

J. A. Ojeda · O. R. Chaparro

Morphological, gravimetric, and biochemical changes in *Crepidula fecunda* (Gastropoda: Calyptraeidae) egg capsule walls during embryonic development

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Abstract Walls of egg capsules of the gastropod *Crepidula fecunda* Gallardo, 1979 were examined at different developmental stages during the period of intracapsular development of embryos. The weight, biochemical composition, and structural features (using scanning and transmission electron microscopy) of the capsule walls were examined at several intervals during development of the embryos to the hatching stage. Biochemical analyses were also carried out on the intracapsular fluid to identify the possible transfer of organic material from the capsule wall to the intracapsular fluid. The capsule wall is composed principally of organic matter, primarily protein (91%), plus minor lipid and carbohydrate components. The capsule wall consists of a thin, fibrous external layer, which overlies a thicker, spongy inner layer. The spongy layer has almost disappeared by the end of the developmental period, losing about 90% of its thickness. The 40% loss in weight of the capsule walls over the developmental period is due to loss of organic matter as protein. This suggests that the inner layer of the capsules dissolves and/or disintegrates as larval development advances. The levels of dissolved and/or particulate proteins in the intracapsular fluid are much higher than those typical of the seawater surrounding the capsules. This suggests that, as embryonic development proceeds, the inner capsule walls could potentially provide extra nutrients to the embryos.

Introduction

Many marine invertebrates whose eggs undergo partial or complete development in the benthos deposit them in enclosing structures, which range from multilaminated capsules to fragile gelatinous masses and belts (Fretter and Graham 1994). The encapsulation allows benthic development of the embryos and is particularly common in polychaetes and gastropod molluscs (Pechenik 1979). Capsules protect embryos against desiccation and predation (Pechenik 1979), osmotic stress (Pechenik 1982, 1983), microorganisms (Pechenik et al. 1984; Lord 1986), and UV irradiation (Rawlings 1996). In some gastropod species, females may deposit nutritive eggs along with the embryos, which the embryos use as an energy source during development (Gallardo and Garrido 1987; Chaparro and Paschke 1990). Most species that deposit eggs rely on energy reserves within the oocytes, and in some cases also on organic substances dissolved in the capsular fluid bathing the embryos. The importance of intracapsular fluid as a nutrient reserve has been recognized in pulmonate gastropods (Taylor 1973), but little is known concerning its importance for prosobranch gastropods (Pechenik et al. 1984; Moran 1999). It is known, however, that the fluid bathing the embryos may contain amino acids, proteins, and polysaccharides, whether nutritive eggs are present or not (Bayne 1968; De Maheiu et al. 1974; Paschke 1992; Miloslavich 1999). High nutritive value of the fluid for encapsulated embryos of *Nucella lapillus* was observed by Stöckmann-Bosbach and Althoff (1989) and for *Urosalpinx cinerea* by Rivest (1986). The proteins present in the intracapsular fluids of the egg capsules of *Adomelon brasiliensis* arise in part from the dissolution of the internal membrane of the capsule, suggesting that the capsule wall contributes to the organic content of the intracapsular fluid, becoming a potential source of nutrition (De Mahieu et al. 1974). Dissolved organic material may be used as a nutrient source by some larval stages of marine invertebrates (Manahan and Crisp

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J. A. Ojeda · O. R. Chaparro (✉)
Instituto de Biología Marina 'Dr. J. Winter',
Universidad Austral de Chile,
Casilla 567, Valdivia, Chile
E-mail: ochaparr@uach.cl
Fax: +56-63-221455

1983; Jaeckle and Manahan 1989; Welborn and Manahan 1990; Moran 1999).

The egg capsules are acellular, and their morphology and structure are related to their functional roles. Knowledge of the composition of the capsule walls is important in interpreting the origin, function, and evolutionary relationships among the different structural patterns observed (Garrido and Gallardo 1993).

All species of the genus *Crepidula* are characterized by embryonic capsule development and parental brooding of the deposited capsules as part of their reproductive strategy (Hoagland 1977). *Crepidula fecunda* is abundant in Chile, occurring in stacks in intertidal and subtidal habitats (Gallardo 1977). *C. fecunda* is a protandric hermaphrodite (Chaparro et al. 2001), with internal fertilization typical of other prosobranchs in the family Calyptraeidae (Hoagland 1986). It shows mixed larval development, with an initial brooding of encapsulated embryos by the females for about 4 weeks in the natural environment (Saldivia 2000). After liberation of veliger larvae, the planktonic phase lasts about 15 days (Soto, personal communication). The egg capsules are flattened, triangular sacs, each attached to the substrate by a peduncle. The average number of capsules per spawning is 46. The total height of the capsule (including stalk) varies between 5 and 10 mm (Chaparro and Flores 2002). There are 400–1600 eggs capsule⁻¹, the eggs varying from 204 to 238 μm in diameter (Gallardo 1979). The height of the capsule and the number of eggs per capsule are strongly influenced by the size of the female (Chaparro and Flores 2002). Embryos within the capsules are immersed in intracapsular fluid, and there are no nutritive eggs (Gallardo 1979). Thus, these embryos depend for their nutrition on their maternal yolk and possibly on the organic material in the intracapsular fluid.

In this study we examined gravimetric, morphological, and biochemical changes in the capsule walls during embryonic development of *C. fecunda*.

Materials and methods

Stacks of individuals of *Crepidula fecunda* Gallardo, 1979 were collected from the intertidal zone at Yaldad Bay, Chiloé Island, southern Chile (43°08'S; 73°44'W) during the austral spring–summer 1999–2000 and maintained in the laboratory under conditions similar to those in the environment (temperature: $18 \pm 1^\circ\text{C}$; salinity: $30 \pm 1\text{‰}$) until processing. About 250 incubating females of 37–43 mm shell length (greatest anterior–posterior axis) were removed from substrates for study, and the length of each individual was measured to the nearest 0.1 mm with a micrometer caliper. Only egg capsule batches from this female size range were collected, ensuring that all stages of intracapsular development of embryos were represented. The stage of development and lengths of embryos from three to five capsules from each clutch were recorded from images obtained from an inverted microscope fitted with a Pullnix camera. Images were processed with the SCION PC image processing program.

Since there was no information on the duration of each of the intracapsular development stages of *C. fecunda*, it was necessary to establish a time-independent reference variable for standardizing

comparisons between the different egg (embryo) masses. For this, the variable employed was the shell length (μm) of shelled stages (veliger larvae, $> 200 \mu\text{m}$ shell length). Larval stages prior to shell formation were scored as zygotes, trochophores, or those showing initiation of velar development (early veliger).

Analysis of capsule walls

Gravimetric analysis

About 30 batches of capsules were analyzed, representing all stages of intracapsular embryonic development. Embryos from any given batch were all at the same developmental stage. Embryos and intracapsular fluids were removed from capsules of each batch. At least 30 capsules from each clutch were collected on previously washed, ashed, and tared 24-mm glass fiber filters, constituting a representative sample of each clutch. Each sample filter was briefly rinsed with distilled water under a gentle vacuum to remove soluble salts, and enclosed in dry, tared aluminum foil. They were then dried at 60°C for 48 h to constant weight, cooled in a desiccator, and weighed ($\pm 10 \mu\text{g}$). The filters were then ignited in a muffle furnace at 450°C for 3 h, cooled in a desiccator, and reweighed to determine ash content for calculation of ash-free dry weight (AFDW, i.e. organic matter).

Histochemical analysis

For histochemical detection of protein and carbohydrate, three to five capsules from each batch, representing the initial (zygote), intermediate (veliger: 220–270 μm shell length), and advanced stages (veliger: 320–370 μm shell length, prehatching) were fixed in 10% formalin saturated with CaCO_3 . Samples were embedded in paraffin, and blocks sectioned at 4 μm using an E-Leit Minot microtome to obtain horizontal sections through the mid-plane of the capsules. Histochemistry of lipids was carried out on sections of frozen samples. Lipids were detected with Sudan III staining. The Schiff (PAS) reaction was used to detect neutral mucopolysaccharides, and Alcian blue to detect acid mucopolysaccharides. Proteins were detected with hematoxylin-eosin staining. This indirectly indicates the presence of proteins, because hematoxylin attaches to acid proteins and eosin to basic proteins.

Biochemical analysis

At least 30 capsule walls from each batch were pooled for each analysis. Each sample was rinsed briefly with distilled water and lyophilized. The lyophilized samples were analyzed for total lipids, carbohydrates, and proteins. For total lipids, the colorimetric method of Marsh and Weinstein (1966) was employed, using tripalmitin in chloroform as a standard. Total carbohydrates were determined by the colorimetric phenol-sulfuric acid method (Dubois et al. 1956) using glucose as a standard. The capsules were pretreated to extract the total carbohydrates from the capsule walls by boiling the sample in a 5% trichloroacetic acid solution containing 0.1% silver sulfate (Barnes and Heath 1966). Samples for protein analysis were combusted in a Perkin-Elmer CHN analyzer using acetanilide as a standard. The value for total protein was calculated from the nitrogen content using a conversion factor of 5.8 (Gnaiger and Bitterlich 1984).

Scanning electron microscopy (SEM)

Twenty batches were selected, including the initial, intermediate, and advanced stages of intracapsular development. Samples of capsule walls were placed in Eppendorf tubes and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer on ice for at least 2 h. The fixative was then removed using three 25-min rinses with

ice-cold 0.1 M phosphate buffer. The samples were dehydrated in an ethanol gradient series from 50% to 90% ethanol in 10% increments, terminating with two final rinses in 100% ethanol. Complete dehydration was achieved in a Hitachi critical point dryer with CO₂ as a transitional fluid. Samples were affixed to SEM stubs and coated with gold-palladium for observation in a LEO 420 SEM with a PC image output. Images representative of each capsular stage were stored for later analysis.

Transmission electron microscopy (TEM)

Capsules in the four developmental stages described above (zygote, initial, intermediate, and terminal veligers) were selected for study. A total of 20 samples was fixed for 2 h in ice-cold 2.5% glutaraldehyde in seawater, and then washed three times with 0.2 M phosphate buffer on ice, followed by post-fixation for 2 h in 2% osmium tetroxide. Dehydration was carried out in successive baths of 50%, 70%, and 96% ethanol, followed by three 10-min washes in 100% ethanol, and two 5-min washes in 100% acetone. Dehydrated samples were pre-embedded in a 1:1 mixture of acetone and epol-araldite, with final embedding in an epol-araldite mix (24 h under vacuum). Specimens were then placed in an oven at 60°C for 48 h to ensure complete polymerization. Sections of 600 Å thickness were cut with a Sorvall MT-1 ultramicrotome. The ultrafine sections were placed on grids and stained with uranyl acetate and lead citrate for subsequent observation with a Hitachi model H700 TEM. Selected representative fields were photographed.

Biochemical analysis of intracapsular fluids

About 60 batches of capsules with veligers from 220 to 370 µm in shell length were used to quantify total carbohydrates, lipids, and proteins in the intracapsular fluid. No determinations were made for initial developmental stages (without shell) owing to the fragility of the embryos, which disintegrated during manipulation, contaminating the intracapsular fluids. Capsules were immediately placed in 0.45-µm-filtered seawater, thoroughly dried with absorbent paper, and then placed in a petri dish. They were then perforated with a needle to allow escape of intracapsular fluid without damaging the embryos. Each sample, consisting of the pooled fluid from all the capsules in a batch, was placed in an Eppendorf tube of which the bottom had been cut off and replaced with a 100-µm-mesh Nitex screen to retain any embryos or debris present while allowing passage of intracapsular fluid. Each Eppendorf tube was placed in a 10-ml centrifuge tube and centrifuged at 3000 rpm for 20 min to transfer the intracapsular fluid to the centrifuge tube through the Nitex screen. Known volumes of the resulting centrifuged intracapsular fluid were taken with microcapillary pipets, transferred to Eppendorf tubes, and frozen for later biochemical analysis.

Total lipids and carbohydrates were determined on samples of centrifuged intracapsular fluid using the methods described above for capsule walls. Total proteins were determined using the BCA method (Pierce Laboratories), which employs bicinonic acid (BCA) for colorimetric quantification of total proteins. Samples of filtered seawater (0.45 µm) from aquaria containing capsules were analyzed for content of proteins, lipids, and carbohydrates for comparison with values from the capsular fluid.

Results and discussion

Analysis of capsule walls

Gravimetric analysis

The dry weight of the capsule walls of *Crepidula fecunda* decreased significantly with the progress of embryonic

developmental of veliger larvae (Fig. 1; $P < 0.01$). Thus, in the zygote stage, mean (\pm SD) weight of the capsule wall was 0.12 ± 0.01 mg, whereas, in a veliger 344 µm in shell length, the estimated weight was 0.0443 mg.

The organic content of the capsule walls also decreased significantly ($P < 0.01$) during development (Fig. 2). Recently deposited capsules of *C. fecunda* were composed principally of organic matter (68%), but as intracapsular development of embryos progressed, a loss in capsule mass was observed, represented by a 40% drop in organic matter from a mean (\pm SD) initial value of 0.0726 ± 0.0049 mg organic matter in the zygote to a mean of 0.0290 mg per capsule wall when veligers reached 344 µm in shell length. A high percentage of organic matter was also found in capsules of *Conchole-*

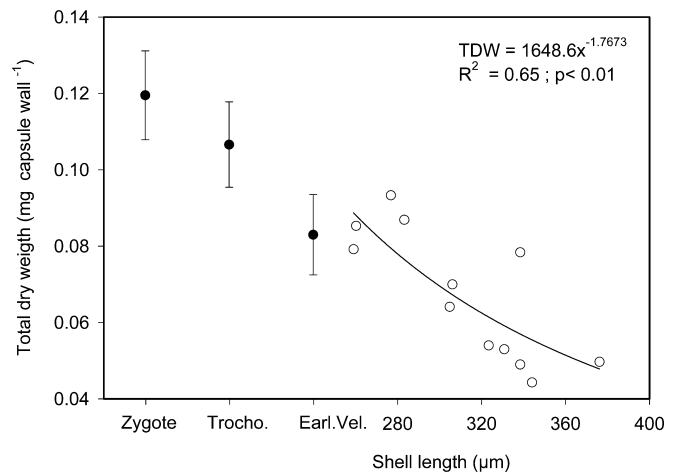


Fig. 1 *Crepidula fecunda*. Variation in dry weight of the capsule wall during capsular development. Filled symbols represent mean values and error bars represent their respective standard deviations. Total samples = 39 [12 samples of intermediate or late veligers (open symbols) and the rest belonging to zygote, trochophore, or early veliger stage]. Each sample had 30 capsule walls

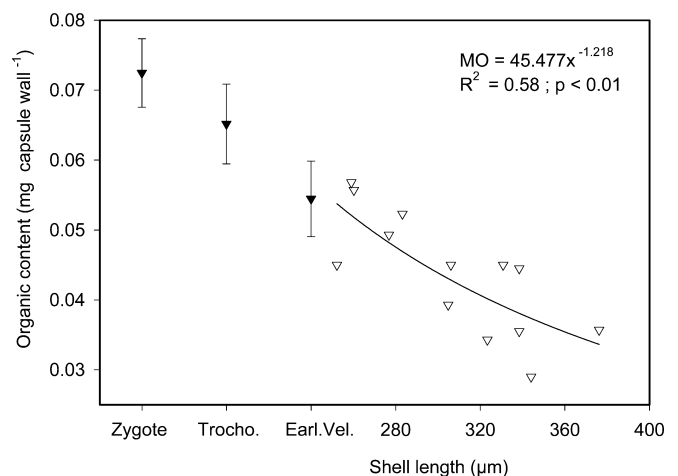


Fig. 2 *Crepidula fecunda*. Organic material content of capsule walls during capsular development. Symbols as in Fig. 1; $n = 42$

pas concholepas by Paschke (1992). In contrast, the inorganic content of capsule walls in *C. fecunda* showed no significant variation over the capsular development period ($P > 0.05$).

Histochemical analysis

The Sudan III did not stain lipids in the capsule walls. Conversely, positive reactions with Alcian blue (blue stain) and PAS (fuchsia stain) indicated the presence of acid and neutral mucopolysaccharides in the capsule walls. Staining with hematoxylin-eosin produced a pronounced red color, indicating the predominance of basic proteins. Bayne (1968) similarly demonstrated the presence of proteins and carbohydrates in the capsule walls of eight gastropod species. Histochemical studies on the neogastropods *Ilyanassa obsoleta* (Sullivan and Mangel 1984) and *Ocenebra erinacea* (Hawkins and Hutchinson 1988) also showed the capsule walls to be formed of proteins and carbohydrates, with no lipids detectable. On the other hand, our biochemical determinations, which were carried out with at least 30 capsules per sample, showed that the capsule walls of *C. fecunda* did contain lipids which were not revealed by histochemical examination of individual capsules.

Biochemical analysis

The protein content declined significantly ($P < 0.01$) with intracapsular embryonic development (Fig. 3), especially during the period in which the veliger larvae grew from 207 μm (approx. 40 μg protein capsule⁻¹) to 365 μm (approx. 25 μg protein capsule⁻¹) in shell length. Neither total carbohydrate nor total lipid showed any relationship with intracapsular development ($P > 0.05$). Protein was the major constituent of capsule walls, with lipid (4.092 ± 2.462 μg capsule⁻¹; mean \pm SD, $n = 47$) and

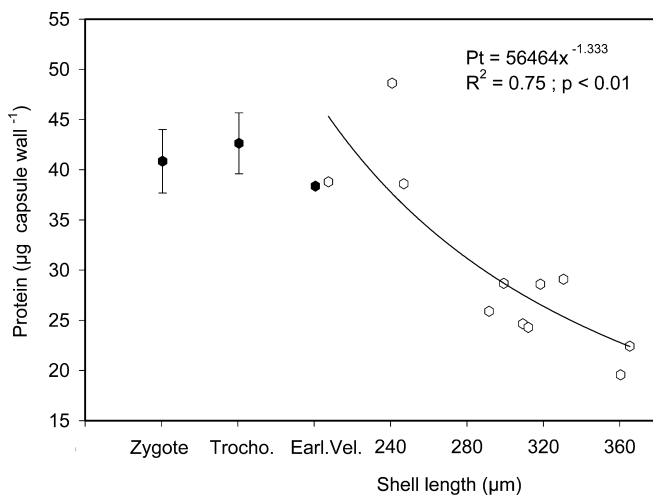


Fig. 3 *Crepidula fecunda*. Variation in protein content of capsule walls during intracapsular development. Symbols as in Fig. 1; $n = 26$

carbohydrate (0.273 ± 0.168 μg capsule⁻¹; $n = 48$) forming their minor constituents.

The very high protein content (>90% of the organic component) of the capsule walls of *C. fecunda* is consistent with data from the whelk *Buccinum undatum*, in which >77% of the capsule is made up of amino acids (Hunt 1966). The complex protein structure of capsule walls has been demonstrated in ultrastructural studies of the protein fibers in egg capsules of *B. undatum* (Flower et al. 1969) and *Cominella maculosa* (Flower 1973). At least two structural proteins are found in capsules of the latter species, and the importance of these proteins accounts for the high protein content of the capsule wall, as suggested by Paschke (1992) for *Concholepas concholepas*. In *C. fecunda* protein always forms the major component of the capsules, although it declines by about 40% in mass during embryonic development.

Scanning and transmission electron microscopy

Two layers formed the capsular wall, a thin, fibrous exterior layer and a thicker internal layer with a loose, spongy consistency (Fig. 4A, B). The latter was in direct contact with the intracapsular fluid containing the embryos.

TEM provided supporting data on the structure of the two capsular layers. The fibrous external layer did

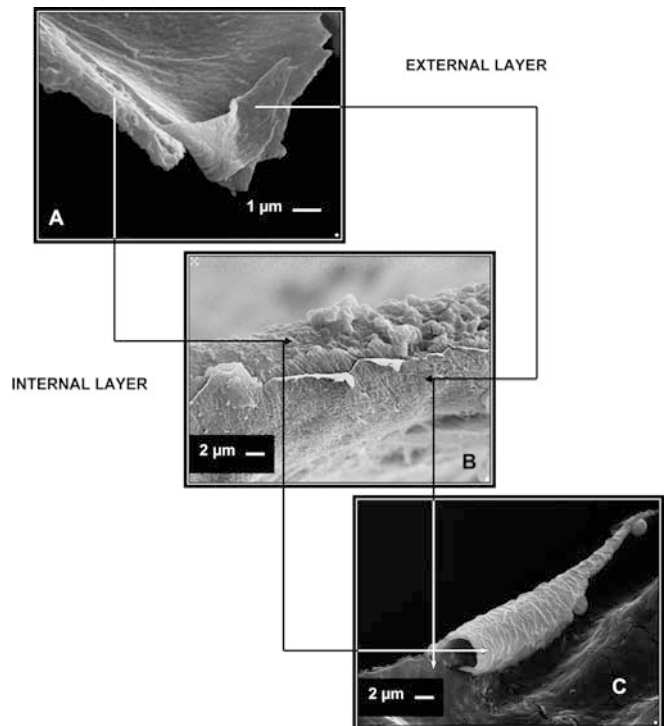
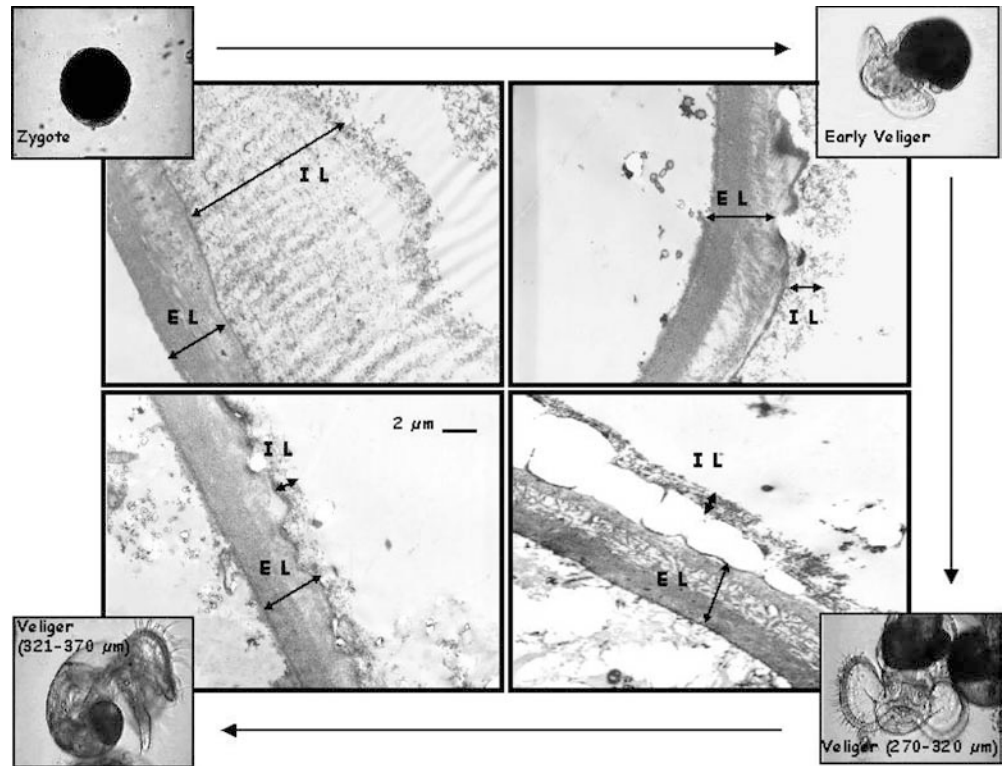


Fig. 4A–C *Crepidula fecunda*. Scanning electron microscopic observations of the egg capsule. **A**, **B** Walls of capsules containing zygotes; **C** wall of capsule containing veliger larvae 360 μm in shell length

Fig. 5 *Crepidula fecunda*. Sections of capsule walls representing different stages of intracapsular development (insets) as observed with transmission electron microscopy (EL external layer; IL internal layer)



not change in thickness over the developmental period, while the internal spongy layer became thinner (Fig. 4A, B, C). The greatest rate of decrease occurred between the zygote and the early veliger (<200 μm in shell length; Fig. 5). The thickness (mean \pm SD) of the capsular wall at the zygote stage was about $11.44 \pm 4.96 \mu\text{m}$, decreasing to $3.28 \pm 0.31 \mu\text{m}$ by the end of development. The external layer was $2.60 \pm 1.21 \mu\text{m}$ thick and remained nearly constant over the entire development period. The inner layer began with a thickness of $7.57 \pm 1.99 \mu\text{m}$, which decreased to $0.83 \pm 0.18 \mu\text{m}$ in the veliger phase (220–270 μm shell length). This decrease was about 70% of the initial value, representing about 90% of the inner wall of the capsule. By the end of the developmental period the inner wall had nearly disappeared (Fig. 6).

The capsule wall in *C. fecunda* is a laminated structure, which appears to be a common feature in gastropod capsule organization. Optical and electron microscopy of capsule walls in neogastropods (primarily muricaceans and buccineans) has revealed a multilaminated structure, usually consisting of more than four layers (Tamarin and Carriker 1967; Sullivan and Mangel 1984; D'Asaro 1988; Hawkins and Hutchinson 1988; Garrido and Gallardo 1993). This configuration provides resistance to mechanical damage and biological deterioration (Hawkins and Hutchinson 1988). At the beginning of intracapsular development in *C. fecunda*, two layers were observed forming the capsule wall. Although the thin, fibrous external layer showed regions with variable electron densities and patterns of fiber orientation, sub-layers within the

external layer were not identified. The internal spongy layer in contact with the embryos and intracapsular fluid in *C. fecunda* may be comparable to the fourth layer or "albumin sac", a term used by some authors for the inner capsular layer in contact with intracapsular fluid in other gastropods; these layers have been described as very thin (60 nm–2.8 μm in thickness), and as having the function of retaining the intracapsular fluid (Tamarin and Carriker 1967; Sullivan and Mangel 1984; D'Asaro 1988; Hawkins and Hutchinson 1988; Garrido and Gallardo 1993).

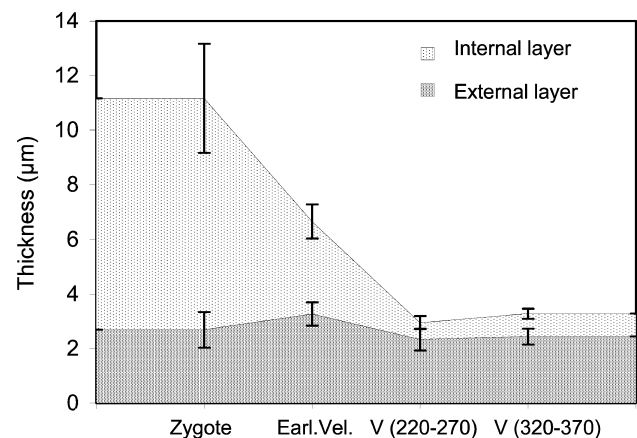


Fig. 6 *Crepidula fecunda*. Variation in thickness (μm) of the capsule wall. Measurements obtained from transmission electron microscopic photographs. Data represent means and standard deviations (Earl. Vel. undergoing shell formation; V veliger, μm shell length)

Biochemical analysis of intracapsular fluid

The intracapsular fluid in the veliger (220–363 μm shell length) contained protein, but no lipid or carbohydrate was detected.

The highest values for protein in the intracapsular fluid were 905 $\mu\text{g protein ml}^{-1}$ fluid at a veliger shell length of 272 μm , and 141 $\mu\text{g protein ml}^{-1}$ fluid at a shell length of 363 μm . There was only $12 \pm 3.02 \mu\text{g protein ml}^{-1}$ ($\pm\text{SD}$) in the surrounding seawater (Fig. 7).

Loss in weight in the capsule wall suggests that material from the inner capsule wall is transferred to the capsular fluid in dissolved or particulate form. This represents a transfer of almost 15 $\mu\text{g capsule}^{-1}$ ($0.015 \text{ J capsule}^{-1}$; Gnaiger 1983) during the early veliger to pre-hatching veliger period in the egg mass of a typical female of shell length 40 mm. Mechanical (Vaughn 1953) or chemical (Pechenik 1975; Sullivan and Bonar 1984; Sullivan and Mangel 1984; Hawkins and Hutchinson 1988) action of embryos against the inner capsular wall or specific portions of it have been proposed as possible mechanisms to explain the thinning of the wall. In *C. fecunda*, reduction in thickness of the capsule wall may be due to unknown mechanical or chemical effects produced by the contained larvae. Another possibility is that the flow of the intracapsular fluid and the friction between shelled larvae and the inner capsule wall could cause inner surface erosion.

Although higher levels of protein were found in the intracapsular fluid than in the surrounding seawater, this study did not establish any trophic function for this protein. Some authors have suggested that bivalve larvae have the capacity to absorb dissolved nutrients such as amino acids from seawater through the epidermis (Manahan and Crisp 1983). Protein uptake has also been recorded for encapsulated larvae of gastropods,

e.g. *Nerita picea* (Rivest and Strathmann 1994) and *Littorina* spp. (*L. saxatilis*, *L. sitkana*, *L. subrotundata*; Moran 1999). Lecithotrophic larvae of the gastropod *Haliotis rufescens* acquire energy by absorbing dissolved amino acids and sugars directly from seawater (Jaekle and Manahan 1989; Welborn and Manahan 1990). Some organic components of the intracapsular fluid may be incorporated by pinocytosis into integumental cells of the cephalic region and a small portion of the fluid internalized by ectodermic larval kidneys in *Nucella lapillus* (Fioroni et al. 1985) and *Searlesia dira* (\cong *Lirabuccinum dirum*) (Rivest 1980). The use of dissolved organic material has also been described for gastropod embryos, including *Adelomelon brasiliiana* (De Mahieu et al. 1974) and *N. lapillus* (Stöckmann-Bosbach and Althoff 1989). The protein content of the intracapsular fluid in *N. lapillus* decreases during capsular development, in part due to uptake by the embryos. The presence of proteins in the intracapsular fluid of *A. brasiliiana*, and the decline due in part to uptake by enclosed embryos, has been demonstrated by De Mahieu et al. (1974). These observations suggest that embryos may use intracapsular fluid proteins as a source of dissolved and/or particulate extraembryonic nutrition, arising entirely or in part from the inner capsular layer. However, the possibility that protein, at least in part, could be lost to the outside medium must also be considered. Although *C. fecunda* embryos and developing larvae may be unable to absorb dissolved proteins from the intracapsular fluid, encapsulated veligers of this species are certainly able to ingest extraembryonic particles (Chaparro et al. 2002a, 2002b). The spongy, fragile nature of the inner capsular layer as revealed by SEM/TEM in this study suggests that disaggregated particles of this layer may be consumed by larval filtration within the capsules. The present study provides evidence that organic material (dissolved or particulate proteins) present in the intracapsular fluids of *C. fecunda* originates from the inner capsular layer. Future study is required to examine the possibility that this material provides an extraembryonic source of nutrition for the developing larva.

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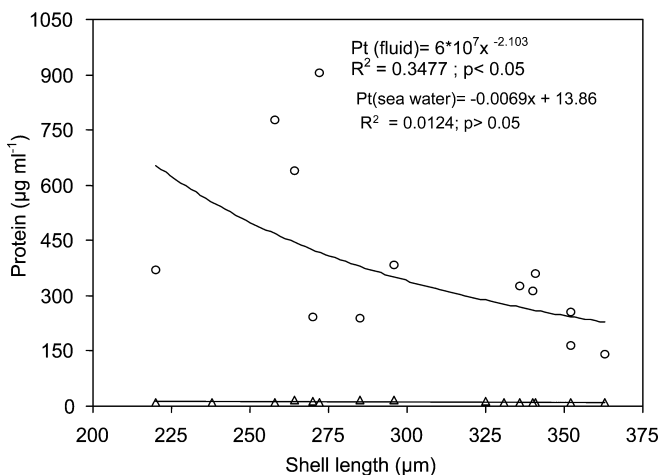


Fig. 7 *Crepidula fecunda*. Protein content of intracapsular fluid (circles) and seawater (triangles) from around egg capsules during intracapsular development. Different batches of capsules represent different veliger stages

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