Structural changes in olive ridley turtle eggshells during embryonic development

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We examined the chemical composition and ultrastructure of the eggshells of olive ridley turtles, *Lepidochelys olivacea*, at various stages of embryonic development (freshly laid, 42 days developed and hatched). The eggshell is mainly composed of calcium carbonate present in its aragonite morph, and serves as a source of calcium for the developing embryo. Gradual utilization of eggshell calcium by the developing embryo was reflected by sequential reduction of calcium content from the fresh eggshell (191±32 mg), through 42 days into development (151±36 mg), to hatching (69±11 mg). Structurally, the shells of olive ridley turtle eggs were composed of an outer inorganic calcareous layer, an inner organic fibrous layer and a thin boundary layer that enclosed the egg contents. Calcium resorption by the developing embryo also brought about remarkable modifications in the structure of the eggshell. Fresh eggshells were composed of closely spaced, organized nodular shell units, disoriented spicules and a dense mat of membrane fibres. After 42 days of incubation, these nodules changed to polygonal units with large inter-spaces and loose fibrilliar membranes, whereas the hatched eggshell appeared amorphous and homogenous with disoriented membrane fibres. A 37% decrease in shell thickness (150±11 μm in fresh to about 100±6 μm in hatched) was observed between laying and hatching. This decrease in shell thickness and structural modifications, with respect to compositional changes, were the effect of calcium resorption from the eggshell by the embryo during its development.

Key words: aragonite, calcium, *Lepidochelys olivacea*, membrane layer, shell units

INTRODUCTION

The generic term "eggshell" refers to all layers of a freshly laid egg external to the albumen. The eggshells of all reptiles and birds are constructed according to a similar basic plan, consisting of an inner organic membrane overlaid by an inorganic calcareous layer, and finally by a thin cuticular layer (Packard & DeMacro, 2004). All marine and some freshwater turtles have flexible-shelled eggs (Packard et al., 1982; Hirsch, 1983; Schleich & Kastle, 1988), and so differ from several other freshwater/terrestrial turtle, crocodilian and avian eggs that have rigid shells. Structural details of sea turtle eggshells are available for green turtles (*Chelonia mydas*; Solomon & Baird, 1976, 1977; Taher et al., 2003), leatherback turtles (*Dermochelys coriacea*; Solomon and Watt, 1985; Solomon & Tippett, 1987; Chan & Solomon, 1989), loggerhead turtles (*Caretta caretta*; Schleich & Kastle, 1988), olive ridleys (*Lepidochelys olivacea*; Sahoo et al., 1996a,b; Wangkulangul et al., 2000), and Kemp's ridley turtles (*L. kempii*, Hirsch, 1983). The eggshells of marine turtles contain more than 20% calcium as a major inorganic constituent. Although reports are available on the possible resorption of eggshell calcium by the developing embryo (Packard & Packard, 1979, 1991; Packard, 1980; Miller & Jones, 1990; Lawniczak & Teece, 2005), the structural changes the eggshell undergoes during embryonic development have not been studied so far. Simkiss (1962) described the eggshell to be a source of calcium for developing leatherback embryos, but found no detectable change in the structure of the eggshell before and after successful incubation. However, studies by Sahoo et al. (1998) in olive ridley turtles have demonstrated that the eggshell provides about 60% of the embryonic calcium requirement. This paper describes the general morphology of olive ridley turtle eggshells, and changes due to calcium utilization at different stages of embryonic development.

MATERIALS AND METHODS

The samples for the present study were collected from Gahirmatha nesting beach, the world's largest rookery for olive ridley turtles, located along the east coast of India. A freshly deposited egg clutch (laid very close to the high tide water mark) containing 127 eggs was collected. The eggs were incubated under laboratory conditions in a BOD incubator at 29.5 °C within 36 hours of oviposition (Silas & Rajagopalan, 1984; Dash & Kar, 1990; Mohany-Hejmadi, 1993). Batches of 10 eggs were wrapped with two layers of moist cotton and kept in different enamel pans. The cotton was changed daily to prevent infection.

To investigate compositional and structural changes, the shells were removed from egg contents, cleaned and air-dried at various stages of development as identified by Miller (1985) and Behera (1989). The stages were 1 (fresh egg), 15, 19, 23, 28, 30, 32, 35 and 38 (hatched) representing 0, 14, 19, 26, 34, 38, 42, 53 and 57 days of incubation, respectively. For each stage, three eggs were removed from the incubated egg clutch. Samples in tripli-

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Table 1. Compositional variation (mean ± standard deviation) in *Lepidochelys olivacea* eggshells at three different stages of embryonic development (*n*=18 each).

Egg shell type	Wet weight (g)	Dry weight (g)	$CaCO_3$ (%)	Ca (mg)	Mg (%)	S (%)	K (%)
Fresh	1.40±0.14	0.76±0.05	52.70±1.26	191±32	0.056±0.005	1.143±0.174	0.049±0.004
42 days developed	0.86±0.08	0.42±0.02	46.10±1.73	151±36	0.054±0.004	0.935±0.152	0.057±0.006
Hatched	0.54±0.03	0.23±0.01	23.85±3.01	69±11	0.044±0.080	0.933±0.104	0.042±0.010

cate were also collected at the above nine developmental stages from five marked nests from their natural environment at Gahirmatha. Thus, for each stage, six sets of samples (one laboratory set and five sets from the wild), each in triplicate, were available. During the study, reductions in eggshell calcium content were noticed from the 34th day of incubation, and structural changes became prominent by day 42, continuing thereafter until hatching (detailed data not presented). Hence, the structural details during three critical stages (fresh, 42 days developed and hatched eggshells) are presented in detail below. For chemical analyses, all 18 samples were used; for mineral phases and structural analyses, 12 samples (two from each clutch) were studied.

Conventional analytical techniques were followed to determine the constituents of eggshells. Triplicate samples were dried at 80 °C in an oven to constant weight and digested following the procedure of Giesey & Weiner (1978). One gram of each sample was heated at 100 °C with 20 ml of concentrated nitric acid, cooled and reheated to dryness with 5 ml of the acid. After cooling, 10 ml of 30% hydrogen peroxide was added and again heated for 30 min until a clear solution was obtained. Distilled water was added to reconstitute the samples, which were then filtered and diluted to 100 ml. We used a standard complexometric titration method for the determination of calcium. We added 10 ml of 20% triethanolamine, 10 ml of 20% hydroxylamine chloride and 15 ml of ammonia to 25 ml of the digestate (Vogel, 1978), which was titrated against 0.02 M ethylene diamine tetrachloro acetic acid (EDTA) using a thymolphthalexone indicator. Magnesium and potassium were determined by an atomic absorption spectrophotometer (Varian 1475) and flame photometer (Systronics-KIII), respectively. The presence of phosphorus was examined by a visible spectrophotometer (Chemito-2500) at 882 nm using the phosphomolybdenum blue complex method (FAO, 1975).

The major mineral phase in the samples was determined from their XRD pattern, using an X-ray automatic diffractometer (PW-1710) operated at 35 kV and 25 mA with CuKα radiation, and their IR spectral pattern using a Jasco FTIR-5300 spectrometer with KBr pressed palate technique. Ultrastructural studies were carried out using a Jeol scanning electron microscope (JSM 35 CF) with working voltage ranging from 8 to 25 kV. For this purpose, samples from the outer surface, radial section and inner surface of the eggshells were mounted on studs with double-sided adhesive carbon tape and coated with gold for 2 min using an ion sputter (JFC-1100).

We conducted tests for homogeneity of variance, one-way ANOVA and Tamhane's post-hoc tests for comparing the differences in eggshell calcium concentration and total eggshell thickness at the three developmental stages using SPSS 15.0 (SPSS Inc., Chicago).

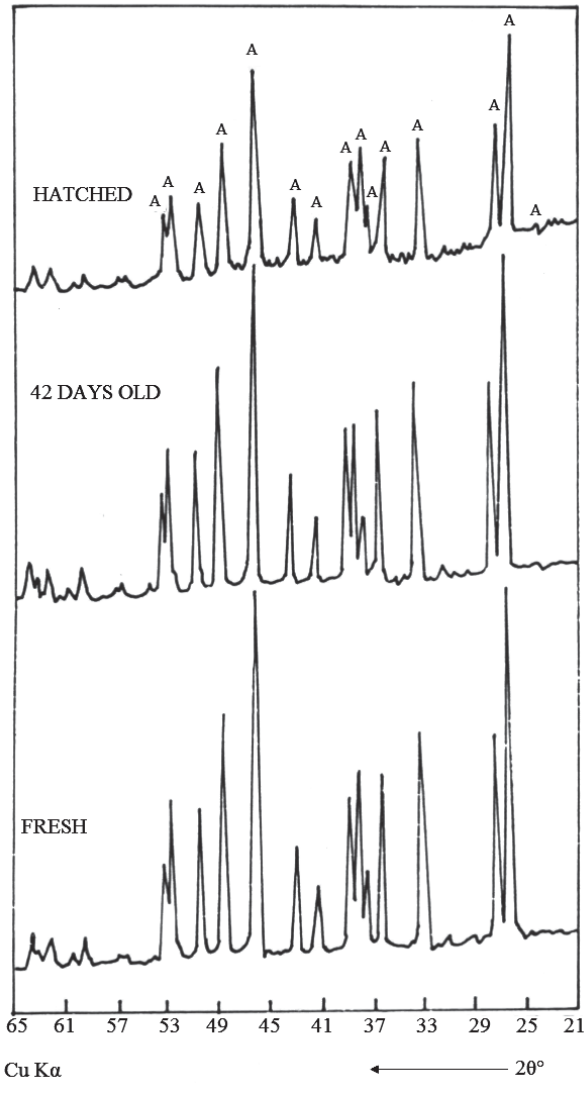

Fig. 1. X-ray diffraction pattern of *Lepidochelys olivacea* eggshells at three developmental stages.

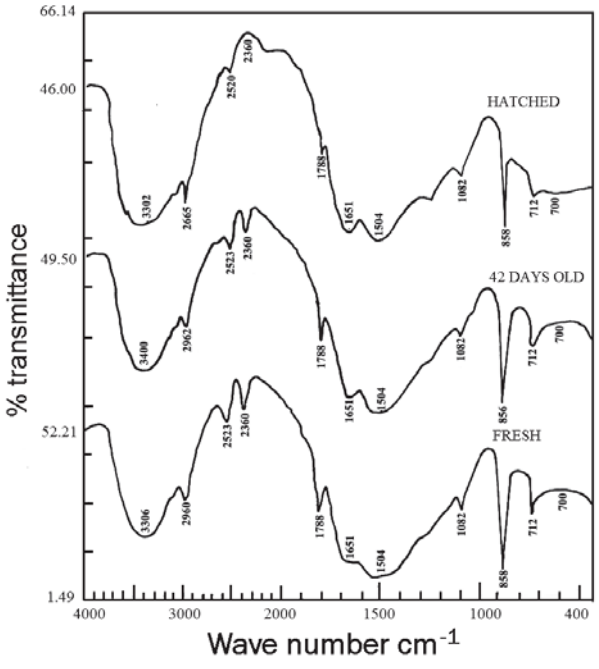

Fig. 2. Infra-red spectral pattern of *Lepidochelys olivacea* eggshells at three developmental stages. The percentage transmittance is from 1.68 to 73.03, 2.08 to 68.96 and 2.58 to 66.14 in fresh, 42 days developed and hatched egg shells, respectively.

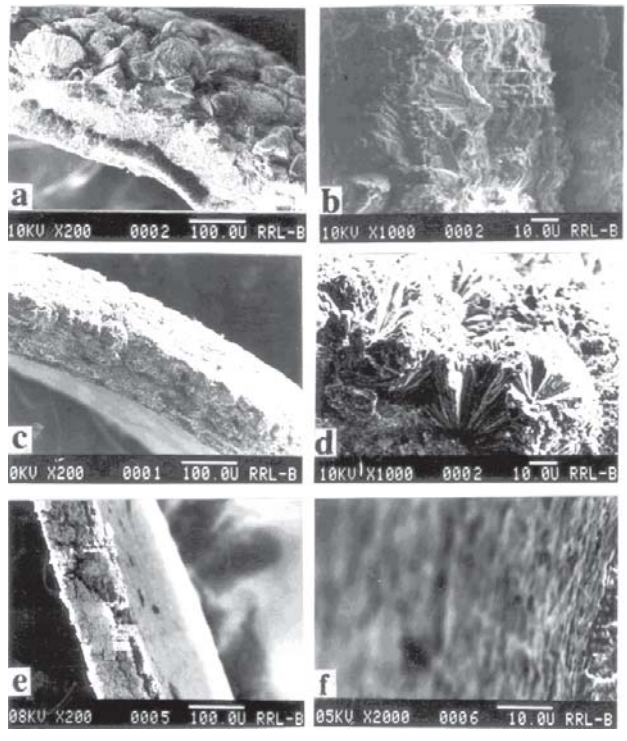

Fig. 3. Scanning electron micrographs showing the radial view of *Lepidochelys olivacea* eggshells at three developmental stages. The mineral layer is towards the upper side of the figure. a) Loosely arranged, irregularly oriented shell units in the calcareous matrix of a fresh eggshell. b) The arrangement of aragonite crystals in a shell unit; the crystals are radiating outward from the primary spherite. c) The calcareous and membrane layers of a day 42 eggshell. d) Enlarged view of c in which the loss of aragonite crystals from shell units is distinctly marked. e) The micrograph of a hatched eggshell showing a thin calcareous layer, completely separated from the shell membrane without discernible shell units. f) Enlarged view of e. Note the calcareous layer that appears amorphous.

RESULTS

Physical and chemical characteristics

Both laboratory-developed and *in-situ* field samples for each stage (see above) produced indiscernible values for the various parameters analysed and were pooled. Olive ridley turtles lay spherical and flexible-shelled eggs (36±3.57 mm diameter, 33±1.25 g weight). The mass of the eggshell ranged from 1.19 g to 1.63 g (1.40±0.14 g) in freshly laid eggs, comprising about 4.27% of the entire egg (Table 1). The eggshell mass reduced to about 0.86±0.08 g by 42nd day of development, and to about 0.54±0.03 g by hatching. Thus, from laying to hatching the eggshell underwent an approximately 61% reduction in its weight.

Calcium carbonate in the fresh eggshell ranged from 50.55 to 54.70%, with a mean value of 52.70±1.26%. There was a 55.6% reduction in calcium carbonate percentage from laying to hatching. Calcium was the major inorganic constituent of the eggshell; the fresh eggshell contained about 191±32 mg of calcium. The shells of eggs incubated for 42 days contained 151±36 mg, an amount which had been reduced to 69±11 mg by hatching. A gradual reduction in calcium content was recorded, although the major changes in the mass of the eggshell and its calcium content occurred towards the end of incubation (one-way ANOVA and Tamhane's post-hoc test, $P<0.05$ for pairwise comparisons between all stages, Table 1). Other elements such as magnesium, sulphur and potassium were present in trace amounts and no change was observed in their concentration during development (Table 1). Phosphorus was totally absent in the eggshell.

Mineralogical characteristics

X-ray diffraction. The "dA^0" values and relative intensities (I/I_0) of the x-ray diffractogram peaks were interpreted in terms of different mineral phases. The eggshell was constituted solely of aragonite (orthorhombic calcium carbonate) crystals (Fig. 1). All the XRD peak positions matched well with the values reported in the JCPDS (1980) data book. Closer examination of XRD patterns revealed a gradual fall in the relative peak intensities from fresh to 42 days old to hatched shells. This clearly indicates that the aragonite content gradually decreased with development, in parallel with the sequential reduction of calcium carbonate content (Table 1).

Infra-red analysis. The infra-red spectra of olive ridley eggshells (Fig. 2) showed four peaks in the 2000–400 cm^{-1} region and three peaks in the 4000–2000 cm^{-1} region. The four distinct and sharp peaks in the former appeared at

Table 2. Differences in the characteristics of *Lepidochelys olivacea* eggshells at three different stages of embryonic development (*n*=12).

Egg shell characteristics	Fresh	42 days developed	Hatched
Total shell			
Netted	Netted	Netted	
Thickness (μm)	150±11	110±5	100±6
Calcareous layer			
Thickness (μm)	80±6	50±4	Very thin
Shell units	Distinct	Distinct	Absent
Membrane layer			
Thickness (μm)	70±5	70±5	*
Fibre width	Uniform	Variable	Variable
Fibrillar arrangement	Netted	Netted	Netted
Globule	Absent	Present	Present

*Not measurable

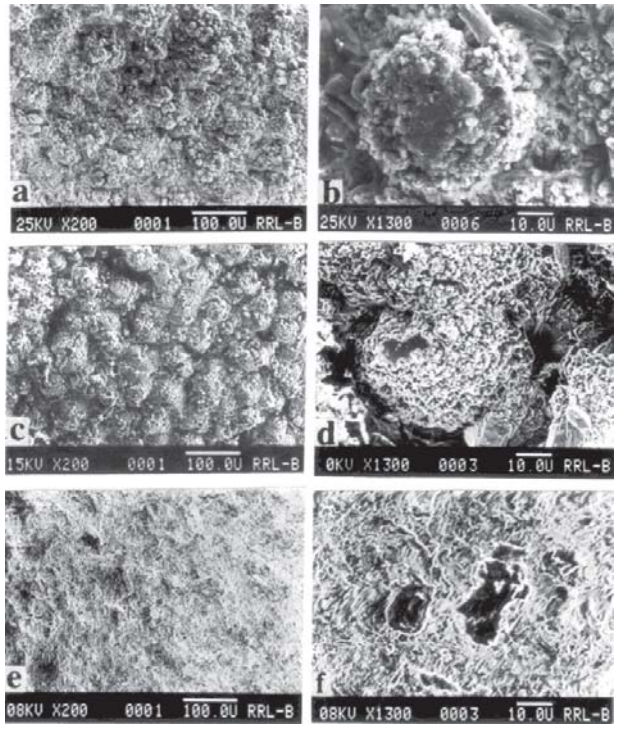

Fig. 4. Scanning electron micrographs showing the upper surface of *Lepidochelys olivacea* eggshells at three developmental stages. a) The fresh eggshell under low magnification exhibiting a coarse granular structure. b) Magnified view of a, focusing on one nodular shell unit. This single unit comprises several micro-nodules. c) The surface structure of an eggshell at day 42, where the shell units are arranged into polygonal fields with more open spaces. d) Enlarged view of c where the nodules appear eroded though the size remains the same. e) Micrograph of a hatched eggshell where no structure is discernible. f) Enlarged view of e. The black patches indicate the areas to which shell units were attached with their primary spherites before incubation.

1788, 1082, 858 and a duplet around 710±1 cm^{-1} in all three shell types. These peaks all matched well with the diagnostic absorption peaks of aragonite (White, 1974). However, their magnitudes gradually decreased from laying to hatching. The low magnitude peak at 1788 cm^{-1} in fresh shells gradually changed to a small and insignificant peak in hatched shells. This decrease in peak intensity demonstrated a lowering of aragonite (calcium carbonate) content in the above order. In the 4000–2000 cm^{-1} region, the absorption peaks around 2960 (duplet), 2520 (triplet) and 2360±2 cm^{-1} were characteristics of the carbonate phase. The magnitude of these peaks showed a minor decrease from fresh to 42 days old indicating thereby relatively lower carbonate content in the later. In hatched shells, a small duplet appeared around 2960 cm^{-1}. However, the peaks at 2520 and 2360 cm^{-1} present in fresh shells became insignificant and later disappeared by hatching. This was indicative of a loss of appreciable volume of CO_2 in hatched shells. These observations supported the XRD findings and were substantiated by a corresponding decrease in calcium carbonate contents of these three eggshell types in the similar order (Table 1).

Structural characteristics

The scanning electron microscope study revealed that the eggshell was composed of an outer crystalline calcium carbonate layer and an inner organic shell membrane followed by a smooth boundary layer. A cuticle was absent from the shell surface. The fresh shell was about 150±11 μm thick (Fig. 3a, Table 2), with the calcareous layer being 80±6 μm. The thickness of the membrane layer was variable in different regions. The calcareous matrix had a single layer of loosely arranged, irregularly oriented shell units. Each shell unit was formed by a spherulitic aggregate of needle-like aragonite crystals that radiated from a central core, basal knob or primary spherite (Fig. 3b). The crystals radiated towards the outer surface as well as towards the lateral sides. The primary spherite was enclosed by the membrane fibres and thus both layers were closely associated with each other. By day 42, the primary spherite appeared to be separated from the membrane layer (Fig. 3c). Both layers remained distinct though the calcareous layer, which was reduced to about 50±4 μm. Individual shell units were marked by the loss of some of their crystals (Fig. 3c,d). The primary spherite was exposed at the point of loss of crystals. By hatching, the shell membrane became almost completely separated from the calcareous matrix (Fig. 3e,f). Total shell thickness reduced to about 100±6 μm (Table 2) in which the inorganic layer appeared as a thin covering over the shell membrane (Fig. 3e). Shell units were not visible at this stage. Thus, the eggshell underwent about a one-third reduction in thickness (calcareous layer) from laying to hatching. Tamhane's post-hoc test showed the changes

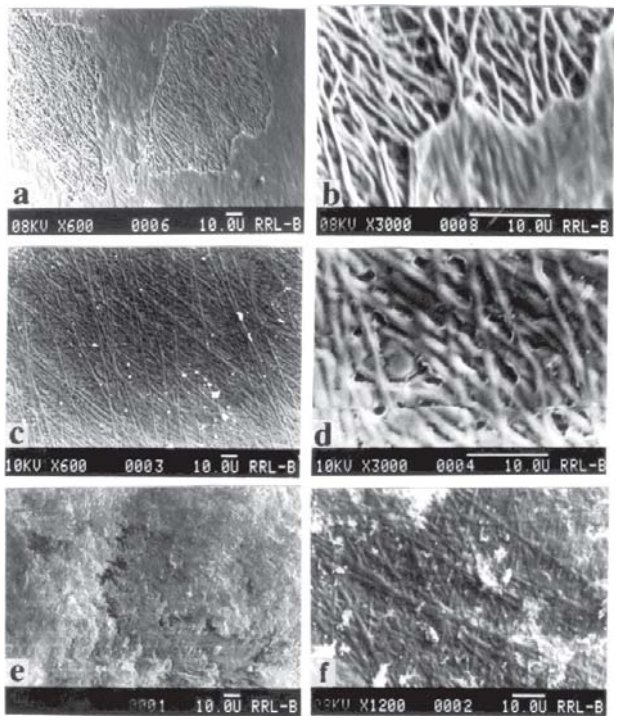

Fig. 5. Scanning electron micrographs showing the lower surface of *Lepidochelys olivacea* eggshells at three developmental stages. a) The fresh eggshell, covered by a thin layer through which the inner network of fibres can be seen. b) An enlarged view of the fibrillar network shown in a. The layer consists of branched, ramified and interwoven fibrils exhibiting a netted structure. c) Micrograph of an eggshell at 42 days development where the fibrils are more or less straight, with indistinct globules embedded in them. d) Magnified view of c, which reveals the membrane fibres to have variable thickness. e) Micrograph of a hatched eggshell, which constitutes a dense, more or less structureless layer. f) Enlarged view of c showing haphazardly arranged fibrous network with occasional globules.

in total eggshell thickness at the three developmental stages were significant at $P<0.05$.

The outer calcareous surface of the fresh eggshell was made up of nodular shell units and exhibited a rough granular structure (Fig. 4a). These units were not uniformly distributed over the shell surface, and gave a pattern of more or less ovoid fields of differentially oriented crystals with pore-like open spaces among the shell units. Each nodule was composed of several globular micronodules of around 50 μm diameter, and possessed a coarse granular surface (Fig. 4b). Besides micronodules, spicules of varying size formed another structural component of this eggshell type. The spicules filled the open spaces between the nodules, rendering the structure of the shell compact. At day 42, the shell units were arranged in polygonal fields with more open spaces among them (Fig. 4c). Although the sizes of nodules remained more or less same, they were not intact (Fig. 4c,d). Some parts of nodules were eroded, a feature that could be seen even at lower magnification (Fig. 4c). Neither micronodules within the nodular units nor spicules in the open spaces were visible. The shell units of eggs at day 42 were somewhat loosely arranged, having large open spaces in the mineral layer, whereas the calcareous layer in the hatched eggshell became completely disorganized, without any discernible structure; the shell units and spicules were no longer distinguishable, and the entire surface appeared homogenous with occasional granules (Fig. 4e,f). The black patches in Fig. 4f are the exposed areas to which shell units were attached with their primary spherites.

The mineral layer was followed by an organic shell membrane or *membrana testacea*, the thickness of which ranged from 60 to 90 μm in the fresh shell. The membrane was made up of a thick, dense mat of interwoven fibres. The fibres were branched, ramified and exhibited a netted structure (Fig. 5a,b). In 42-day-old eggshells (Fig. 5c, d), the membrane fibres appeared more or less straight, and were of variable thickness with minute but distinct globules embedded within them. Such a fibrillar network in the hatched eggshell was generally not observed at lower magnification (Fig. 5e), probably because of dense, structureless cover. However, at higher magnification (Fig. 5f), a haphazardly arranged fibrous network with few globules became apparent.

The inner surface of the shell membrane, i.e. the surface facing the egg contents, is bounded by a smooth, structureless boundary layer. The covering layer is very thin and the membrane fibres are visible through it (Fig. 5a, b).

DISCUSSION

Marine turtle eggshells have compositional and structural characteristics comparable to those of other contemporary chelonians (Hirsch, 1983). Calcium was the major inorganic constituent of the egg contents (shell, yolk and albumen) and hatchlings of olive ridley turtles. Other trace elements were of minor significance. It is, therefore, unlikely that minerals other than calcium contribute to shell composition and function. Along with other inorganic constituents, calcium has been investigated in green (Solomon & Baird, 1976), leatherback (Solomon & Watt, 1985; Chan & Solomon, 1989) and olive ridley turtle (Sahoo et al., 1998) eggshells. The present report highlights that calcium is the major and most important inorganic constituent of the eggshell. The 191 mg of calcium in the freshly laid eggshell of olive ridley turtles were reduced to 151 mg by day 42 and to 69 mg by the time of hatching, an observation that is also supported by their XRD and IR patterns. Thus, the eggshell provides about 60% of its calcium content to the developing embryo, whereas the egg content (yolk and albumen) contributes 40% of the required amount. These results run counter to values given by Simkiss (1962), who reported that leatherback turtle egg contents provide only 25% of the embryonic calcium requirement, while the remaining 75% is presumably obtained from the eggshell. Jenkins & Simkiss (1968) and Packard et al. (1977) con-

sider that, amongst egg-laying reptiles, there are two ways to meet the calcium need of a developing embryo. Squamates obtain their calcium exclusively through egg yolk, without the need for an external mineral supply. Chelonian and crocodilian egg contents, on the other hand, provide only a fraction (usually 20–25%) of the calcium required by the hatchling, and the balance may be reabsorbed from the eggshell. Thus, the eggshell acts as a secondary source of the embryonic calcium requirement after yolk and albumen.

After providing a substantial amount of calcium, structural changes in the eggshell become obvious. In hatched eggshells, the calcareous matrix is present as a thin covering, but does not exhibit any organized structure and is amorphous. This is in sharp contrast to the fresh eggshell, in which an array of nodular units with spicules is present. A 37% reduction in the thickness of the calcareous layer from laying to hatching was observed due to calcium resorption, which is initiated at about day 40 of incubation. Gradual calcium utilization also results in structural changes in the eggshell. At hatching, the eggshell looses its matt-white appearance and becomes slightly grey and friable. Earlier studies reported the eggshells to be passive in relation to the calcium contribution to the developing embryo (Simkiss, 1962). Solomon & Collins (1986) observed structural changes in fresh and hatched crocodile (*Crocodylus niloticus*) eggshells, but did not put forward any reason for it. Here we recorded distinct structural changes in the hatched eggshell over fresh shells, which we attribute to utilization (60%) of the eggshell's calcium by the developing embryo.

The primary spherite plays a dual role: it acts as the site for further crystal growth for calcium uptake. The tip of the shell units (primary spherite) gets isolated from the shell membrane when calcium is resorbed from the inner side of the eggshell. When the embryo starts resorbing calcium after approximately day 40, minor flakings at the point of attachment of the two layers become evident. By day 57 (hatching), an almost complete separation of the mineral layer from the underlying shell membrane was noticed. Freeing of the shell membrane was also seen in common snapping turtles (C*helydra serpentina*) by the 35th day of incubation (Packard, 1980) and in domestic fowl by the 15th or 16th day (Taylor & Simkiss, 1959). This exfoliation is the consequence of removal of calcium from the eggshell by the embryo. The detachment of the calcareous layer from the shell membrane in hatched eggshells is generally observed in birds (Taylor & Simkiss, 1959; Simkiss, 1967; Bellairs & Boyde, 1969), crocodiles (Ferguson, 1982) and other chelonia (Packard & Packard, 1979; Packard, 1980). It is generally assumed that resorption of calcium from the inner surface of the shell by an avian embryo weakens the attachment between the tip of a shell unit and the remainder of the unit, leading to separation of the two (Simkiss, 1967; Bellairs & Boyde, 1969; Taylor & Simkiss, 1959).

It may be surmised that during embryonic development marine turtle eggshells undergo gradual changes in shell structure due to calcium utilization. This is important from several perspectives. Eggshell thinning facilitates the easy emergence of the hatchling, and allows increased gaseous exchange as development proceeds. Studies of eggshell structure and composition are also important in relation to the effect of organochlorine pesticides and the effect of thinning (which has been studied extensively in birds). Such studies are also essential in crocodiles and turtles, where the calcareous shell matrix is used as a calcium reserve for the developing embryo.

ACKNOWLEDGEMENTS

Thanks are due to Dr Robert Jehle and Dr Robert Brown for critically editing the manuscript, and to the Forest Department, Government of Orissa for permission to carry out the work in the Protected Area. The research was supported by a grant from the Department of Ocean Development, Government of India to IMMT, where GS was working as a fellow.

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Accepted: 27 July 2009