

Calcium Source for Protoconch Formation in the Florida Apple Snail, *Pomacea paludosa* (Prosobranchia: Pilidae): More Evidence for Physiologic Plasticity in the Evolution of Terrestrial Eggs

by

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Abstract. The calcified capsule of terrestrial eggs of several gastropods is known to provide calcium as well as structural support for the embryo. This study examined capsular ultrastructure and intracapsular calcium content of the terrestrial eggs of the aquatic Florida apple snail, *Pomacea paludosa*. The calcium content of intracapsular material did not increase during embryogenesis of *P. paludosa*, and little erosion of the capsule was evident by scanning electron microscopy. Initial intracapsular calcium concentration was 745 mM, far higher than values reported for other gastropods. These results indicate that the embryo of *P. paludosa* is independent of capsular calcium. Reliance of the embryo on intracapsular, rather than capsular, stores of calcium for protoconch formation is a strategy not recognized before among gastropods but is one that has a parallel among squamate reptiles.

INTRODUCTION

Many families of gastropods have independently evolved calcified terrestrial eggs. The more heavily calcified eggs are cleidoic, and those of a few species have been directly or indirectly shown to resorb calcium from the eggshell (TOMPA, 1980). Uncalcified eggs are non-cleidoic and absorb calcium from the extracapsular environment. TOMPA (1980) described the diversity of embryonic calcium dynamics in gastropods. He proposed that calcified egg capsules evolved in terrestrial gastropods to provide not only structural support to the capsule but also calcium for embryonic shell (protoconch) formation. Furthermore, he hypothesized that, despite the diversity of adaptations for embryonic calcium provision in gastropods, intracapsular concentrations of calcium must remain low in the newly oviposited egg to prevent toxicity. In the present study, we report the changes in intracapsular calcium content during

embryogenesis of the Florida apple snail, *Pomacea paludosa* (Say, 1829). Unlike other species previously studied, the terrestrial embryos of this aquatic, prosobranch snail do not depend on the capsule for calcium; this is a strategy of calcium provision that extends the known range of physiologic plasticity in gastropod eggs.

MATERIALS AND METHODS

Egg clutches of *Pomacea paludosa* were collected from emergent vegetation at ponds in West Melbourne, Florida. Voucher specimens of adult snails (IRCZM 065:02878) and of clutches and an adult shell (IRCZM 065:02879) are deposited at the Indian River Coastal Zone Museum, Harbor Branch Oceanographic Institution, Fort Pierce, Florida.

Freshly laid (jellied) clutches were collected in early morning and processed upon return to the laboratory. Individual eggs were teased from the extracapsular jelly and transferred to a dish of distilled water. The capsule was slit, spread open, and evacuated of contents (egg fluids,

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Table 1

Changes in dry weight and calcium content of *Pomacea paludosa* during intracapsular development. Each sample consists of pooled intracapsular contents of 10 newly oviposited eggs or of pooled shells or pooled bodies from 10 hatchlings. "Shell" of hatchlings consists of the protoconchs and opercula; "body" includes all soft parts. Values are $\bar{x} \pm \text{SD}$.

Sample (n = 4)	Dry weight (mg/egg)	Calcium content	
		(mg/egg)	(% dry weight)
Egg	5.64 $\pm$ 1.166	1.03 $\pm$ 0.286	18.3
Hatchling			
Shell	2.90 $\pm$ 0.037	0.933 $\pm$ 0.0229	32.2
Body	1.78 $\pm$ 0.113	0.033 $\pm$ 0.0038	1.8
Total	4.68 $\pm$ 0.109	0.967 $\pm$ 0.0224	20.7

sensu FOURNIÉ & CHÉTAIL [1984]; including zygote, perivitelline albumen, perivitelline sac, and intracapsular jelly [BAYNE, 1966]) by pipette. When properly removed, the albumen and zygote were invested by a membrane; a sample was discarded if the membrane ruptured during evacuation. For each of four clutches, the contents of 10 eggs were pooled for analysis.

Old clutches with well-calcified capsules and the opaque white color of late-stage eggs (PERRY, 1973) were collected from the field and held in the laboratory in dry beakers until hatching. Adherent pieces of calcified capsule were removed from the protoconch (body shell at hatching) and mantle cavity. Hatchlings were dissected to separate the soft parts from the protoconchs and opercula. Because moderate desiccation greatly decreased the tendency of the soft parts to separate cleanly from the protoconchs and opercula, snails were dissected within 2 hr after hatching. Body parts of 10 hatchlings were pooled for each of four sets of samples, each set consisting of a pooled sample of soft parts and a pooled sample of protoconchs and opercula.

Samples were dried to constant weight at 68°C in acid-cleaned beakers and digested in 1 mL 6 N HCl for 2 hr at 68°C. Cooled samples were brought to 10 mL with 6 N HCl, diluted serially with 0.4% La₂O₃ to reduce ionization interference, and analyzed for calcium with a Perkin-Elmer model 4000 atomic absorption spectrophotom-

eter with background correction. Values were corrected also by analysis of blanks that were similarly processed.

Capsular material for scanning electron microscopy was prepared from freshly laid eggs and from eggs hatched in the laboratory. Pieces of capsule were treated with 5.25% NaOCl to remove remnants of the intracapsular contents, air-dried, mounted on aluminum stubs, sputter-coated with gold-palladium alloy, and examined with a Zeiss Novascan 30 scanning electron microscope for physical evidence of calcium resorption from the inner capsular layer.

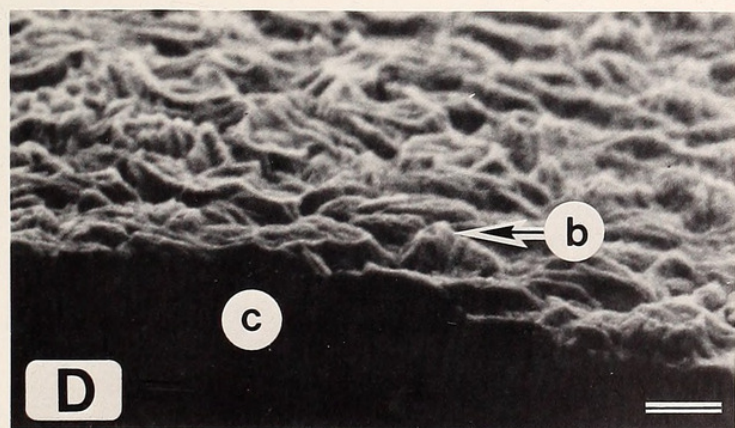
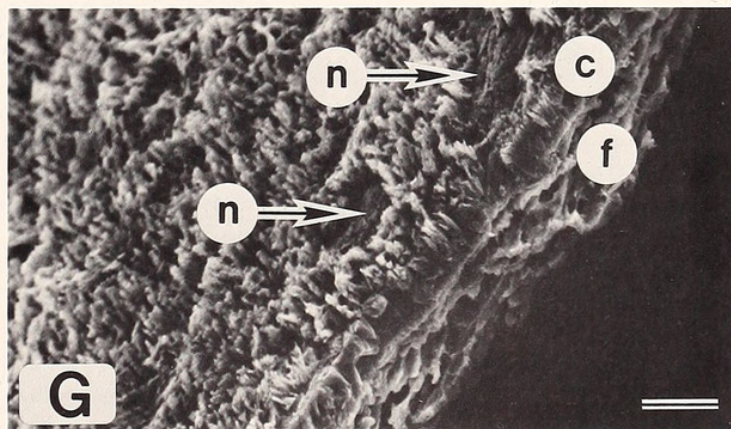
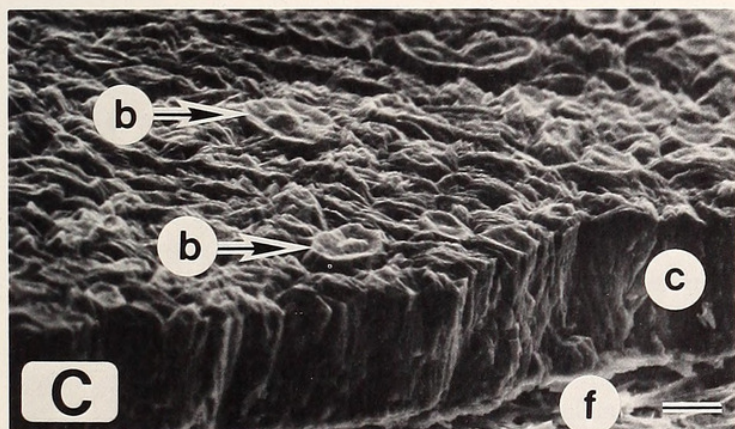
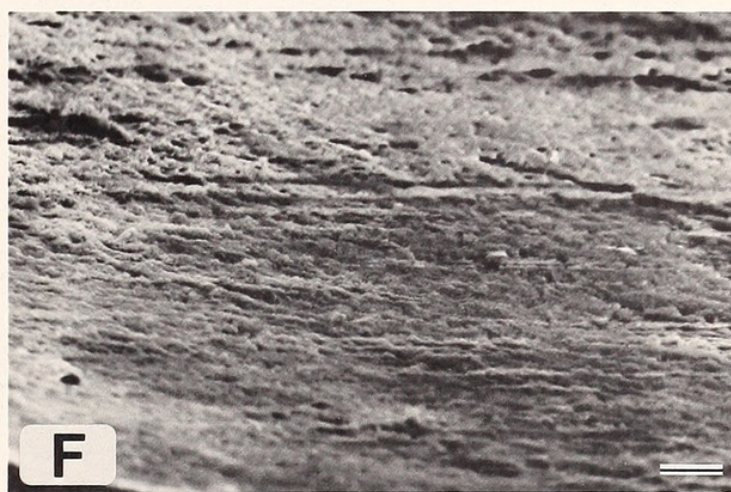
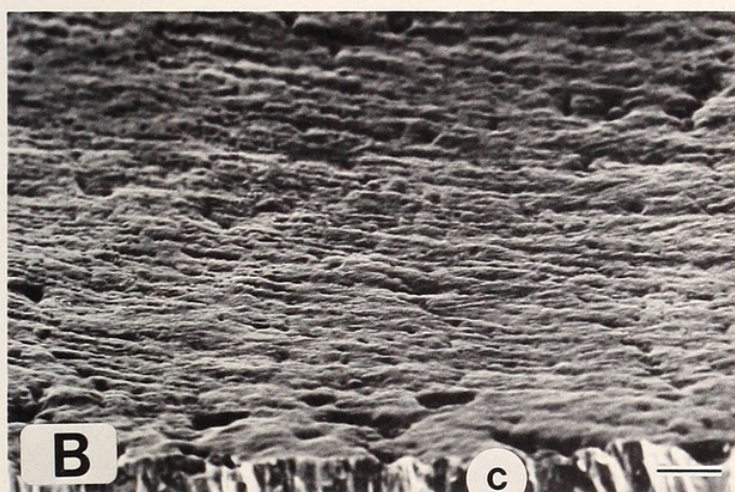
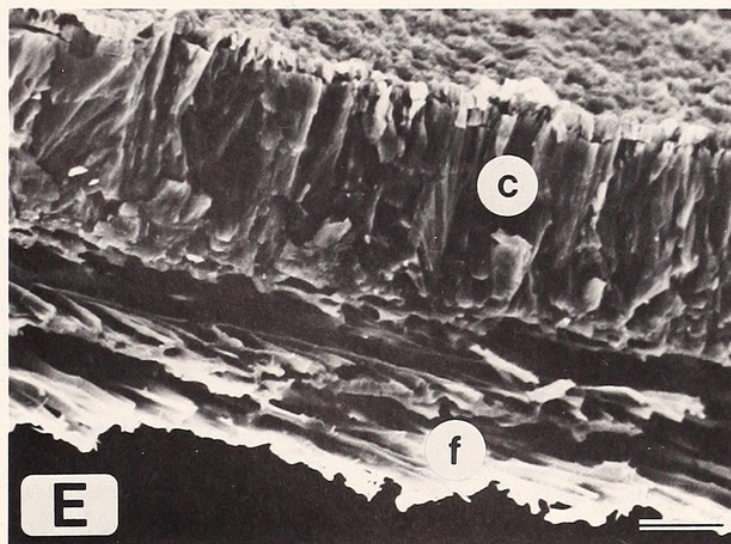
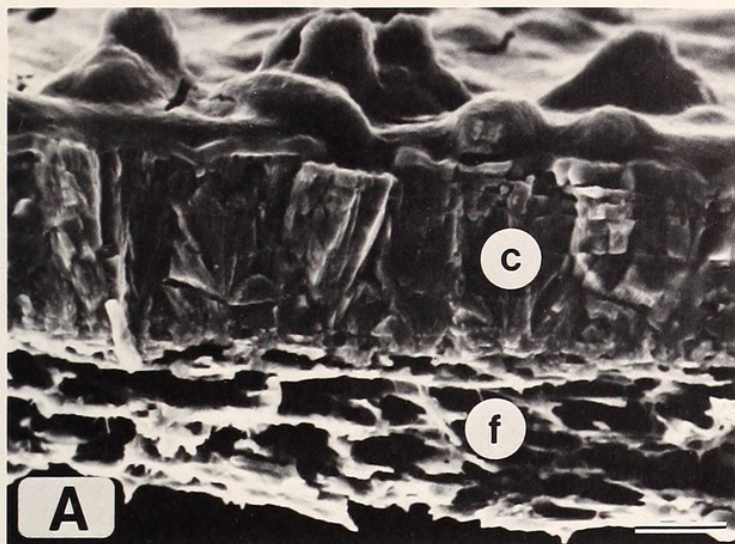
RESULTS

The mean dry weight of intracapsular fluids of the newly oviposited egg was 5.6 mg, of which 1.0 mg (18%) was calcium (Table 1). Assuming a spherical egg with a diameter of 4.0 mm, the intracapsular concentration of calcium was 745 mM. During approximately 3 weeks of development from oviposition to hatching, dry weight of the intracapsular contents decreased 17% (Table 1). Because the weights of contents of the freshly laid eggs were highly variable among clutches, the loss in weight was not statistically significant ($t = 1.640$, $df = 3$, $P > 0.05$). Calcium content of the intracapsular material decreased slightly (6%); but this, too, was not statistically significant ($t = 0.460$, $df = 3$, $P > 0.05$). The amount of calcium expressed as percent dry weight of the egg fluids or the hatchling was similar (18% and 21%, respectively). At hatching, most of the calcium (97%) was in the protoconch and operculum.

The inner, crystalline layer of the thick, calcified capsule changed little in physical appearance during embryogenesis; but its structural integrity seemed to be modified, perhaps by degradation of the organic matrix and mineral recrystallization, making it susceptible to disaggregation by NaOCl (Figure 1). Although thickness of the crystalline layer varied among eggs, the layer did not thin noticeably by the time of hatching, nor did the fracture surface change appearance (Figure 1A, E). Brief (<1-min) treatment with NaOCl only partly removed the organic membrane lining the inside of the capsule (Figure 1B, F). In newly oviposited eggs, longer (20-min) treatment with NaOCl completely removed the membrane, exposing the proximal free surfaces of the capsular crystals (Figure 1C); integrity of the balanoid facets of the crystals was maintained when

Figure 1

Appearance of the fracture and inner surfaces of the egg capsule of *Pomacea paludosa* at oviposition (A–D) and hatching (E–H). A, E: fracture surface of inner, crystalline layer (c) and middle, fibrous layer (f) of the capsule. B, F: oblique views of inner surface of capsule near a fracture surface, treated <1 min with NaOCl; much of inner organic membrane remains. C, G: oblique views of inner surface after 20-min treatment with NaOCl; n, naked patches of deeper crystalline layer exposed by loss of fine, surficial crystals; b, representative balanoid facets. D, H: oblique views of inner surface after 40-min treatment with NaOCl. Scale bars: A, C–E, G–H, 10 μm ; B, F, 20 μm .



treatment was extended to 40 min (Figure 1D). In hatched eggs, 20-min treatment with NaOCl exposed a layer of fine, vertically oriented crystals, patches of which broke free and revealed a deeper, more stable surface (Figure 1G); 40-min treatment removed all fine crystals, and the deeper layer appeared to have a negative image of the balanoid facets (Figure 1H).

DISCUSSION

Calcium dynamics in somatic and reproductive physiology have been studied in snails nominally assigned to *Pomacea paludosa* (MEENAKSHI *et al.*, 1974, 1975; WATABE *et al.*, 1976; MEENAKSHI & WATABE, 1977). The snails differ, however, from *P. paludosa* in egg size, capsular ultrastructure, clutch size and morphology, hatching time, and protoconch size (PERRY, 1973; McCabe & Turner, unpublished data). Moreover, they differ in calcium dynamics during embryogenesis (see below). In the absence of voucher specimens (Watabe, pers. comm.), we only speculate that their material from local pet shops (MEENAKSHI *et al.*, 1974) was the Brazilian *P. bridgesi* (Reeve, 1856), which is marketed commercially (THOMPSON, 1984; STARMÜHLNER, 1989).

Members of the Pilidae, Neritidae, and 36 families of pulmonates lay terrestrial eggs with calcified capsules (TOMPA, 1980). In reviewing available information on embryonic calcium dynamics in gastropods, TOMPA (1980) concluded that calcium for the hatchling is derived predominately from the capsule in cleidoic, calcified eggs and from extracapsular sources in non-cleidoic, poorly or uncalcified eggs. In *Anguispira alternata* (Say, 1816), the intracapsular contents at oviposition contain only 1% of the calcium of the 43-day embryo, and calcium content of the capsule is correspondingly reduced during embryogenesis (TOMPA, 1975). Only 6.7% of the calcium of the protoconch is attributable to the intracapsular fluids of *Pomacea* sp. (MEENAKSHI & WATABE, 1977). Hatchling *Veronicella ameghini* (Gambetta, 1923) derives 22% of its calcium from intracapsular fluids (TOMPA, 1980). In the present study, on the other hand, we found that embryonic calcium requirements of *P. paludosa* are met entirely from intracapsular sources. Without clear presentation of data or methodology, FOURNIÉ & CHÉTAIL (1984) indicated that *Deroceras reticulatum* (O. F. Müller, 1774) also is independent of capsular calcium; but BAYNE's (1966) equally brief account of a more qualitative analysis reported a reduction of capsular calcium.

TOMPA (1980) hypothesized that an osmotic or other toxic effect precluded the storage of sufficient intracapsular calcium to support embryogenesis of cleidoic eggs. But intracapsular calcium concentrations can be high in newly oviposited eggs: 3.8 mM in *Veronicella ameghini* (TOMPA, 1980); 9.1 mM in *Anguispira alternata* (calculated from TOMPA [1975]); 37 mM in *Deroceras reticulatum* (FOURNIÉ & CHÉTAIL, 1984); 183 mM in *Pomacea* sp. (calculated from MEENAKSHI *et al.* [1974: fig. 8] and MEENAKSHI &

WATABE [1977]); 745 mM in *Pomacea paludosa* (present study). Alternatively, FOURNIÉ & CHÉTAIL (1984) predicted that most of the calcium in intracapsular fluids is organically bound; this would seem to be the case for all species cited above if physiologic concentrations of free calcium are typically 10^{-3} mM (ECKERT *et al.*, 1988). The calcium-rich egg of *P. paludosa* represents an extreme in the range of strategies by which gastropods provide calcium for embryogenesis.

In addition to chemical analyses of capsules and intracapsular material, evidence for calcium resorption from the capsule comes from direct observation of changes in its crystalline structure. The number of calcium carbonate spherules in the partly calcified capsule of *Veronicella ameghini* decreases during embryogenesis (TOMPA, 1980). TOMPA (1979) demonstrated an observable loss of crystals in the capsule of *Stenotrema leaii* (Ward in Binney, 1840) and reported similar losses to occur in *Varohadra yeppoonensis* (Beddome, 1897) and *Helicodiscus parallelus* (Say, 1821). Heavy erosion of the initially thick inner crystalline layer in *Pomacea* sp. progresses to the thin, outer monolayer of spherules by the time of hatching (MEENAKSHI & WATABE, 1977). Erosion in *P. paludosa*, on the other hand, is superficial and does not contribute measurably to calcium dynamics of the embryo; changes in surface morphology and structural integrity of the inner capsular layer might only serve to weaken the capsule in preparation for hatching.

Reliance on eggshell calcium for embryogenesis has been demonstrated in insects (MOSCONA, 1948), gastropods (TOMPA, 1980), reptiles (PACKARD *et al.*, 1977), and birds (BOND *et al.*, 1988). The degree of reliance, however, varies widely. For example, embryos of altricial birds erode the eggshell mammillae less than do embryos of precocial birds (BOND *et al.*, 1988); and squamate reptiles rely on calcium stores in the yolk, whereas crocodilians and chelonians rely on eggshell calcium (PACKARD *et al.*, 1977). Mechanisms for calcium provision in terrestrial eggs of gastropods are more diverse (TOMPA, 1980) than for avian and reptilian eggs. The present study extends this diversity among gastropods further to include total reliance on calcium stores in the intracapsular fluids, a mechanism previously considered maladaptive (TOMPA, 1980).

ACKNOWLEDGMENTS

We thank Robert P. Trocine of Florida Institute of Technology (FIT) for assistance with atomic absorption spectroscopy; Patricia A. Linley of the Harbor Branch Oceanographic Institution (HBOI), Electron Microscopy Center, for assistance with scanning electron microscopy; Paula M. Mikkelsen, HBOI, Indian River Coastal Zone Museum, for helpful comments on the manuscript and for assistance with gastropod nomenclature; and Duane E. De Freese, FIT, for his review of the manuscript. This study was supported in part by a Sigma Xi Grant-in-Aid of Research to C. M. McCabe.

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