidae	<i>Platysternon megacephalum</i>	212 \pm 9	288 \pm 28	IV	
	Geoemydidae	<i>Cyclemys dentate</i>	565 \pm 19	645 \pm 24	V	
		<i>Cuora flavomarginata</i>	264 \pm 31	328 \pm 12	IV	
		<i>Cuora galbinifrons</i>	210 \pm 30	328 \pm 41	IV	
		<i>Cuora mouhotii</i>	300 \pm 15	462 \pm 46	V	
		<i>Geoemyda japonica</i>	96 \pm 7	175 \pm 5	III	
		<i>Geoemyda spengleri</i>	161 \pm 3	250 \pm 16	IV	
		<i>Mauremys annamensis</i>	174 \pm 13	422 \pm 31	IV	
		<i>Mauremys japonica</i>	105 \pm 10	188 \pm 13	III	
		<i>Mauremys mutica kami</i>	164 \pm 10	309 \pm 10	IV	
		<i>Mauremys reevesii</i>	215 \pm 5	324 \pm 18	IV	
		<i>Orlitia borneensis</i>	585 \pm 22	758 \pm 71	V	
		<i>Sacalia quadriocellata</i>	229 \pm 25	428 \pm 77	IV	
Testudinidae		<i>Chenonoidis carbonaria</i>	444 \pm 11	517 \pm 41	VI	
		<i>Chersine hermanni hermanni</i>	236 \pm 9	298 \pm 31	IV	
		<i>Chersine hermanni boettgeri</i>	197 \pm 5	254 \pm 23	IV	
	<i>Dipsochelys dussumieri</i>	473 \pm 32	672 \pm 48	V		
	<i>Geochelone elegans</i>	424 \pm 21	505 \pm 20	VI		
	<i>Geochelone platynota</i>	410 \pm 12	559 \pm 54	VI		
	<i>Geochelone sulcata</i>	335 \pm 16	493 \pm 18	V		
	<i>Indotestudo elongata</i>	239 \pm 7	383 \pm 23	V		
	<i>Indotestudo forsterii</i>	405 \pm 23	543 \pm 26	V		
	<i>Kinixys belliana nogueyi</i>	435 \pm 23	497 \pm 28	V		
	<i>Malacochersus tornieri</i>	186 \pm 5	401 \pm 24	V		
	<i>Pxyis arachnoides arachnoides</i>	617 \pm 16	673 \pm 12	V		
	<i>Testudo graeca ibera</i>	270 \pm 8	349 \pm 10	V		

*Family and genus are arranged according to the Turtle Taxonomy Working Group (2010).

**Length is expressed by the mean \pm SD of five shell units or five shell pieces from a single egg.

The TEM observation of shell units showed that the proteinaceous fibrils were arranged radially and in several layers, and that their inner tips were firmly attached to the underlying shell membrane (Fig. 2F, G). We call such a structure the mammillary core, using an analogy with that of birds. In the matrix of the shell membrane, fibers were arranged randomly in the plane of the membrane and these planes were stacked, leaving small spaces between them (Fig. 2F). The limiting membrane of the shell membrane was perforated by rather large canals (Fig. 2H).

Type II eggshells were also observed in the eggs of the sea turtle *Eretmochelys imbricate* (Cheloniidae) and the West African mud turtle *Pelusios castaneus* (Pelomedusidae).

Type III: Eggshells of the Florida box turtle *Terrapene carolina bauri* (Emydidae) were 162 μm in thickness in cross section, and had a calcareous shell 100 μm thick. Crystals of calcium carbonate were organized into regularly and discretely arranged shell units (Fig. 3A, B). Thus, the outer surface view by SEM showed a clear boundary between shell units (Fig. 3C).

Under TEM, there was a distinct layer outside the mammillary cores (Fig. 3D). It consisted of an array of fine fibrils that ran perpendicular to the shell surface (Fig. 3E). We call this the palisade layer, after Mikhailov (1997). The precise relationship between the palisade layer and the mammillary cores was

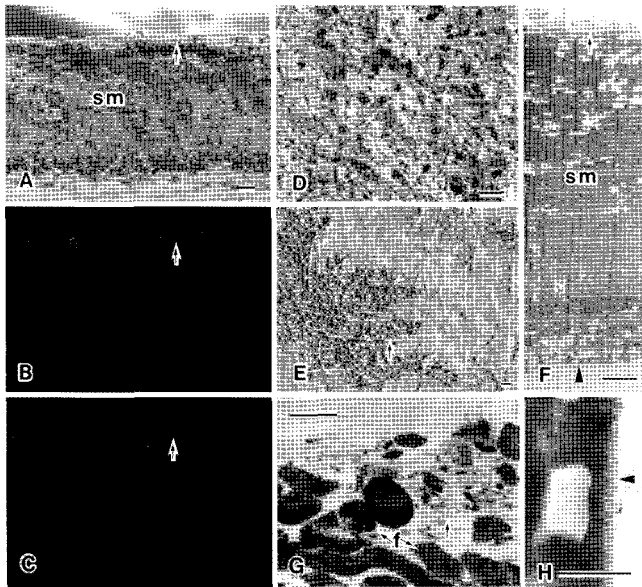


Fig. 1. Scanning (A–E) and transmission (F–H) electron micrographs of *Pelusios sinuatus* eggshells. (A–C) cross section, showing signals of Ca (B) and P (C) in the calcareous layer (arrow). (D) Outer surface of eggshell. (E) Inner surface of a limiting membrane, showing a smooth sheet and its disruption (arrow). (F) A whole cross section view of a decalcified eggshell. The arrow indicates a matrix of the calcareous layer, and the arrowhead indicates a limiting membrane, respectively. (G) The matrix of the calcareous layer (unlabeled arrow), consisting of vesicles and particles. (H) A limiting membrane perforated with canals (arrowhead). f, fiber of shell membrane; sm, shell membrane. Bars are: 10 μm in (A–F); 1 μm in (G, H).

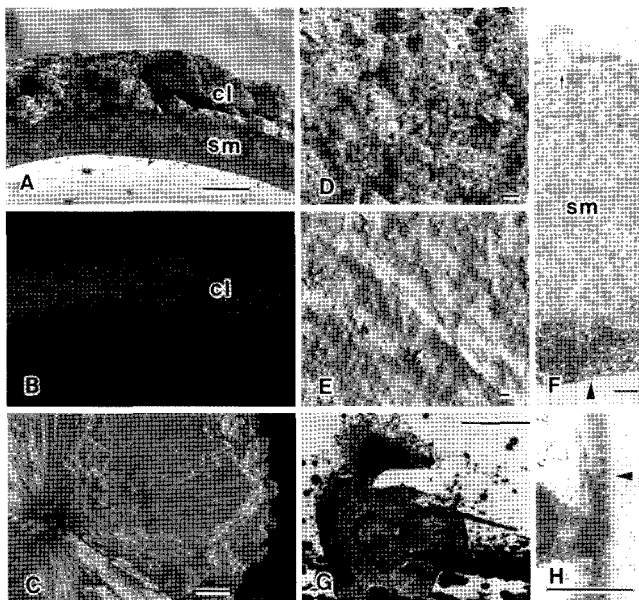


Fig. 2. Scanning (A–E) and transmission (F–H) electron micrographs of *Caretta caretta* eggshells. (A, B) Cross section, showing Ca signals in the calcareous layer (cl). (C) A calcareous shell unit of radiating crystal arrays. (D) Outer surface of an eggshell with many pores. (E) Inner surface of a limiting membrane, showing a smooth sheet. (F) A whole cross-section view of a decalcified eggshell. The arrow indicates a mammillary core and the arrowhead indicates a limiting membrane, respectively. (G) The mammillary core consisting of radially arranged fibrils in several layers. (H) The limiting membrane perforated by many canals (arrowhead). sm, shell membrane. Bars are: 100 μm in (A); 10 μm in (C–G); 1 μm in (H).

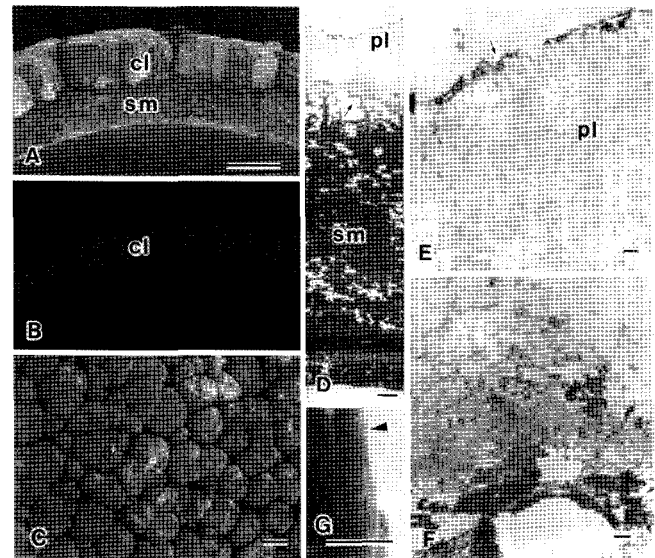


Fig. 3. Scanning (A–C) and transmission (D–G) electron micrographs of *Terrapene carolina bauri* eggshells. (A, B) Cross-section, showing Ca signals in the calcareous layer (cl). (C) Outer surface of an eggshell showing a clear boundary between shell units. (D) A whole cross-section view of a decalcified eggshell. The palisade layer (pl) is located at each mammillary core (arrow). (E) Palisade layer consisting of an array of fine fibrils that run perpendicular to the shell surface. The arrow indicates an accumulation of electron-dense materials at the outermost surface. (F) Mammillary core consisting of radially arranged fibrils. (G) A limiting membrane perforated by canals (arrowhead). Egg white remains at the innermost surface. sm, shell membrane. Bars are: 100 μm in (A–C); 10 μm in (D); 1 μm in (E–G).

unclear, as bubbles produced during decalcification with EDTA destroyed the connection between the two. However, a single line of crystal growth from sites of nucleation to the outermost shell surface indicated a close connection between the fibrils of the palisade layer and those of the mammillary cores (Fig. 3F).

Very electron-dense materials were observed at the outermost surface of the eggshells (Fig. 3E). Such materials were also observed in the eggshells of the other types of, but not all, turtle eggshells. At the innermost surface of the shell membrane, canals were present in the limiting membrane (Fig. 3G).

Other than *T. carolina bauri*, turtles with eggshells of type III were the common snapping turtle *Chelydra serpentina* (Chelydridae), yellow-blotched map turtle *Graptemys flavimaculata*, Colombian slider *Trachemys callirostris callirostris*, and red-eared slider *T. scripta elegans* (Emydidae), and the Japanese pond turtle *Mauremys japonica* and Ryukyu leaf turtle *Geoemyda japonica* (Geoemydidae) in the present study.

Type IV: Eggshells of the stripe-necked musk turtle *Sternotherus minor peltifer* (Kinosternidae) were 233 μm thick in cross section, and had a calcareous shell 154 μm thick. The calcareous shell layer was formed of tightly packed shell units (Fig. 4A, B). The interlocking of crystallites of adjacent shell units rendered the calcareous layer rigid and the outermost surface of the eggshell smooth (Fig. 4D). The interlocking sites were buried by a mixture of com-

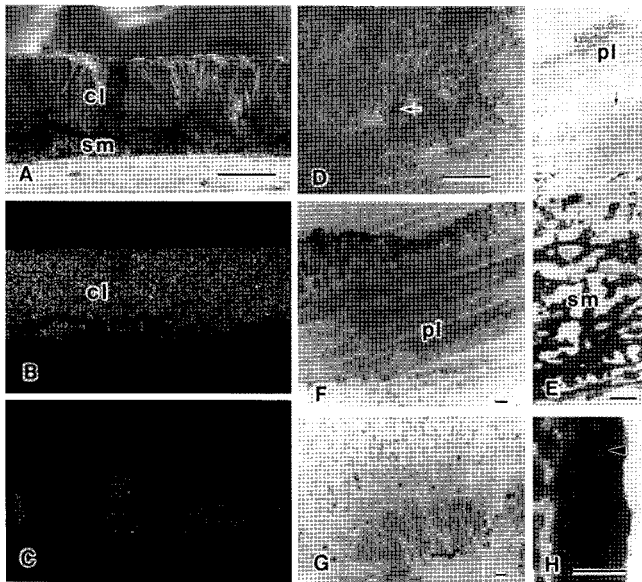


Fig. 4. Scanning (A–D) and transmission (E–H) electron micrographs of *Sternotherus minor peltifer* eggshells. (A–C) Cross-section showing signals of Ca (B) and P (C) in the calcareous layer (cl). (D) Outer surface of an eggshell showing a smooth surface with a pore (arrow). (E) A whole cross section view of a decalcified eggshell. A single sheet of the palisade layer (pl) is located above the mammillary core (arrow). (F) The palisade layer with fibrils running perpendicular to the shell surface. Sub-layers may be seen owing to an oblique section of neighboring shell units. (G) Mamillary core of a radial arrangement of fibrils. (H) A limiting membrane perforated by canals (arrowhead). Remaining egg white adheres to the right. sm, shell membrane. Bars are: 100 μm in (A–D); 10 μm in (E); 1 μm in (F–H).

pounds of calcium and phosphorus (Fig. 4B, C).

The TEM observations showed a palisade layer, which exhibited unity in a single eggshell; when decalcified, the palisade layer had a tendency to separate as a single sheet from the remaining mammillary cores and shell membrane (Fig. 4E–G). The spaces in the meshwork of the shell membrane matrix were huge. Canals running through the limiting membrane were present (Fig. 4H).

Type IV eggshells were observed in the eggs of most turtles belonging to Kinosternidae and Geoemydidae in the present study (Table 1). However, the presence of phosphorus was limited to the Eastern mud turtle *Kinosternon subrubrum*, striped mud turtle *K. baurii*, yellow mud turtle *K. flavescens* and stripe-necked musk turtle, all belonging to the Kinosternidae.

Type V: Eggshells of the Western hinge-back tortoise *Kinixys belliana nogueyi* (Testudinidae) were 497 μm thick in cross section, and had a calcareous shell 435 μm thick. With SEM observation, there was an interruption in the crystal disposition of shell units (Fig. 5A, B). This suggested that matrices of a different nature were present at regions outside and inside the boundary. Cracks in the section seemed to occur at different crystal planes of the shell units. The outer surface of the eggshells was rather smooth, so the existence of pores was apparent (Fig. 5C).

Using TEM, the matrix of the calcareous layer was thick and differentiated into two palisade layers (Fig. 5D). We call

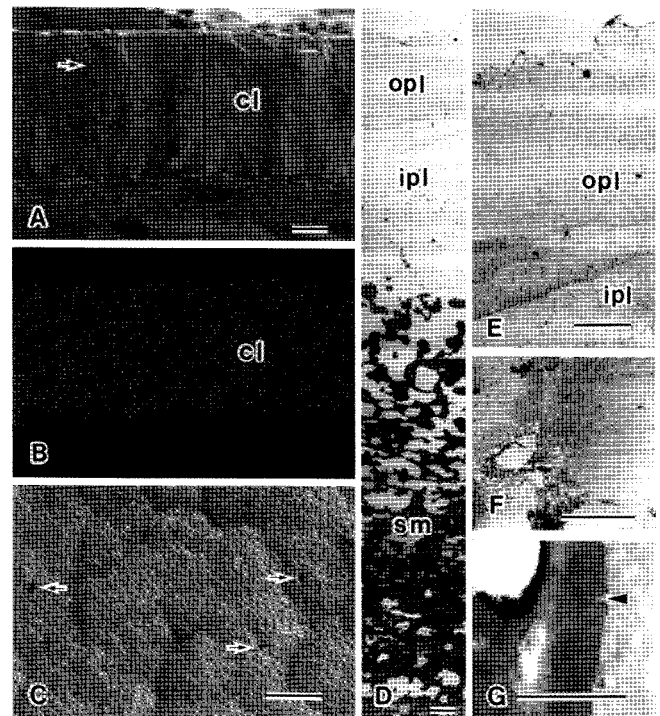


Fig. 5. Scanning (A–C) and transmission (D–G) electron micrographs of *Kinixys belliana nogueyi* eggshells. (A, B) Cross section showing Ca signals in the calcareous layer (cl). The arrow indicates an interruption in the crystal disposition of shell units. (C) Outer surface of eggshell with pores (arrows). (D) A whole cross section view of a decalcified eggshell. The palisade layer is separated into two sheets, outer (opl) and inner (ipl). (E) The outer and inner palisade layers both consisting of fibrils running perpendicular to the egg surface. (F) The mammillary core. (G) A limiting membrane perforated with canals (arrowhead). sm: shell membrane. Bars are: 100 μm in (A–C); 10 μm in (D–F); 1 μm in (G).

these the outer palisade layer and the inner palisade layer, and they led to the mammillary core. Fibrils of both layers ran perpendicular to the egg surface (Fig. 5E). Mammillary cores consisted of a radial array of fibrils on the outside of the shell membrane (Fig. 5F). Canals running through the limiting membrane seemed to be narrow and twisted because regular disposition was not observed (Fig. 5G).

Type V eggshells were observed in the eggs of most turtles belonging to Chelidae and tortoises belonging to Testudinidae, and in some turtles of other families, in the present study (Table 1).

In most eggshells of type V, the crystallization of calcium carbonates occurred consecutively through the mammillary cores and the inner and outer palisade layers, whereas in the eggshells of the Geoffroy's toad-headed turtle *Phrynops geoffroanus*, black spiny-necked swamp turtle *Acanthochelys spixii*, Branderhorst's turtle *Elseya branderhorsti* (Chelidae) and the common Asian leaf turtle *Cyclemys dentata* (Geoemydidae), the crystallization was interrupted at the boundary between the inner and the outer palisade layers, and the second nucleation seemed to occur at the outer palisade layer.

Type VI: The eggshells of three tortoises, the red-footed tortoise *Chelonoidie carbonaria* (Fig. 6A), Indian star tor-

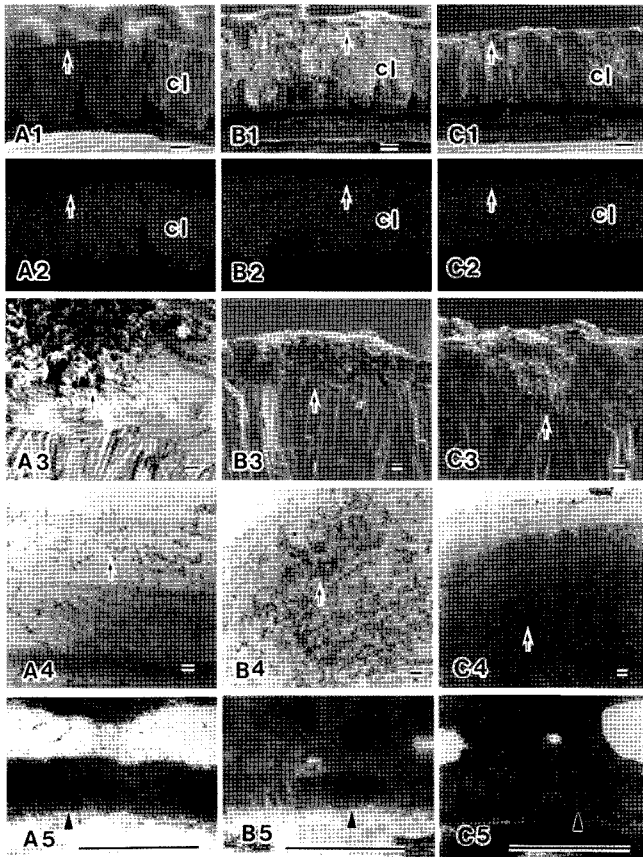


Fig. 6. Electron micrographs of *Chelonoidis carbonaria* (A), *Geochelone elegans* (B) and *G. platynota* (C) eggshells. (A₁–C₁, A₂–C₂) Scanning electron micrographs of cross sections showing Ca signals in calcareous layers (cl). Arrows indicate a layer of the cuticle that is distinct from the underlying layer. (A₃–C₃) Enlarged view at the cuticle layer (arrows). (A₄–C₄) Transmission electron micrographs of cross sections of the cuticle layer showing an accumulation of vesicles and fibrils (arrows). (A₅–C₅) Limiting membranes perforated with canals (arrowheads). Bars are: 100 μm in (A₁–C₁); 10 μm in (A₃–C₃); 1 μm in (A₄–C₄) and (A₅–C₅).

toise *Geochelone elegans* (Fig. 6B) and the Burmese star tortoise *G. platynota* (Fig. 6C) had a thin cuticle layer, similar to that of birds, at their outermost surface. The cuticle layer was calcified (Fig. 6A₁–C₁, A₂–C₂). However, it was apparently different from the underlying calcareous layer at the outer palisade layer in the SEM images (Fig. 6A₃–C₃): The cuticle materials of the red-footed tortoise formed small crystal bodies that were separated from neighbors and easily removed from the palisade layers (Fig. 6A₃), while those of star tortoises merely accumulated with an irregular disposition (Fig. 6B₃, C₃). From the TEM view, the cuticle substrate showed an accumulation of particles and small vesicles that were connected by fine fibrils (Fig. 6A₄–C₄). Canals in the limiting membrane were present at the innermost surface of the shell membrane (6A₅–C₅). Characteristics of type VI eggshells, other than the cuticle layer, were mostly the same as those of type V eggshells.

Observations with X-ray diffractometry

When the X-ray diffraction analysis was done at the inner surface of eggshells, no specific signal was observed

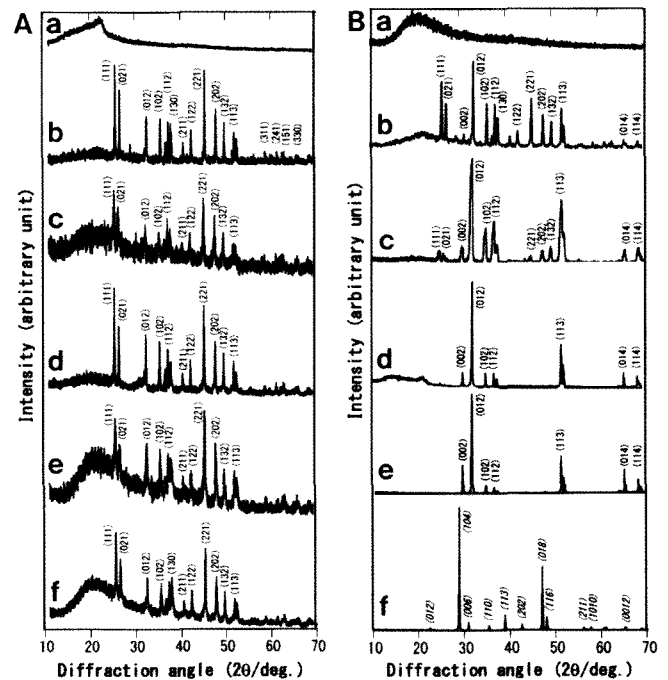


Fig. 7. X-ray diffraction profiles of eggshells measured at the inner (A) or the outer surface (B). The planes of aragonite diffraction peaks are shown in regular font, and those of calcite are in italic font. Line a, type I eggshell of *Pelusios sinuatus*; line b, type II eggshell of *Caretta caretta*; line c, type III eggshell of *Terrapene carolina bauri*; line d, type IV eggshell of *Staurotypus triporcatus*; line e, type V eggshell of *Kinixys belliana nogueyi*; line f, type VI eggshell of *Chelonoidis carbonaria*.

in the type I eggshells of *P. sinuatus*, whereas the profiles of all other types of eggshell were aragonite crystal calcium carbonates (Fig. 7A); the main peaks were {111}, {021}, {012}, {102}, {112}, {130}, {122}, {221}, {202}, {132} and {113}. The differences in intensity were small among these peaks, and this pattern was mostly the same in eggshells of types II to VI.

The analyses at the outer surface of eggshells revealed no peaks in type I eggshells of *P. sinuatus* (Fig. 7B, line a). However, the analyses in eggshells of types II to V showed profiles that were basically of aragonite form, but variously deformed; in the type II eggshell of *C. caretta*, their outer-surface profiles were mostly the same as those at their inner surface (line b); in the type III eggshell of *T. carolina bauri*, peaks {111}, {021}, {122}, {221}, {202} and {132} were reduced in intensity, but peaks {002} and {014} appeared, and peak {012} was the highest (line c); in the type IV eggshells of the Mexican giant musk turtle *Staurotypus triporcatus* (Kinosternidae), peaks {111}, {021}, {221}, {202} and {132} completely disappeared, but peak {002} was intensified to a level comparable to the intensity of peaks {102} and {112} (line d); in the type V eggshells of *K. belliana nogueyi*, the peak intensity of {002} was higher than those of {102} and {112} (line e). This was increased to a level comparable to the intensity of {012} in the eggshells of the Roti island long-necked turtle *Chelodina mccordi* (Chelidae), pig-nose turtle *Carettochelys insculpta* (Carettochelyidae), common Asian leaf turtle *C. dentata* and Bornean river turtle *Orlitia*

borneensis (Geoemydidae), and pancake tortoise *Malacochersus tornieri* and common spider tortoise *Pyxis arachnoids arachnoids* (Testudinidae) (lines not shown).

Analyses at the outer surface of eggshells of type VI produced profiles of calcite crystals of calcium carbonates (Fig. 7B, line f); the main peaks were {104}, {006}, {110}, {113}, {202}, {018} and {116}. When analysis was done on the eggshells from which the cuticle layer was removed, the profile was of aragonite crystals, indicating that the calcite profile of type VI eggshells originated from calcium carbonates in the cuticle layer.

DISCUSSION

Chelonian eggshells were classified into six types on the basis of the matrix structure of their calcareous layers in the present study, and are schematically represented in Fig. 8; the calcareous layer of type I eggshells consisted of small vesicles and particles, and was mineralized with calcium and phosphorus non-crystal compounds. That of type II eggshells consisted of shell units only of the mammillary core layer made of radially arranged fibrils, and was calcified with calcium carbonates of aragonite crystals. That of type III eggshells consisted of shell units of the mammillary core layer plus a palisade layer which was consecutively arranged by fibrils, calcified in the form of aragonite crystals, and of which the shell unit maintained individuality; that of type IV eggshells consisted of the same matrix structure as that of type III eggshells, but its shell units were tightly packed with each other. That of type V eggshells consisted of shell units of the mammillary core layer plus two palisade layers, all calcified with aragonite crystals. That of type VI eggshells consisted of a type V calcareous layer plus a cuticle layer which was calcified with calcite crystals.

Eggshell structure may be related to the environment of the nest in which turtles and tortoises lay eggs. Most chelonian eggs are covered with some substrate at nest sites (Ewert, 1979; Ehrenfeld, 1979; Kuchling, 1999; Ackerman and Lott, 2004; Dinkelacker and Miller, 2008). Therefore, two factors may be tested in the evolution of eggshells: regulation of water and/or gas permeability and resistance to the weight of the covering.

Sufficient water is necessary for eggs to develop. The limiting membrane of the shell membrane faces the egg white, which includes water. This membrane was commonly perforated by canals in all types of chelonian eggshells. In addition, in type I eggshells the membrane itself was absent in several places, indicating poor potential to retain water compared with that of avian eggs in which the limiting membrane is constructed by homogeneous, dense material with no canals (Yoshizaki and Saito, 2002; Yoshizaki et al., 2004; Mao et al., 2006). The main body of the shell membrane is made up of roughly parallel, intertwining fibers (Yoshizaki et al., 2004), suggesting a similar low potential for waterproofing. However, at least in types I to III eggshells, the fibers bound very closely and the spaces in the meshwork were small. In these types of eggshells, the shell membrane likely plays the main role in retaining water. The calcareous material was organized into discrete shell units and numerous

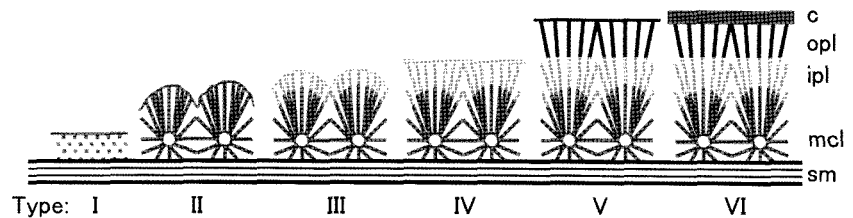


Fig. 8. Schematic representation of the matrix structure of chelonian eggshells. c, cuticle; ipl, inner palisade layer; mcl, mammillary core layer; opl, outer palisade layer; sm, shell membrane. Explanation in text.

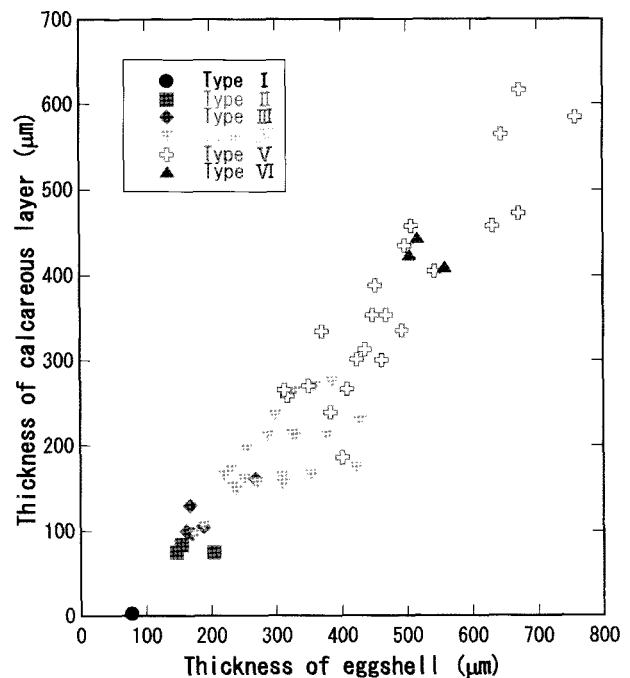


Fig. 9. Scatter plot of eggshells regarding the thickness of the calcareous layer and eggshell. Data come from Table 1.

pores penetrated the calcareous layer in types II and III eggshells, whereas in types IV to VI eggshells, the calcareous layer was formed of tightly packed shell units with a few pores open in the layer. The eggshells of types IV to VI had the most elaborate form of waterproofing in chelonian eggs.

According to the classical criteria used to classify eggshells from the viewpoint of shell hardness (Packard and Packard, 1980), type I eggshells have a parchment shell, types II and III eggshells have a pliable shell (some type III eggshells are a rigid shell) and types IV to VI eggshells have a rigid shell. The thickness of the calcareous layer of chelonian eggs in Table 1 is plotted against the thickness of the whole eggshell in Fig. 9. Eggshells of each type seemed to distribute as a group, and these groups were roughly separated from each other. Figure 9 also shows that the thickness of the calcareous layer and/or the eggshell increased along with an increase in the type number. As a result, the hardness could be represented by the thickness of the calcareous layer, and the thickness determined by the composition of the shell matrices. The X-ray diffraction profiles of the inner surfaces of eggshells were of aragonite crystals of calcium carbonates in all shell units of types II to VI egg-

shells. The intensities of their main peaks were mostly similar, suggesting a random disposition of crystal faces, probably at the mammillary cores. On the other hand, the profiles observed at the outer surfaces of eggshells varied from the profile of the random crystal disposition in type II eggshells of sea turtles to the profile of some specifically intensified peaks in type V tortoise eggshells. Since calcium crystallization builds up from the mammillary core to the palisade layer, and since crystal growth toward the side of shell unit is restricted by neighbors, the crystal planes are arranged in a single direction, as the thickness of the shell unit increases, with the exception of some turtles, *P. geoffroanus*, *A. spixii*, *E. branderhorsti* (Chelidae) and *C. dentata* (Geoemydidae), in which the second crystallization nucleation occurs at sites on the inner palisade layer; their X-ray diffraction profiles at the outer surfaces of the eggshells are like those of numbers III–IV. Therefore, the thickness of the shell unit is approximately parallel to the length of the matrix fibrils of the palisade layer.

The cuticle is found in all bird eggshells (Burley and Vadehra, 1989). The matrix of the cuticle layer is composed of vesicles in a variety of sizes, and if calcified, calcite crystals or a mixture of calcite and vaterite crystals are formed (Kusuda et al., 2011), but if phosphorus is present together with calcium, hydroxyapatite is formed in the domestic fowl *Gallus gallus* (Dennis et al., 1996), or no crystals are formed in the Japanese quail *Coturnix japonica*, the greater flamingo *Phoenicopterus ruber roseus* and the Humboldt penguin *Spheniscus humboldti* (Kusuda et al., 2011). Its function is speculated to be controlling the passage of water and gases, as a lubricant to facilitate egg rotation in the oviduct, and to maintain eggshell strength (Kusuda et al., 2011). The cuticle is absent in the eggshells of most turtles and tortoises, in which the surface of the eggshell is demarcated with outermost matrix materials of unknown nature. The cuticle was found in the type VI eggshells of three tortoises in the present study, with a matrix composed of minute vesicles and particles calcified with calcite crystals.

The eggshells of most lizards and all snakes are categorized as a parchment shell that is thin, highly extendable and with little or no calcareous layer (Packard et al., 1982). The mammillary core is absent in their eggshells and the crystal form is calcite if calcified (Kusuda and Yoshizaki's unpublished data). These eggs are tightly coupled to the hydric environment, with eggs exposed to favorable hydric conditions exhibiting higher hatching success (Packard et al., 1982). In contrast, the eggshells of all crocodylians are categorized as a rigid shell that is non-compliant, and are calcified with calcite crystals, the characteristics being similar to those of birds (Silyn-Roberts and Sharp, 1985, 1986). These eggs are independent of the hydric environment by minimizing the potential for water loss (Packard et al., 1982). Chelonian eggshells are widely differentiated in morphology from a parchment shell similar to that of snakes to a rigid shell similar to that of crocodylians. The sensitivity of eggs to variations in the hydric environment (Packard and Packard, 1980; Packard et al., 1982) and the water vapor conductance of eggshells (Packard et al., 1979) settle similarly at intermediate values between those of the two groups. The evolution of the chelonians may be not only an adaptation for terrestrial life, but for egg incubation on land as well.

Table 1 suggests that the evolution of chelonian eggshells occurred independently at the level of a suborder (between Pleurodira and Cryptodira). Most extant species of the Testudines produce eggs with a rigid shells such as types IV–VI eggshells in the present study, whereas a few produce eggs with a soft shell, types I–III eggshells. From an evolutionary viewpoint, the turtles of Pelomedusidae, Chelydridae and Cheloniidae, which produce soft-shelled eggs, may maintain primitive characteristics of egg integuments, but some species of Emydidae and Geoemydidae, which similarly produce soft-shelled eggs, may reverse to produce primitive-type integuments after acquisition of a rigid shell. Tortoises of Testudinidae all produce eggs with a rigid shell. In particular, the eggshell structure categorized as type VI of *C. carbonaria*, *G. elegans* and *G. platynota* is very similar to that of avian eggs. This may indicate a reproductive adaptation to land living by Testudines. However, no distinct interrelationship between the eggshell type and adult habitat is apparent in turtles at present. It is necessary to further investigate eggshells of many species of Testudines and the detailed environment of their nests.

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