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# Calcium metabolism in olive ridley turtle eggs during embryonic development

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**Abstract**

Analyses of calcium, magnesium, sulphur, potassium and phosphorus content of the eggshell, yolk-albumen and embryos of olive ridley turtle, *Lepidochelys olivacea*, have been carried out at various stages of embryonic development. Calcium is the major inorganic constituent in the egg (shell and yolk-albumen) and embryos. Other elements are present either in trace or in minute trace amounts. The egg contents (yolk and albumen) provide only 40% of the embryonic calcium requirement of the hatchling. The remaining 60% is provided by the eggshell. The eggshell also undergoes a similar reduction in its calcium content from laying to hatching. Elements other than calcium present in the yolk-albumen are sufficient for normal embryonic development. The movement of calcium from the eggshell to the embryo starts at about the 40th day of development at 29.5°C. Birds, turtles and crocodiles use their eggshell as the secondary source of embryonic calcium requirement. This dependence on the eggshell varies in different groups which is highest in birds and lowest in crocodiles. 

*Keywords:* Calcium; Egg; Eggshell; Embryo; Embryonic development; Hatchling; Olive ridley turtle; Yolk-albumen

## 1. Introduction

The egg has a complex physical structure and chemical composition. Its complexity is related to its role of protecting and sustaining the developing embryo. However, its composition in relation to the developing embryo is not yet fully understood even in the most widely studied species-the chicken. Foreign substances like fat dyes, Evan's Blue or natural pigments like carotenoids when fed or injected are deposited in yolk, yet are not required by the embryo. Thus, many egg components may be completely unnecessary for embryonic development. At the same time some other substances present in the egg may not be adequate for successful development thus necessitating an external source. The knowledge of precise body composition of the hatchling would make it possible to determine this. This metabolism of different elements is an interesting phenomenon in chemical embryology.

Calcium is one of the most important constituents in the eggs and embryos of vertebrates. The developing embryos of higher vertebrates have three main sources (yolk, eggshell and maternal blood stream) to meet their calcium requirements. The eggshell has been stated as a source of calcium for birds [8], crocodiles [6] and turtles [22]. Reptiles have a unique calcium metabolism during their embryonic development due to their calcareous body coverings like the scaled integument in lizards and the carapace and plastron in chelonians. The calcium content of yolk, albumen and embryo have been studied in squamates [3,7,15], crocodiles [6,11] and chelonians [9,10,14,22] (for review see [13]). Squamate egg yolks are rich in calcium whereas those of chelonians and crocodilians provide only a fraction of it. However, little information is

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Table 1
Shell, yolk and albumen mass (mean ± S.D.) in the freshly laid eggs of *L. olivacea* ($n = 20$, ten laboratory developed and ten field developed)

| | Wet weight (g) | Percentage of wet weight | Dry weight (g) | Percentage of dry weight |
|---|---|---|---|---|
| Shell | 1.40 ± 0.14 | 4.27 | 0.76 ± 0.05 | 10.87 |
| Yolk | 19.20 ± 0.89 | 58.38 | 5.99 ± 0.16 | 85.69 |
| Albumen | 12.30 ± 1.07 | 37.35 | 0.21 ± 0.03 | 3.00 |
| Total | 32.89 ± 1.25 | 100.00 | 6.99 ± 0.20 | 100.00 |

available on the calcium metabolism in these groups. Further, the sources of extra calcium of the embryo which might be needed for its successful development are not known for any of the reptiles. This paper describes the calcium metabolism and sources of extra calcium of the embryo during its development in olive ridley turtle, *Lepidochelys olivacea*, the most abundant sea turtle in the world.

## 2. Materials and methods

A freshly deposited olive ridley turtle egg clutch containing 120 eggs was collected during the first mass nesting (30 January 1994) of the 1994 season from the Gahirmatha rookery along the east coast of India (Orissa state), the largest rookery for this turtle in the world. The eggs were transported in plastic baskets to the laboratory at Utkal University, Bhubaneswar located 220 km away. Ten freshly laid eggs were immediately separated into shell, yolk and albumen mechanically by opening the eggs. The remaining eggs were rinsed with tap water (without changing the orientation), drained, marked, measured, weighed and incubated under laboratory conditions in a BOD incubator at 29.5°C within 36 h of oviposition. Batches of ten eggs each were wrapped with two layers of moist cotton and kept in different enamel pans. The cotton was changed daily to prevent infection.

The laboratory incubated eggs were separated into shell, yolk-albumen and embryo at various stages of development as identified by Behera [1]; the stages being; 1, 15, 19, 23, 28, 30, 32, 35 and 38 representing; 0, 14, 19, 26, 34, 38, 42, 53 and 57 days of incubation, respectively. Stage 1 represented the freshly laid egg and stage 38 represented the hatched stage.

Separate sets of air-dried eggshell samples were used for physical and chemical analyses. Physical analyses such as moisture content (1 hour heating at 107°C), volatile matters (2 hours heating at 700°C) and residual matters were carried out in a coal analyser (Fisher-490). The volatile matters include moisture, gasses such as carbon dioxide, sulphur dioxide, sulphur and potassium. The residual matters mostly consist of the oxides of calcium and magnesium. Chemical analyses were performed for the estimation of calcium, magnesium, sulphur, potassium and phosphorus. The shell, yolk, albumen and hatchlings of different stages were dried to constant mass at 80°C in an oven. The samples were digested following the method of Giesey and Weiner [5] with a minor modification. About 20 ml of concentrated nitric acid was needed for complete digestion of the turtle samples. About 1 g of the sample was placed in 20 ml of concentrated nitric acid and heated at 100°C until dry, cooled, placed in 5 ml of acid and again heated until dry. After cooling, 10 ml of 30% hydrogen peroxide was added to the digest 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.

Calcium content was estimated by standard complexometric titration method using thymolphthalexone as an indicator which is sensitive to calcium and manganese only [25]. This is the most suitable method for determination of calcium up to 0.1% sensitivity in the presence of negligible quantities of manganese. To 25 ml of the digest, 10 ml of 20% triethanolamine, 10 ml of 10% hydroxylamine chloride were added followed by 15 ml of ammonia. This was titrated against 0.02 M EDTA (ethylene diamine tetrachloro acetic acid) disodium salt using thymolphthalexone as an indicator. Magnesium was determined by atomic absorption spectrophotometry (Varian-1475) and potassium by flame photometry (Systronics-K-III). Phosphorus was determined spectrophoto-metrically (Chemito-2500). The characteristic blue colour was developed with a phosphorus molybdenum complex with ascorbic acid and the colour intensity was measured at 882 nm [25].

All analyses were repeated with samples collected from nests developed in the natural environment at Gahirmatha rookery. Both laboratory developed and in situ field samples produced identical results and hence, the data presented here represent both analyses. Since eggs from different clutches laid by different females were collected for analyses, the data can be generalized for olive ridley turtles.

## 3. Results

The weights of shell, yolk and albumen in the freshly laid egg are given in Table 1. The wet and dry weights

Table 2
Compositional changes (mean ± S.D. and average) in *L. olivacea* eggshells during development ($n = 20$)

| | Moisture (%) | Volatile matters (%) | Residual matters (%) | $CaCO_3$ (%) | Ca (%) | Mg (%) | S (%) | K (%) |
|---|---|---|---|---|---|---|---|---|
| Fresh | 6.30 ± 0.35<br>5.78–6.83 | 70.60 ± 0.47<br>69.87–71.35 | 29.40 ± 0.47<br>28.65–30.13 | 52.70 ± 1.26<br>50.55–54.70 | 21.08 ± 0.50<br>20.22–21.88 | 0.056 ± 0.005<br>0.050–0.065 | 1.143 ± 0.174<br>0.917–1.480 | 0.049 ± 0.004<br>0.042–0.057 |
| 42 days old | 7.23 ± 0.31<br>6.72–7.75 | 74.09 ± 0.40<br>73.36–74.58 | 25.91 ± 0.40<br>25.33–26.64 | 46.10 ± 1.73<br>43.62–49.01 | 18.44 ± 0.69<br>17.45–19.60 | 0.054 ± 0.004<br>0.048–0.062 | 0.935 ± 0.152<br>0.793–1.254 | 0.057 ± 0.006<br>0.048–0.069 |
| Hatched | 11.24 ± 0.32<br>10.65–11.71 | 39.02 ± 1.12<br>87.22–90.16 | 10.98 ± 1.72<br>9.84–12.78 | 23.35 ± 3.01<br>18.35–27.55 | 9.35 ± 1.20<br>7.34–11.02 | 0.044 ± 0.008<br>0.031–0.058 | 0.933 ± 0.104<br>0.800–1.083 | 0.042 ± 0.010<br>0.028–0.058 |

of the spherical and flexible-shelled calcareous egg are about 32.89 g and 6.99 g, respectively. The eggshell weighs about 1.40 g (4.27%) in fresh and 0.76 g (10.87%) in dry samples. The yolk is the major constituent both in wet (19.20 g, 58.38%) and dry (5.99 g, 85.69%) eggs. The albumen which forms about 37.35% (12.3 g) of the fresh egg, constitutes only 3% (0.21 g) in the dried condition.

Analyses are presented for three critical stages, fresh, 42 days old and hatched, since chemical analyses of egg constituents in the above mentioned stages have indicated that appreciable changes in the calcium content of the eggshell occur at about 40th day of embryonic development.

Calcium is the major inorganic constituent of the eggshell (Table 2) while magnesium, sulphur and potassium are present in trace amounts and phosphorus is absent. The moisture content in fresh eggshells varies from 5.78 to 6.83% with a mean of 6.30% (Table 2). Volatile matters include moisture, gases such as carbon dioxide (from calcium carbonate, $CaCO_3 = CaO + CO_2$ at 520°C); magnesium carbonate, ($MgCO_3 = MgO + CO_2$ at 350°C), sulpur dioxide, sulphur (boiling point 119°C) and potassium (boiling point 63.65°C). The residual matters mostly consist of oxides of calcium and magnesium. Calcium constitutes about 21.08% in the fresh eggshell whereas magnesium (0.056%), sulphur (1.143%) and potassium (0.049%) are present in minute quantities.

The eggshell undergoes drastic changes in its calcium content from laying (21.1%) to hatching (9.4%); a reduction of 55–64% (Fig. 1(a)) of the total calcium content of the eggshell (Table 2). As residual matters contain mostly the oxide of calcium, a similar decrease (62–65%) is also noticed during this period (Fig. 1(b)). Moisture and volatile matters increased by 78.4 and 26.1%, respectively (Fig. 1(c and d)). The decrease in calcium content and residual matters starts approximately after 40 days of incubation. About 12.6% decrease in calcium content, 11.9% decrease in residual matters, 14.8% increase in moisture and 4.9% increase in volatile matters are observed in the eggshell after 42 days after oviposition. However, no change in the composition of other elements (manganese, sulphur and potassium) occurred during this period.

The level of various elements on dry weight basis in fresh yolk-albumen, in 42 days old embryo and hatchling is presented in Table 3. The yolk and albumen were analysed together due to the difficulty in separating them. Calcium percentage in fresh yolk-albumen, which weighs about 31.5 g, ranged from 0.50% (32 mg) to 0.99% (63 mg) with a mean of 44 mg. A rough separation of yolk (19.20 g) and albumen (12.30 g) showed that 37 mg calcium is present in the yolk fraction while only 7 mg were found in the albumen fraction. Unlike the eggshell, most other elements are present in higher proportion in the yolk-albumen fraction; magnesium 0.12% (7 mg), sulphur 0.22% (13 mg) and potassium 0.59% (36 mg). Phosphorous, which is absent in the eggshell, is present in appreciable amounts with a mean percentage of 0.17% (10 mg) in this fraction.

The 42 day old embryo (wet weight 10.5 g, dry weight 1.63 g) contains about 2.25% (36 mg) calcium, 0.07% (1.2 mg) magnesium, 0.22% (3.7 mg) sulphur, 0.50% (8 mg) potassium and 0.10% (1.7 mg) phosphorous. The freshly emerged hatchling (wet weight 17.5 g, dry weight 2.92 g) contains about 2.93–4.56% (mean 3.68%) calcium, which amounts to about 108 mg. The mean percentage of other constituents in the fresh hatchling are magnesium 0.12 (3.4 mg), sulphur 0.35 (10 mg), potassium 0.59 (17 mg) and phosphorous 0.15 (4 mg).

### 3.1. Calcium metabolism

Calcium metabolism in the olive ridley egg during development is presented in Table 4. About 235 mg calcium is present in the freshly laid egg. The major percentage (81.30%) of calcium is present in the eggshell. Together yolk-albumen contain calcium in the range 34–51 mg. The yolk-albumen mass thus constitutes only 18.7% of the total calcium of the egg. The 42 days old embryo has about 36 mg and the eggshell at this stage has 151 mg calcium. Calcium in the freshly emerged hatchling ranged from 84 to 130 mg with a mean value of 108 mg, which is about 3.7% of the dry weight of the hatchling (2.92 g).

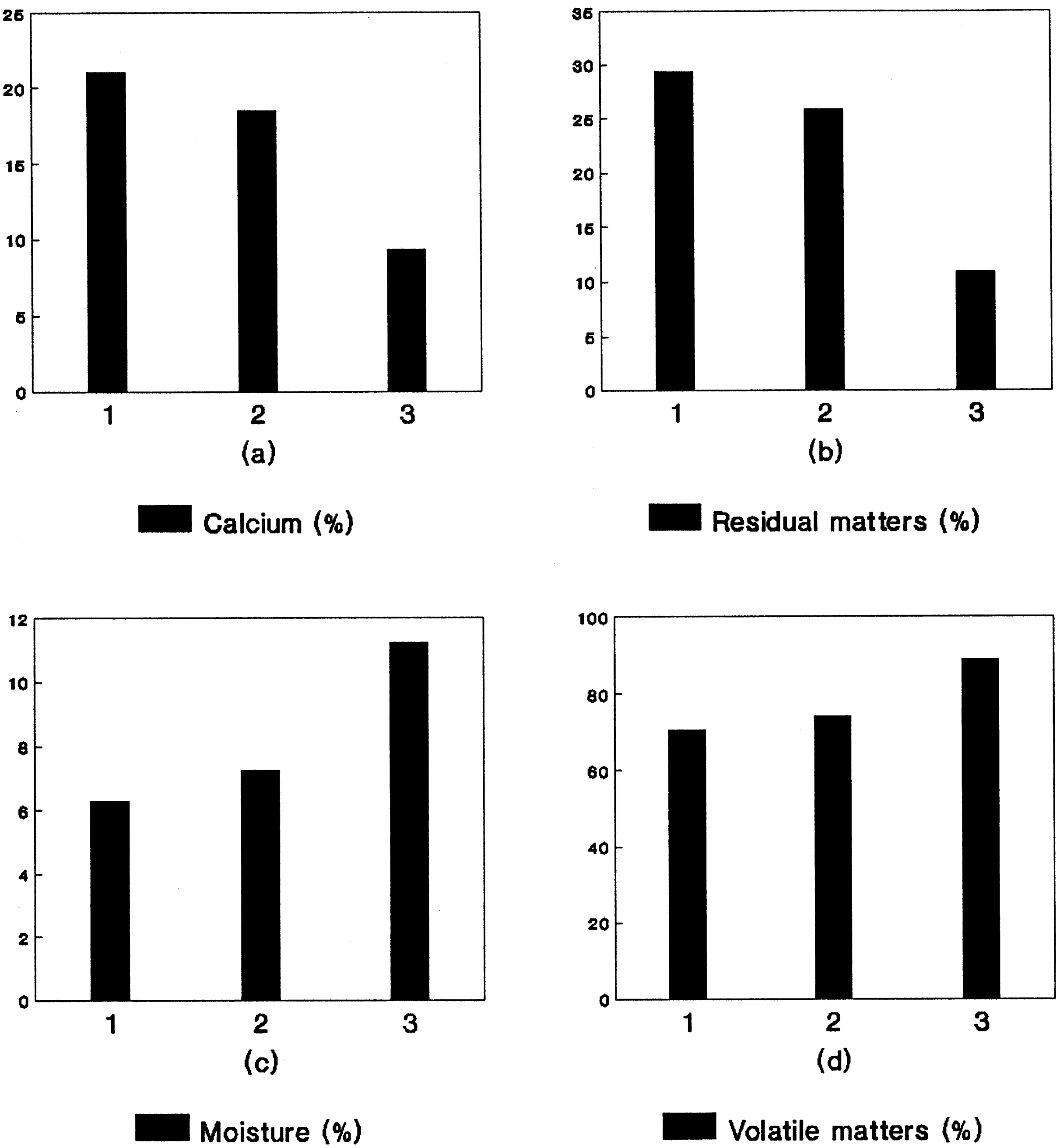


Fig. 1. Changes in *L. olivacea* eggshell constituents during embryonic development.

The amount of calcium present in the yolk-albumen fraction (44 mg) meets only 40% of the total amount of calcium needed by the hatchling (108 mg) for its normal development. The remaining 60% is provided by the eggshell. The fresh eggshell has about 191 mg calcium whereas the eggshell at hatching has only 69 mg. Thus, the eggshell undergoes 64 and 55% reduction in its calcium content and calcium percentage, respectively from laying to hatching.

The movement of calcium from the eggshell to the hatchling starts at around the 40th day of incubation. The 36 mg calcium present in the 42 days old embryo is comparable to that in the yolk-albumen fraction. The shell is reduced in its calcium content from 191 mg (fresh) to 151 mg (42 days old), a 21% reduction. This means that before complete drainage of calcium from yolk-albumen, the embryo begins to utilize the eggshell calcium reserve.

## 4. Discussion

The material and energy of a turtle egg is first utilised for embryonic development within the egg and then

Table 3
Distribution of different elements (both in mg and percentage composition) (mean ± S.D., range) in fresh yolk-albumen, 42 day old embryo and fresh hatchling of *L. olivacea* ($n = 20$)

| | Ca | | Mg | | S | | K | | P | |
|---|---|---|---|---|---|---|---|---|---|---|
| | (mg) | (%) | (mg) | (%) | (mg) | (%) | (mg) | (%) | (mg) | (%) |
| Fresh yolk + albumen | 44 ± 9<br>32–63 | 0.71 ± 0.15<br>0.50–0.99 | 7 ± 1<br>6–9 | 0.12 ± 0.01<br>0.09–0.15 | 13 ± 2<br>11–17 | 0.22 ± 0.03<br>0.19–0.28 | 36 ± 4<br>29–41 | 0.59 ± 0.06<br>0.49–0.67 | 10 ± 1<br>8–12 | 0.17 ± 0.01<br>0.15–0.19 |
| 42 day old embryo | 36 ± 4<br>30–42 | 2.25 ± 0.27<br>1.83–2.50 | 1.2 ± 0.19<br>1.0–1.6 | 0.07 ± 0.01<br>0.05–0.09 | 3.7 ± 1.28<br>1.5–4.6 | 0.22 ± 0.07<br>0.10–0.33 | 8 ± 2<br>5–11 | 0.50 ± 0.15<br>0.28–0.69 | 1.7 ± 0.26<br>1.4–2.2 | 0.10 ± 0.01<br>0.08–0.13 |
| Fresh hatchling | 108 ± 14<br>84–130 | 3.68 ± 0.51<br>2.93–4.56 | 3.4 ± 0.05<br>3–4 | 0.12 ± 0.01<br>0.11–0.14 | 10 ± 2<br>7–15 | 0.35 ± 0.07<br>0.26–0.48 | 17 ± 4<br>11–24 | 0.59 ± 0.12<br>0.40–0.80 | 4 ± 1<br>3–5 | 0.15 ± 0.03<br>0.10–0.18 |

presumably for hatchling maintenance and growth. The hatchlings of some species emerge from the nest a short time after hatching, others delay emergence for months [4]. The amount of stored material and energy required by hatchlings may, therefore, vary among species considerably. The varying requirements for hatchling survival may be reflected in the amount of yolk and albumen fractions of the egg. Marine turtles are known to delay emergence for several days and so require an extensive energy reserve [4].

The olive ridley eggs are yolk-rich (58.4% yolk), confirming previous data [20]. A comparison of different egg components in domestic fowl, leatherback turtle and olive ridley turtle shows that chelonian eggs contain more yolk (58.4% in olive ridley and 46.8% in leatherback) than avian eggs (31.5% in domestic fowl). Available data on egg contents suggest that leatherback eggs [22] contain less yolk than loggerhead eggs [24], and we found that the yolk percentage in olive ridley eggs is higher than these two marine turtle species.

### 4.1. Calcium content of the eggshell

The results of the present study demonstrate that calcium is an important constituent of the eggshell (21%) as also observed in green turtle eggshells (20%) [23]. Elements other than calcium are present in negligible amounts. Sahoo et al. [18] also reported that elements other than calcium are present in trace amount in olive ridley eggs and embryos. It is therefore unlikely that minerals other than calcium would contribute significantly to eggshell composition and function.

Table 4
Calcium content (mg; mean ± S.D., range) (dry weight basis) of *L. olivacea* eggshells and embryos during development ($n = 20$)

| | Shell | Yolk-albumen/embryo/hatchling |
|---|---|---|
| Fresh | 191 ± 32<br>147–238 | 44 ± 5<br>35–51 |
| 42 day old | 151 ± 36<br>141–171 | 36 ± 4<br>30–42 |
| Hatched | 69 ± 11<br>52–85 | 108 ± 14<br>84–130 |

### 4.2. Yolk as a calcium source

A comparative analysis shows that the weight of wet yolk per gram of offspring in olive ridley egg (0.9) is comparable to that in leatherback turtle (1.2), but considerably lower than domestic fowl (1.9). When calcium content of the egg is compared, large variability is observed in different species of turtles. Turtle eggs contain more calcium than that of birds. Olive ridley yolk has double calcium concentration (1.92 mg $g^{-1}$ yolk) than the yolk of leatherbacks (0.91 mg $g^{-1}$ yolk). Some turtles have large yolks and some have yolk very rich in calcium, but these two characteristics need not occur in the same species [21].

### 4.3. The extra embryonic calcium source

Among reptiles, there are three ways to meet the calcium requirement of the developing embryos [7,16]. Squamate egg yolks provide an extensive store of minerals for which the embryos develop without needing an external calcium supply. In contrast, chelonian and crocodilian egg contents provide only a fraction (usually 20–25%) of the calcium required by the hatchling. The balance is thought to be resorbed from the eggshell. The third is viviparity.

In sea turtles, the absorption of minerals (calcium/magnesium) from the environment (sea water/nesting beach sand) was postulated by Simkiss [22]. The main difficulty to this concept is that all eggs in a clutch are not in contact with the sand and the limited amount of water absorbed per egg during incubation. Further, an

Table 5
Calcium content (mg) of the eggs and hatchlings of different reptilian groups

| Calcium | Snake | Crocodile | Turtles | | Bird |
|---|---|---|---|---|---|
| | *Vipera berus* [7] | *Crocodylus noveguineae* [6] | *Dermochelys coriacea* [22] | *Lepidocyelys olivacea* (present study) | *Gallus domesticus* [8] |
| Egg contents (yolk + albumen) | 25 | 124 | 34 | 44 | 23 |
| Hatchlings | 12–16 | 280 | 138 | 108 | 120 |
| Increase | 0.8*x* | < 2.4*x* | < 4*x* | 2.5*x* | < 5.2*x* |

external magnesium source is not required for olive ridley development as the yolk-albumen contains about 7 mg whereas the hatchling needs only 3.4 mg. Bustard and Greenham [2] postulated the high $CaCO_3$ rich beach sand of some nesting grounds as a possible calcium source. The coral sand of the Heron beach (Queensland) is in excess of 99% $CaCO_3$ which is relatively insoluble. The $CO_2$ released by the metabolism of the egg mass forms carbonic acid which, on reaction with $CaCO_3$, forms the much more soluble calcium bicarbonate. But the validity of this experiment in the natural environment is doubtful as the amount of $CO_2$ released from the egg mass is insufficient to convert the insoluble $CaCO_3$ to its soluble calcium bicarbonate form. This demonstrates that the eggshell is the only possible alternative for embryonic calcium requirement.

The eggshell has been stated as a source of calcium for developing embryos. Studies on chemical composition of eggshells during development is not available for most birds and reptiles except for a crocodile [6] and a squamate [3]. Cox et al. [3] thought that the alteration in squamate eggshell composition results either from the action of one or more extrinsic (both abiotic, e.g. leaching by soil moisture or abrasion by soil particles and biotic agents, e.g. microbial degradation) or intrinsic (calcium uptake by the embryo) factors. However, Sahoo et al. [19] reported structural changes in olive ridley eggshells during development due to calcium uptake by the embryo.

Karashima [9] observed that the original egg contents of *Thalassochelys corticata* contained 302 mg ash whereas the hatchling contained 382 mg ash. He concluded that the 21% increase in ash content was due to calcium and magnesium which might be obtained presumably from the eggshell. The results of the present study reveal that the olive ridley egg contents are not sufficient to meet the entire calcium demand of the hatchling. Further, the complete ossification of olive ridley [17] and leatherback [22] hatchlings along with the calcareous body coverings of turtles suggest a large calcium requirement by the embryo during its development.

The embryonic calcium requirement and its availability for some reptiles and birds is summarized in Table 5. The olive ridley eggshell provides about 60% of the embryonic calcium requirement, a figure that is lower than the 75% provided by leatherback turtle [22]. Calculation of Jenkin's [6] figure gives a 41% contribution by crocodilian eggshells whereas in chickens, the contribution is about 80% [8,12,21]. Further, olive ridley eggshells undergo a 55.6% reduction in their calcium content during incubation. This reduction is comparable to the extra calcium requirement (60%) of the hatchlings.

Packard [13] reviewed the calcium contribution of the eggshell to the embryos in various groups of oviparous, amniotic vertebrates (snakes, lizards, fresh water chelonians and archosaurians). According to him, embryos in these groups obtain calcium from both the yolk and the eggshell, but the relative importance of these sources varies in different groups depending on the degree of the calcification of the eggshell. Eggs of most lepidosaurians are characterized by calcium-rich yolks and calcium-poor eggshells, where as those of other oviparous amniotes have relatively calcium-poor yolks and calcium-rich eggshells. This difference in calcium contents of egg components enables lepidosaurian embryos not to rely on the shell as a source of calcium, whereas embryos of chelonians and archosaurians obtain most of their calcium from the eggshell.

Thus, in birds, turtles and crocodiles, the eggshell acts as a supplementary source of calcium for the developing embryo in the same order of importance. But how embryos remove calcium from the eggshell and how they control this process have not been elucidated for any species.

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