

Sources of calcium for the agamid lizard *Psammophilus blanfordanus* during embryonic development

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Abstract. We determined the sources of calcium for the developing embryo and the parallel changes in eggshell structure in the Indian agamid lizard *Psammophilus blanfordanus*. The developing eggs were opened at 0 (freshly laid), 10, 20, 30, 35, 38, and 40 days of incubation and at hatching (day 41) and subjected to chemical and structural analyses. The oval and flexible-shelled eggs had undergone significant changes in size (40% increase in length, 68% increase in breadth and 315% increase in weight) from laying to hatching. The fresh eggshell contained 2.76 mg (12.51%) calcium whereas the hatched eggshell had only 1.02 mg (7.20%), or a 63% reduction from its original content. The yolk + fluids fraction provides only 0.47 mg to the 1.76 mg of calcium in the hatchling, the rest being resorbed from the eggshell during development. The fresh eggshell (62 µm thick) had a rough granular structure in its calcareous layer with near uniform rectangular/polygonal fields made up of globules of varying sizes. The membrane layer had a multilayered mat of interwoven, irregularly oriented and bifurcated, fibres of uneven thickness. The spherical globules were absent at several places in the hatched eggshell as a result of eggshell calcium utilisation by the developing embryo. Hence, like that of most reptiles, the eggshell of *Psammophilus blanfordanus* also acts as a secondary source of calcium for the developing embryo. The embryo utilizes the eggshell calcium towards the end of development.

Keywords. *Psammophilus blanfordanus*, embryonic development, eggshell, calcium, ultrastructure.

INTRODUCTION

Eggshell structure of reptiles is diverse, ranging from the small, flexible, parchment-shelled eggs of most squamates to the large, rigid-shelled eggs of crocodylians. It consists of an outer, inorganic layer underlain by an organic (shell) membrane comprised of multiple layers of fibres. The structural units in the calcareous layer are also diverse (Schleich and Kästle, 1988). The eggshell of reptiles plays a complex, but only partially understood, role in development, particularly as a source of inorganic ions (Packard et al., 1992). Some major functions of the eggshell are to accommodate permeability to gas and water,

provide mechanical stability, and to serve as a potential calcium reserve (Schleich and Kästle, 1988).

The eggshells of most oviparous lepidosaurians are fibrous and poorly calcified (Packard et al., 1982a, b; Packard and Demarco, 1991; Thompson et al., 2001). The inorganic (mineral) content of the shell is usually restricted to the outermost portion and is comprised mainly of calcium carbonate in the form of calcite (Packard et al., 1982a, b, Deeming, 1988).

Yolk provides a considerable amount of calcium during embryonic growth for most oviparous reptiles whereas the eggshell calcium supplements that of yolk late in the incubation period (Packard et al., 1984, 1985; Shad-

rix et al., 1994; Packard and Clark, 1996; Stewart et al., 2004; Sahoo et al., 2009). As embryos of oviparous squamates depend heavily on the yolk as a source of calcium, their calcium mobilisation pattern has been proposed as the most appropriate model for functional characteristics of the common ancestor of oviparous amniotes (Packard and Seymour, 1997; Stewart et al., 2004). The morphological changes in eggshell structure are indeed caused by the mobilisation of eggshell calcium by the developing embryo.

The present work describes the eggshell calcium utilization pattern and the parallel structural changes in the eggshell of an agamid lizard, *Psammophilus blanfordanus*, during various stages of embryonic development.

MATERIAL AND METHODS

Five clutches of *Psammophilus blanfordanus* eggs (n= 12-14) were collected on campus of North Orissa University from nests just after oviposition in July, 2013 and brought to the laboratory. The eggs were measured, weighed and numbered. Two eggs from each clutch were reserved for chemical analyses. The remaining eggs of each clutch were incubated in separate plastic boxes at ambient temperature (33° C average, range = 28–38° C) with sandy soil as the medium of incubation. The soil medium was rehydrated at regular intervals. The development of two more clutches (n = 13 and 12) were also observed in their original nests from the time of oviposition. Laboratory eggs were maintained at similar hydric condition as those in the field.

One egg from each clutch from both field and laboratory studies was removed on days 10, 20, 30, 35, 38, and 40 of incubation. Eggs (total of eight sample days – freshly laid, 10, 20, 30, 35, 38, 40 day and hatched) were washed in tap water; egg dimensions and weight were measured and eggs were separated into shell and egg components which included yolk, albumen and embryo including the extraembryonic membranes) and subjected to chemical and structural analyses.

Chemical analysis

Determination of inorganic composition requires conversion of the solid samples to liquid form through acid digestion. The samples (eggshell and egg components: yolk, albumen and embryo) were dried to constant weight at 80 °C in an oven and digested following the method of Geisey and Weiner (1978) with a slight modification. Pre-weighed samples (about 1 g) were heated to 100°C until dry with 20 ml of concentrated nitric acid, cooled and again heated until dry with 5 ml of the acid. After cooling, 10 ml of 30% hydrogen peroxide was added to the digestate and heated for 30 minutes until a clear solution was obtained. Distilled water was added to reconstitute the samples that were then filtered and diluted to 100 ml.

Calcium estimation was done gravimetrically using calcon indicator (0.4 g solid calcon dissolved in 100 ml methanol).

To 10 ml of the digestate, 20 ml distilled water, 2-3 ml NaOH solution and 3-4 drops of calcon were added to make the solution alkaline. This solution was titrated against 0.02 M EDTA. Samples with very low levels of calcium (e.g., egg components) were analyzed using an atomic absorption spectrophotometer (Perkinelmer, Analyst 200) with standard solutions of 1 ppm, 2 ppm, 4 ppm and 10 ppm of calcium ($r = 0.995074$). Magnesium in the digestate was analyzed by an atomic absorption spectrophotometer and potassium by a flame photometer (Systronics, Model K-III). Phosphorus was determined with a spectrophotometer (Varian, Model – Carry 100). The characteristic blue colour was developed with a phosphomolybdenum complex and ascorbic acid and the intensity of the colour was measured at 882 nm.

Structural analysis

The structure of eggshells (fresh, 10 day, 20 day, 30 day, 35 day, 38 day, 40 day) was determined with a scanning electron microscope (JEOL, JSM 35 CF) with working voltage of 5-15 kv at magnifications ranging from 100 to 1500x. The eggshell samples were air dried and broken into pieces suitable for microscopic observations. For SEM study, outer surface, radial section and sub-surface samples were mounted in stubs with double-sided adhesive carbon tape and coated with gold for 2 minutes by an Ion sputterer (JFC-1100). The thickness of the fiber and mineral layers was measured using the scale bar on the photomicrographs.

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

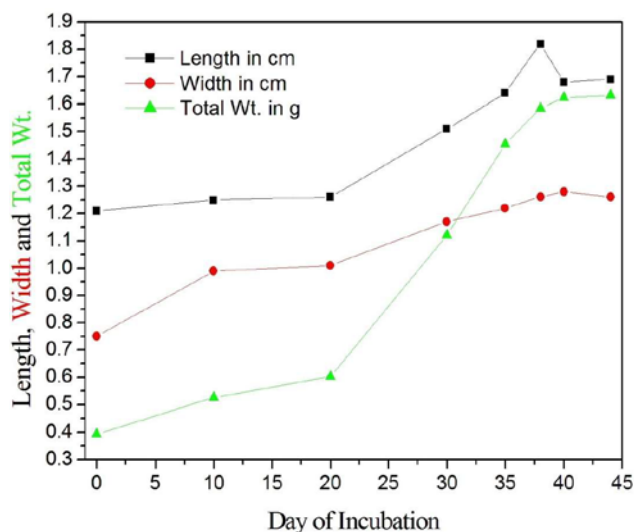
RESULTS

Physical characteristics

The physical and chemical composition of *Psammophilus blanfordanus* eggs from both laboratory and *in-situ* field samples were similar and were therefore pooled for analysis (Table 1). The incubation period varied from 41-42 days (laboratory eggs) to 44 days (field eggs). At oviposition, eggs were 1.21 ± 0.46 cm long, 0.75 ± 0.27 cm in diameter, and weighted 0.393 ± 0.021 g, n =65); variation in linear dimensions and weight among clutches was not significant (one-way ANOVA and Tamhane's post-hoc test, $P < 0.05$). The wet weight of eggshells ranged from 0.037 to 0.048 g (0.042 ± 0.004 , n =10). The average wet weight of the egg contents was $0.351 \text{ g} \pm 0.019$ (88.4%) whereas the eggshell formed 11.6% of the total egg weight. The fresh egg contained about 62.8% water. The total dry weight varied from 0.132 to 0.166 g (0.146 ± 0.007 g). The shell weight varied from 0.020 to 0.027 g and the egg content

Table 1. Changes in physical and chemical composition of *Psammophilus blanfordanus* eggs during embryonic development (n = 7 per sample date, field and laboratory eggs combined).

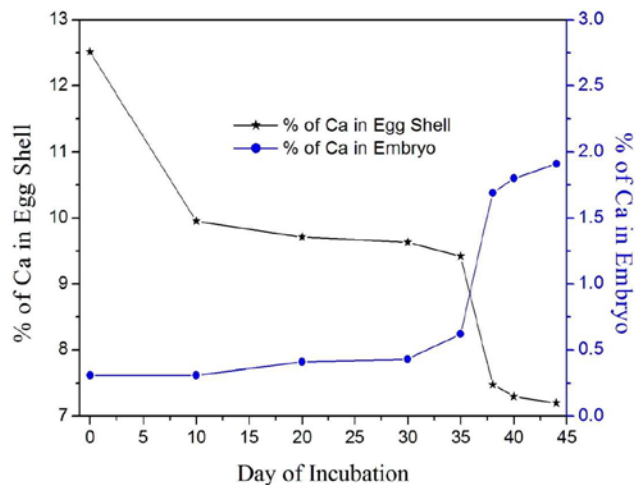
Day of incubation	Wet weight (g)			Dry weight (g)			Calcium content (dry weight)			
	Total wt.	Shell	Egg component	Total wt.	Shell	Egg component	Eggshell		Egg contents including embryo	
							mg	%	mg	%
Freshly laid	0.393	0.042	0.351	0.146	0.022	0.124	2.76	12.51	0.47	0.31
10 day	0.526	0.044	0.482	0.145	0.022	0.123	2.19	9.95	0.47	0.31
20 day	0.603	0.051	0.602	0.144	0.021	0.123	2.04	9.71	0.55	0.41
30 day	1.120	0.269	0.851	0.122	0.020	0.102	1.73	9.63	0.59	0.43
35day	1.454	0.211	1.243	0.139	0.017	0.122	1.61	9.42	0.74	0.62
38 day	1.586	0.127	1.459	0.159	0.015	0.143	1.05	7.48	1.38	1.69
40 day	1.623	0.176	1.447	0.167	0.014	0.153	1.03	7.30	1.54	1.80
Hatched	1.631	0.087	1.544	0.102	0.014	0.088	1.02	7.20	1.76	1.91

**Fig. 1.** Changes in the size of *Psammophilus blanfordanus* eggs as a function of the day of incubation.

from 0.113 to 0.147 g. On dry weight basis, the eggshell composed 15% and the egg components 85% of the total egg weight.

The eggs of *P. blanfordanus* increased in size and weight from laying to hatching (Fig. 1). The eggs increased 40% in length (from 1.21 cm at laying to 1.69 cm at hatching); 68% in width (0.75 cm at laying to 1.26 cm at hatching); and 315% in weight (from 0.393 g at laying to 1.631 g at hatching) (Table 1).

Calcium content of the eggshell and the egg components at different days of incubation are presented in Table 1 and Fig. 2. The amount of other minerals like

**Fig. 2.** Percentage calcium in eggshell and egg contents + embryo of *Psammophilus blanfordanus* eggs through incubation.

magnesium (0.79 mg in fresh egg contents and 0.75 mg in hatched embryo) and potassium (2.68 mg in fresh egg contents and 2.23 mg in embryo) did not change during development. The fresh eggshell contained 2.76 mg of calcium that formed 12.51% of the total eggshell. The fresh egg components contained only 0.47 mg of calcium (0.31% of the total egg). Thus, 3.23 mg calcium was present in the freshly laid egg.

The calcium content of 35 day incubated eggshell (1.61 mg, 9.42%) was comparable to that at 30 days. The calcium contents of 38, 40 day developed and that of the hatched eggshell was almost comparable (one-way ANOVA and Tamhane's post-hoc test, $P < 0.05$ for pair wise comparisons between all stages, Table 1).

The yolk fraction of the fresh egg contained 0.47 mg of calcium (0.3% of the egg content). The calcium content of the hatchling increased over the embryonic period. The freshly emerged hatchling contained 1.76 mg of calcium (1.91%).

Calcium utilisation pattern

The total calcium content of the freshly laid egg was 3.23 mg of which the eggshell contained 2.76 mg and the egg component 0.47 mg. The calcium in the egg component increased with the growth of the embryo. Significant alternations were observed in calcium content of the eggshell from laying to hatching (Fig. 2). The eggshell calcium content decreased slowly from day 10 to day 38 (Table 1). The mobilization of calcium from the eggshell was slow up to 35 day of incubation. However, a quantum jump was observed thereafter up to 40 days of incubation. A parallel gradual increase in calcium content of the egg component was also observed during development. The hatched eggshell (1.02 mg) contained about 63% less calcium than that of the fresh eggshell (2.76 mg).

Structural characteristics

The fresh eggshell

Surface morphology: The outer surface of the eggshell exhibited a rough granular structure (Fig. 3A). The surface was organized into regular rectangular and/or polygonal fields of almost uniform size. The fields were arranged in a parallel fashion. The boundaries of such fields appeared to be elevated. The outer surface at higher magnification (Fig. 3B) also did not reveal any definite crystal structure. Rounded/spherical globules of varying sizes made up the entire thickness of the calcareous layer. Peripheral margins of the polygonal fields were formed by larger globules whereas the central area was formed by smaller ones.

Cross Section: The thickness of the shell was about 62 μm (membrane layer 50 μm , calcareous layer 12 μm) (Fig. 3C). The calcareous layer did not show any discernible structure in this view. Figure 3D reveals the demarcations of the polygonal calcareous fields on the surface of the egg. The thick membrane layer appeared to have layers of fibers.

Inner surface: The inner surface of the egg shell was bounded by a very thin smooth boundary layer (Fig. 3E and F). It revealed irregularly placed globules/spheres of varying sizes. The underlying fibres of the membrane layer were not visible because of a smooth covering layer.

The hatched eggshell

Upper Surface: The smooth outer surface at lower magnification (Fig. 4A) revealed clear grooves arranged in a regular parallel pattern. The grooves were formed due to absence of larger globules from the boundaries of the polygonal areas that were present on the fresh eggshell. Fig. 4B depicts irregular absence of smaller globules from inside the fields. The absence of globules from the outer surface exposed the underlying membrane fibers.

Inner surface: The inner surface with a thin shell membrane (Fig. 4C) revealed the underlying fibers to be distinctly visible showing their arrangement. The membrane layer contained a mat of interwoven, irregularly oriented uneven fibrils that were bifurcated at intervals (Fig. 4D-F). The fibrils were arranged into several layers.

DISCUSSION

The eggshells of *P. blanfordanus* are typical of the flexible shelled eggs of squamates (Packard et al., 1982c). The calcareous layer is thin and the shell membrane contributes the majority of shell thickness. Calcium carbonate crystals are present as calcite, the form of calcium carbonate found in vast majority of lacertilian eggs (Packard et al., 1982a, 1982b; Packard and Hirsch, 1986). Also, the eggs increased in size throughout incubation with uptake of water from the substrate, which is typical of flexible shelled squamate eggs in favorable hydric environments (Packard et al., 1980, 1982a; Andrews and Sexton, 1981). Some of the eggs tripled their mass during incubation. Water uptake is essential for normal embryonic development and survival for many squamate embryos with flexible-shelled eggs (Muth, 1980; Tracy, 1980).

The most important function of the egg is related to embryonic nutrition (Bellairs, 1964; Cook, 1968; Ackerman, 1991; Linville, 2010) for which it contains all the necessary materials. Any loss of egg components may seriously affect embryonic development within the egg and hatchling maintenance and growth.

Embryos of oviparous lizards have two potential sources of calcium for developmental requirements; calcium sequestered in yolk during vitellogenesis and calcium deposited on the eggshell by oviducal secretions. Packard (1994) reviewed the calcium contribution of the eggshell to the embryos in various groups of oviparous, amniotic vertebrates (snakes, lizards, etc.). According to her, the degree of calcification of the eggshell is very important as it acts as a source of embryonic calcium in these groups of animals. *Psammophilus blanfordanus* eggshell is poor in calcium in comparison to other species (Stewart and Ecay, 2010) and has a thin calcareous

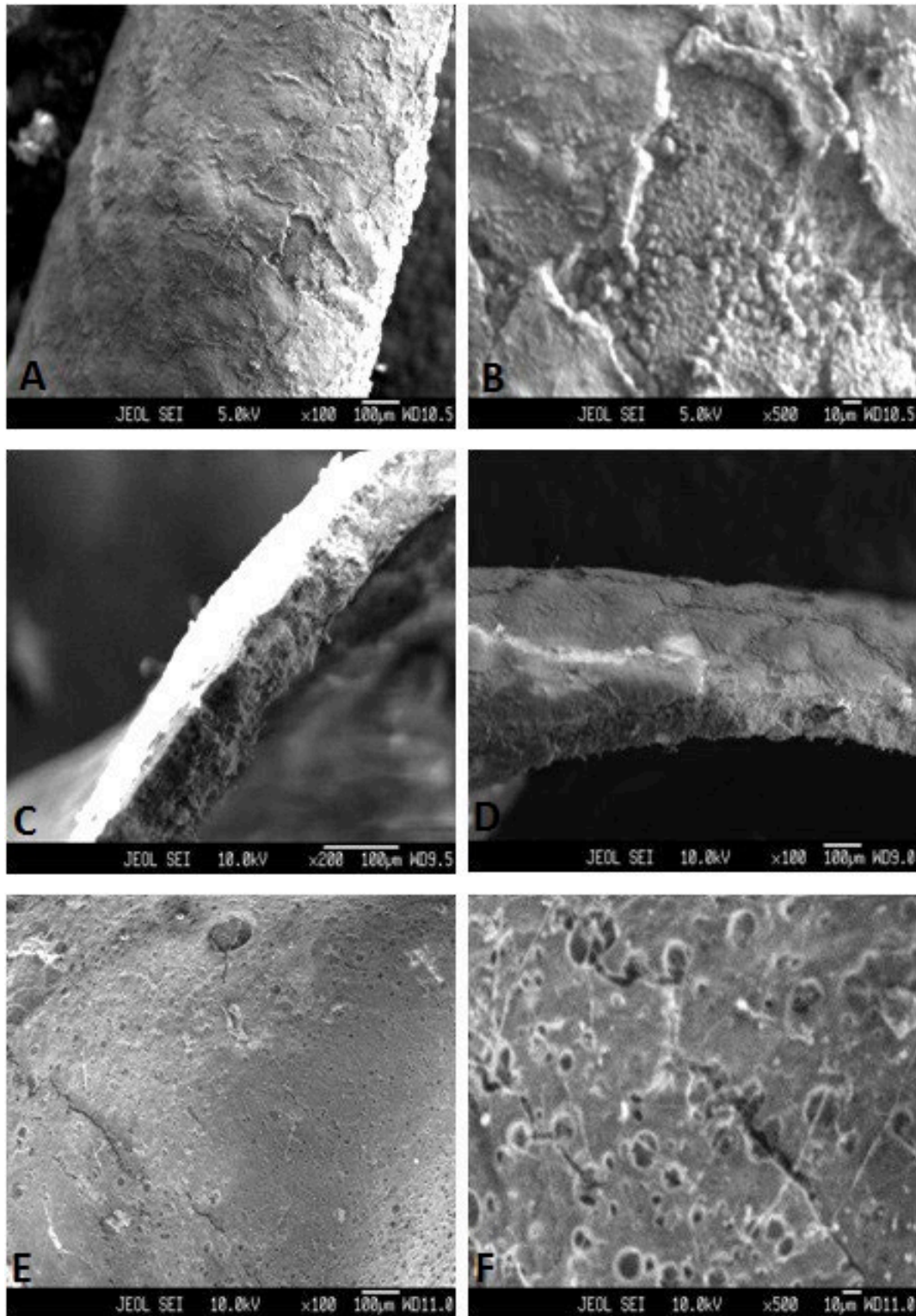


Fig. 3. Scanning electron micrographs of freshly laid eggshells (A & B upper surface, C & D cross section, E & F inner surface) of *Psammophilus blanfordanus*: (A) Upper surface at low magnification showing regular nearly polygonal fields on a smooth surface. (B) Part of calcareous layer removed to show the inner surface. (C) Much of the thickness of the shell is made up of membrane fibres with a thin calcareous layer. (D) The cross section slightly tilted showing the irregular field-like arrangement on the surface of the eggshell. (E & F) The inner surface is covered by a smooth inner membrane (boundary layer). Globules of varying sizes are embedded on this surface that faces the egg contents.

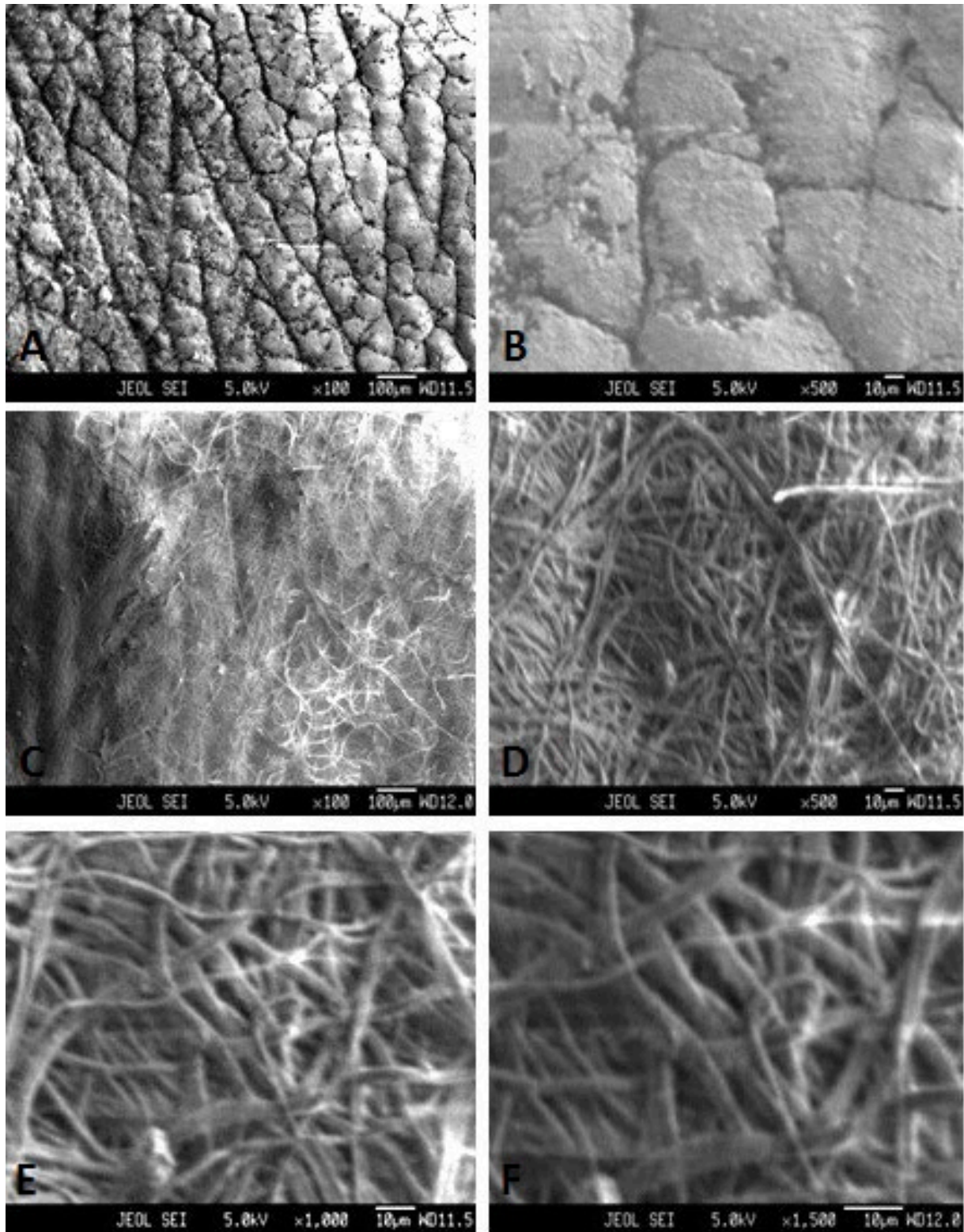


Fig. 4. Scanning electron micrograph of hatched eggshell of *P. blanfordanus*. (A) Upper surface: Larger globules on the boundary of the irregular fields (see Fig.3A) are absent. This has made the formation of clear grooves on the surface. (B) Enlarged view of (A) smaller globules are also absent at places. (C) Inner surface: The thin boundary membrane present on the inner surface exposes the inner membrane fibres. (D-F) Inner surface, enlarged view showing the arrangement of ramified and somewhat curved fibres of uneven thickness on the membrane layer.

layer, yet it provides a considerable proportion of calcium to the developing embryo. The freshly laid egg contains 3.23 mg of calcium (2.76 mg in shell and 0.47 mg in egg component), whereas the embryo needs 1.7 mg of calcium for its complete development. The embryo initially utilized the calcium of the yolk fraction for its development. This means the embryo required 1.29 mg more calcium from sources other than yolk. At the same time the eggshell had undergone 1.74 mg reduction in its calcium content over the entire embryonic period. This suggests that the embryo has utilized this much amount of calcium from the eggshell. Thus, the embryo used calcium from two sources: initially from the yolk followed by the eggshell. The calcium requirement of the embryo and loss of that of the eggshell was comparable. According to Abdel-Salam et al. (2006), concentration of calcium in the eggshell of reptiles is higher than that of sodium/magnesium and calcium distribution in the eggshell differs before and after hatching. In the present study, concentrations of minerals like potassium and magnesium in the egg contents of a freshly laid egg did not alter through incubation. The eggshell provides about 63% of its calcium to the developing embryo, the rest being supported by the yolk.

The eggshell had undergone considerable structural changes from laying to hatching. The calcareous layer formed distinct grooves due to the absence of larger globules. Besides, smaller globules were also absent exposing the underlying membrane fibres. Absence of globules was caused due to the resorption of calcium by the developing embryo from the calcareous layer. However, no change was observed in the thickness of the shell membrane and in the arrangement of the membrane fibres. Embryos of most oviparous reptiles obtain calcium from both yolk and eggshell, but patterns of calcium mobilization vary (Packard and Packard, 1984; Packard, 1994). Oviparous squamate reptiles generally have lightly calcified eggshells and embryos mobilize most calcium from yolk, whereas embryonic turtles, crocodilians and birds are highly dependent on calcium from the eggshell. However, in *P. blanfordanus*, 63% of embryonic requirement is supplied by the eggshell.

Thus, the eggshell composition and structure of *Psammophilus blanfordanus* fall within the range reported for other species in this group. The role of eggshell as a calcium source in lizards is little studied. Future research into this field is important to understand the complex role the eggshell played in development of the embryo. The broad similarities in chemical composition and structure of eggshells of agamid lizards (Packard and Demacro, 1991; Osborne and Thompson, 2005; Stewart and Ecay, 2010) suggest a conserved evolutionary strat-

egy in eggs of lizards, but more species must be studied to confirm such a conclusion.

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