Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2018) **84**: 469–479. doi:10.1093/mollus/eyy045
Advance Access publication date: 22 September 2018

Enveloping walls, encapsulated embryos and intracapsular fluid: changes during the early development stages in the gastropod *Acanthina monodon* (Muricidae)

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(Received 28 February 2018; editorial decision 14 August 2018)

ABSTRACT

Encapsulation of embryos in marine gastropods affords protection for the developing young, whether or not parental care takes place. The capsule wall is laminated and its dimensions change during development. Dissolution of the capsule wall releases dissolved organic matter (DOM) into the intracapsular fluid, providing a nutritional source for the embryo. The capsule wall of *Acanthina monodon* is composed of three layers, a thin outer layer with projections to the exterior, a thicker intermediate layer containing vacuoles and a thin inner layer that gives rise to the hatching plug. During embryonic development the capsule wall lost 27.2% of its initial mass owing to a pronounced thinning of the outer layer, especially of the projections. The organic fraction in the capsule wall (>85% of total mass) decreased by 23.3%, mostly due to the loss of 52.8% of the protein originally present. The internal layer of the capsule wall became 35.2% thinner during embryonic development, and in capsules containing embryos in the prehatching stage the surface area of a section through the plug was reduced to 2% of its original value. Total protein concentration in the intracapsular fluid decreased by 54% during embryonic development. The dry weight of the encapsulated juveniles immediately before hatching was 96% greater than that of the eggs from which they developed. Total protein concentration increased by 148% between the egg and the advanced veliger stages, then decreased in the prehatching juvenile, presumably as a result of the energy cost of metamorphosis, which occurs within the capsule before the juveniles are released. During development, material was lost from the exterior surface of the outer layer of the capsule wall. In contrast, the inner layer partially dissolved and disintegrated into the intracapsular fluid, providing the developing embryos with a secondary source of nutrition, mainly protein, during the later stages of encapsulation, when the material from the nurse eggs was exhausted.

INTRODUCTION

Benthic marine invertebrates exhibit a wide range of life-history patterns. Many species possess a planktonic larval stage that permits dispersal and finally settles on the bottom, where metamorphosis is completed (Thorson, 1950; Vance, 1973; Scheltema, 1986). Some species with benthic development in capsules lack a planktonic larval stage entirely and instead hatch as juveniles that resemble miniature adults. Early development takes place either partially or completely within specialized structures that surround or confine the embryo or mass of embryos until the hatching stage is reached. Whatever the stage at hatching, when a female encapsulates groups of embryos there are more possible nutritional sources to support development of the embryos and larvae than if they were to develop individually in the plankton. The enveloping structures may be solid multilayered capsules or fragile gelatinous ribbons (Fretter & Graham, 1994), secreted by the female and deposited on the substrate. The capsule or ribbon mass may or may not be protected directly by the female (Thorson, 1950; Pechenik, 1986; Collin, 2003; Chaparro *et al.*, 2008, 2011).

Encapsulation of embryos on the bottom is a mode of development especially common in polychaetes and gastropod molluscs (Pechenik, 1979). Although chemically complex and energetically expensive (Pechenik, 1986), it protects the embryos (see review by Przeslawski, 2004) from desiccation and predation (Pechenik, 1979), osmotic stress (Pechenik, 1982, 1983), microorganisms (Pechenik, Chang & Lord, 1984; Lord, 1986) and UV radiation (Rawlings, 1996). Encapsulation also increases the probability that

the progeny remain in the same locality as their parents (Chapman, 1965; Gibbs, 1968; Rivest, 1983) and ensures a greater survival rate of the embryos, which compensates for the high costs of this reproductive strategy (Pechenik, 1986).

Reproduction by means of encapsulation requires energy expenditure not only in gametogenesis but also in the production of structures to isolate the developing embryos from the external environment (Chaparro & Flores, 2002). Furthermore, in some species the female invests in supplementary, extraembryonic sources of nutrition for the embryos, for example nurse eggs and/or nurse embryos (Gallardo & Garrido, 1987; Chaparro & Paschke, 1990; Collin, 2003) and dissolved organic compounds secreted into the intracapsular fluid (Ojeda & Chaparro, 2004; Bigatti *et al.*, 2014), particularly amino acids, proteins and polysaccharides (Bayne, 1968; De Mahieu, Penchaszadeh & Casal, 1974; Miloslavich, 1999; Bigatti *et al.*, 2014). The nutritional role of intracapsular fluid has been demonstrated in various families of gastropods, such as the Muricidae (*Nucella lapillus*, Stöckmann-Bosbach & Althoff, 1989; *Urosalpinx cinerea*, Rivest, 1986), the Volutidae (*Odontocymbiola magellanica*, Bigatti *et al.*, 2014) and the Calyptraeidae (*Crepidula fecunda*, Ojeda & Chaparro, 2004). Although the dissolved organic nutrients in the intracapsular fluid are often secreted directly by the female during the formation of the capsule (Miloslavich, 1999; Marin *et al.* 2003; Bigatti *et al.*, 2014), in some cases they originate from the capsule wall, especially by release of proteins and amino acids during dissolution of the innermost layer, which is in direct contact with the intracapsular fluid (De Mahieu *et al.*, 1974). As embryonic development proceeds, this nutrient flow from the capsule wall to the intracapsular fluid usually results in a decrease in the mass of the capsule wall, especially in the organic component. When the encapsulated embryo reaches the veliger stage, the larval shell or the velar cilia can erode minute amounts of material from the inner layer of the capsule wall by physical contact, facilitated by its spongy and fragile consistency (Ojeda & Chaparro, 2004; Segura *et al.*, 2010), allowing the dissolution of organic molecules, which results in thinning of the capsule wall.

There is considerable evidence that the capsule wall is a significant source of nutrition for developing embryos in marine invertebrates that exhibit encapsulation (De Mahieu *et al.*, 1974; Ojeda & Chaparro, 2004; Brante, Fernandez & Viard, 2009). According to Gallardo (1980), direct development is an advanced mode of reproduction from the point of view of energy utilization, particularly when encapsulation of the embryos is accompanied by the provision of nurse eggs. Furthermore, in muricid gastropods this extraembryonic yolk facilitates rapid intracapsular development and earlier hatching of the juveniles, thereby minimizing the period of exposure of the capsules and embryos to mortality in the intertidal zone (Gallardo, 1979).

In this paper we consider the muricid *Acanthina monodon* (Pallas, 1774), in which development is completed entirely within capsules deposited on intertidal rocks by the female and then abandoned. This species is common in the lower intertidal and shallow subtidal (Osorio, Atria & Mann, 1979; Reid & Osorio, 2000) and widely distributed along rocky coasts in Chile (Gallardo, 1979; Valdovinos, 1999; Gallardo & Penchaszadeh, 2001). The period of encapsulation varies from 55 to 65 days at temperatures from 14 °C to 11 °C, respectively (Gallardo, 1979), and oocyte yolk is supplemented by nurse eggs (nonviable eggs) within each capsule. The developing embryos consume all the nurse eggs when they reach the trochophore stage. Approximately 7% of the eggs deposited in the capsules continue embryonic development (O. Chaparro, unpublished observations). No information is available about the morphological and biochemical changes that occur in the capsule wall during embryonic development in *A. monodon*, or about the potential nutritional contribution of the products of dissolution of the inner layer that accumulate in the intracapsular fluid. Intracapsular development of embryos occurs over weeks or months, with exposure to changes in the external environmental conditions and in the internal milieu. This study examines changes in the capsule wall and intracapsular fluid that provide nutrition for the embryos and also act as a buffer between the external environment and the embryos during development.

Table 1. Shell length (μm) in various developmental stages of *Acanthina monodon*.

Development stage	Abbreviation	Shell length (μm)
Egg*	egg	Not measured
Trochophore*	troch	Not measured
Early veliger	early vel	500–750
Advanced veliger	Adv vel	790–920
Juvenile prehatching	juv pre-hatch	>1,000

*Stages not measured owing to absence of shell.

MATERIAL AND METHODS

Sample collection

Capsules of *Acanthina monodon* were collected between January and March 2017 from the rocky intertidal at Calfuco, southern Chile (39°46′50″S, 73°23′34″W). Approximately 400 capsules were collected from different places and clutches, potentially produced by different females. The capsules were maintained in constantly aerated filtered seawater in 6-l tanks at the Calfuco Marine Laboratory under conditions corresponding to the natural environment (temperature 16 ± 1 °C, salinity 30 ± 1). Seawater was changed daily and each capsule individually cleaned with a brush to minimize growth of epibionts such as fungi, bacteria and protozoans. Capsules with embryos at different stages of development were maintained under these conditions for a maximum of one week until they were used. Five developmental stages were identified for analytical purposes (Table 1).

Gravimetric analysis of capsules and embryos

The capsule wall was separated from the enclosed embryos in approximately 250 capsules from 50 different egg masses. The embryos from each capsule were photographed with a digital camera under a dissecting microscope and the images captured with Micrometrics SE Premium software. For stages with a shell, shell length was measured in 10 embryos selected at random from each capsule (Image J software) and a mean value calculated.

For each developmental stage, five of the empty capsules (i.e. capsule walls) were rapidly washed in distilled water to remove residual salts, then placed on a preweighed, numbered piece of aluminium foil. The loaded foil pieces were oven-dried (48 h at 60 °C), cooled in a desiccator, weighed, combusted in a muffle furnace (3 h at 450 °C) to remove organic material, cooled again in a desiccator and reweighed. The dry weight and ash-free dry weight of the capsules were obtained by difference.

The same procedure was used to determine dry weight and ash-free dry weight of embryos at each stage, except that glass-fibre filters (Advantec GA55, 24 mm diameter) were used instead of aluminium foil, and all the embryos in a single capsule were counted, then placed on a filter. Filters loaded with embryos were rinsed quickly under vacuum with distilled water to eliminate seawater salts.

Dry weight, dry organic matter and dry inorganic matter were expressed as mean values per capsule or embryo at each developmental stage.

Histology and scanning electron microscopy of capsule walls

Histology: Twenty-nine capsules from different females were selected for each developmental stage (Table 1). Capsules were fixed for 2 h in 7% formalin in filtered seawater, then transferred to fresh 7% formalin and stored in this fixative at 4 °C until used. The samples were dehydrated, cleared and embedded in paraffin wax, and the blocks sectioned at 7 µm according to standard histological practice (Montuenga, Esteban & Calvo, 2009). Longitudinal and transverse sections were prepared and stained with haematoxylin and eosin, and examined with a compound microscope (Olympus BX41) fitted with a digital camera. Images were captured with Micrometrics SE Premium software and analysed with ImageJ. The number of layers in the capsule wall was determined and their thicknesses measured. The length and area of the projections from the external layer of the capsule wall were measured at various stages of embryonic development. Thinning of the hatching plug was measured as a decrease in the area of the plug viewed in a longitudinal section.

Scanning electron microscopy (SEM): Twelve capsules with embryos at different developmental stages were selected and the capsule walls fixed for at least 25 min in 2.5% glutaraldehyde on ice, with occasional agitation. The fixative was removed and the samples washed four times in 0.1 M phosphate buffer on ice (10 min per wash) before storage in buffer at 4 °C (Rivest, 1992; Ojeda & Chaparro, 2004). Samples were then dehydrated in an ethanol series (50–90% in steps of 10%), rinsed twice in absolute ethanol and processed in a critical point dryer with CO_2 as the transition fluid. Each dry specimen was mounted on a sample holder, coated with gold and examined by SEM (Zeiss Oxford x-act).

Protein concentration in the capsule walls, intracapsular fluid and embryos

Four capsules from each stage of development were dissected and the capsule walls, intracapsular fluid and embryos separated, providing three samples per capsule. A total of 50 samples for each component (fluid, embryos, walls) were stored at –80 °C to await analysis. Where the quantity of material from a capsule was too small for analysis, samples from several capsules from different clutches at the same stage of development were pooled. Before analysis, samples of capsule wall and embryos were homogenized with a porcelain pestle and mortar containing liquid nitrogen, then sonicated in Tris HCl buffer (pH 6.9). Total protein was determined with a Pierce BCA (bicinchoninic acid) protein assay kit, following the directions of the manufacturer. Albumin was used as a standard. In brief, samples were transferred to a microplate, reagents added and the plate read in a PC-controlled microplate reader (Biochrom Anthos Zenyth 200 with ADAP software). Data were expressed as mg protein per mg dry tissue (mg protein per ml in the case of intracapsular fluid).

Statistical analysis

Levene's test showed that variances were not homogenous for the variables dry weight of the capsule wall, dry weight of organic material in the capsule wall, protein concentration in the embryos, hatching plug area and thickness of capsule walls, so the data were transformed before statistical analysis. We used one-way ANOVA followed by a post-hoc multiple range test (Tukey HSD), with the stages of embryonic development as a fixed independent variable. All statistical procedures were carried out with STATISTICA software.

RESULTS

Gravimetric analysis of capsules and embryos

The dry weight of the capsule wall of *Acanthina monodon* decreased by 27.2% as embryonic development proceeded, from an initial value of 2.634 ± 0.433 (mean ± SD, $n = 9$) mg capsule^{-1} at the egg stage to a final value of 1.917 ± 0.666 ($n = 13$) mg capsule^{-1} in the prehatching juvenile (Fig. 1A; $F_{4,44} = 5.315$, $P < 0.01$).

The dry weight of the embryo increased twice during development from 0.038 ± 0.008 ($n = 7$) mg embryo^{-1} at the egg stage to 0.076 ± 0.005 ($n = 13$) mg embryo^{-1} in the prehatching juvenile (Fig. 1B; $F_{4,39} = 55.71$, $P < 0.0001$). Presumably, organic material from nurse eggs, capsule wall and capsule fluid all contributed to the increase in weight of the embryos.

The dry weight of organic material in the capsule wall decreased by 23.4% during development, from 2.383 ± 0.386 ($n = 9$) mg capsule^{-1} in the egg stage to 1.827 ± 0.267 ($n = 13$) mg capsule^{-1} in the prehatching juvenile (Fig. 2, $F_{4,44} = 3.148$; $P < 0.03$). A significant reduction (64%) in the dry weight of inorganic matter in the capsule wall was also observed, from 0.250 ± 0.141

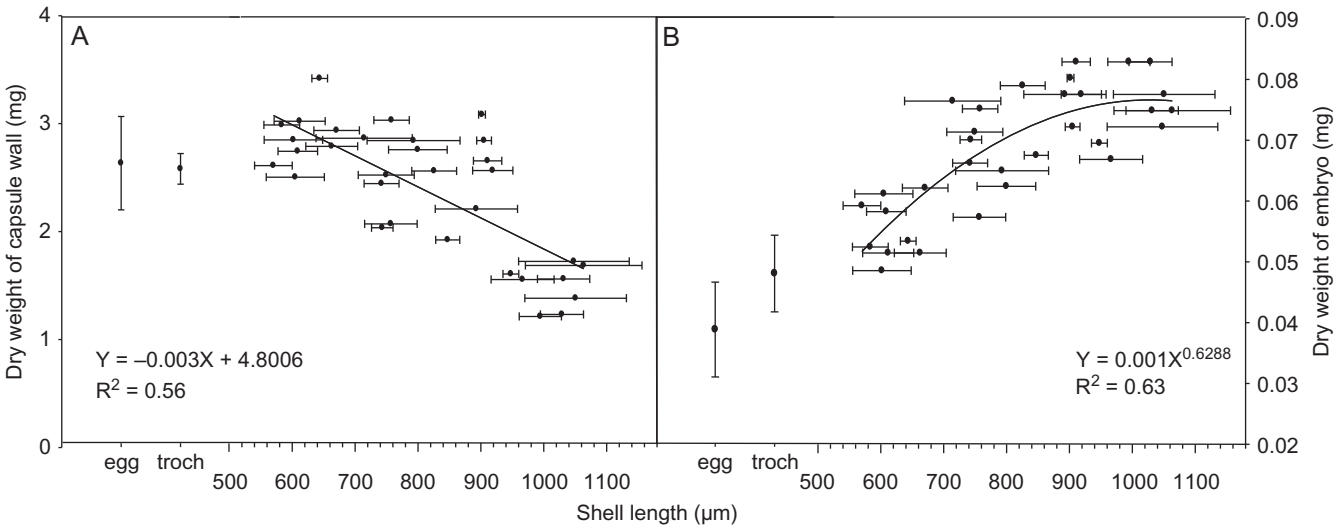

Figure 1. *Acanthina monodon*. **A.** Dry weight per capsule wall as a function of developmental stage or embryo shell length; total $n = 50$. Egg: 2.63 ± 0.43; trochophore: 2.58 ± 0.33 (mean ± SD). **B.** Dry weight (mg) per embryo at different stages of encapsulated development; total $n = 44$. Egg: 0.038 ± 0.008; trochophore: 0.048 ± 0.006. Vertical lines indicate SD for capsule wall and embryo weight at egg and trochophore development stages. Horizontal lines indicate range in shell length. Only data from stages with a shell were used to derive the regression equations.

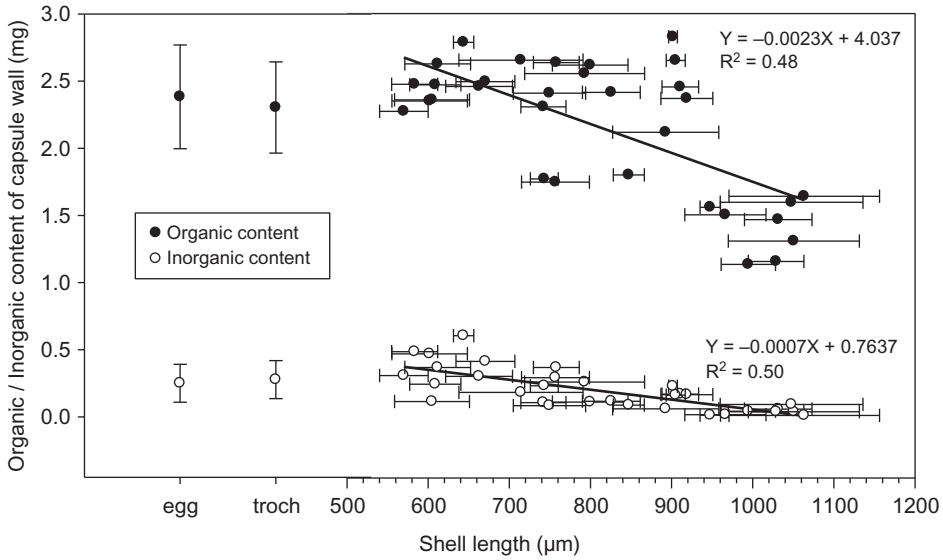

Figure 2. *Acanthina monodon*. Organic and inorganic dry weight per capsule wall at different embryonic stages or embryo shell lengths; total $n = 49$. Organic content: egg 2.38 ± 0.39; trochophore 2.3 ± 0.34 (mean ± SD). Inorganic content: egg 0.25 ± 0.14; trochophore 0.27 ± 0.14. Horizontal lines indicate range in shell length. Vertical lines indicate SD for organic and inorganic dry weight of capsule wall at egg and trochophore stages. Only data from stages with a shell were used to derive the regression equations.

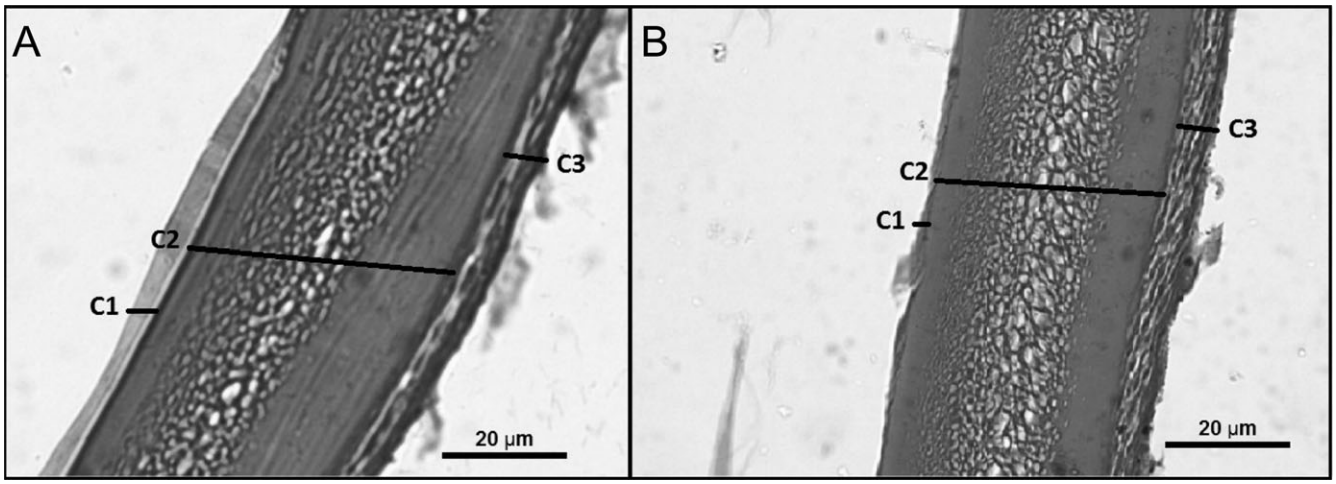

Figure 3. *Acanthina monodon*. Histological longitudinal sections of capsule wall. **A.** Capsule containing eggs. **B.** Capsule containing prehatching juveniles. Abbreviations: C1, internal layer; C2, intermediate layer; C3, external layer. Arrows indicate middle part of intermediate layer, in which vacuoles are present.

($n = 9$) mg capsule^{-1} (egg stage) to 0.090 ± 0.072 ($n = 13$) mg capsule^{-1} (prehatching juvenile) (Fig. 2; $F_{4,44} = 7.126$, $P < 0.002$).

Histology and SEM of capsule walls and associated structures

The capsule wall is composed of three discrete layers (outer, intermediate and inner; Fig. 3). All persist throughout the intracapsular stages of development, but there was a significant decrease in thickness during embryonic development from the egg stage to the prehatching juvenile (Fig. 4A, B; $F_{1,28} = 160.4$, $P < 0.0001$). The thickness of the inner layer decreased by 35.2%, from 2.385 ± 0.311 µm ($n = 4$) in the egg to 1.546 ± 0.342 µm ($n = 10$) in the prehatching juvenile ($F_{3,12} = 5.0993$, $P < 0.0001$). The observed decrease in the thickness of the outer layer from 2.288 ± 0.337 µm ($n = 4$) in the egg to 2.083 ± 0.324 µm ($n = 10$) in the prehatching juvenile was not significant (one-way ANOVA, $P > 0.2$). The outer layer gives rise to projections of the capsule wall (Fig. 5) towards the exterior, which decreased in area and length as development progressed (Fig. 6; area: $F_{4,24} = 25.75$, $P < 0.0001$; length: $F_{4,24} = 34.54$, $P < 0.0001$). The intermediate layer is the thickest of the three and decreased in thickness from 36.968 ±1.717 µm ($n = 4$) at the egg stage to 28.919 ± 2.274 µm ($n = 10$) at the prehatching juvenile stage (decrease of 22%, $P < 0.0001$). The middle part of the intermediate layer is vacuolated and represents 50–60% of its cross-sectional area (Fig. 7), the remainder being composed of denser material flanking the vacuolated region.

The inner layer of the capsule wall forms the hatching plug, which projects into the interior of the capsule and opens to release the juveniles to the exterior (Fig. 8). We recorded a decrease of 98% in the area of a longitudinal section through the plug through the development period, from an initial value of 0.774 ± 0.191 mm^2 ($n = 6$) in the egg to 0.062 ± 0.017 mm^2 ($n = 6$) in the advanced veliger and finally to 0.010 ± 0.004 mm^2 immediately before release of the juvenile (Fig. 9; $F_{3,20} = 218.1$, $P < 0.0001$).

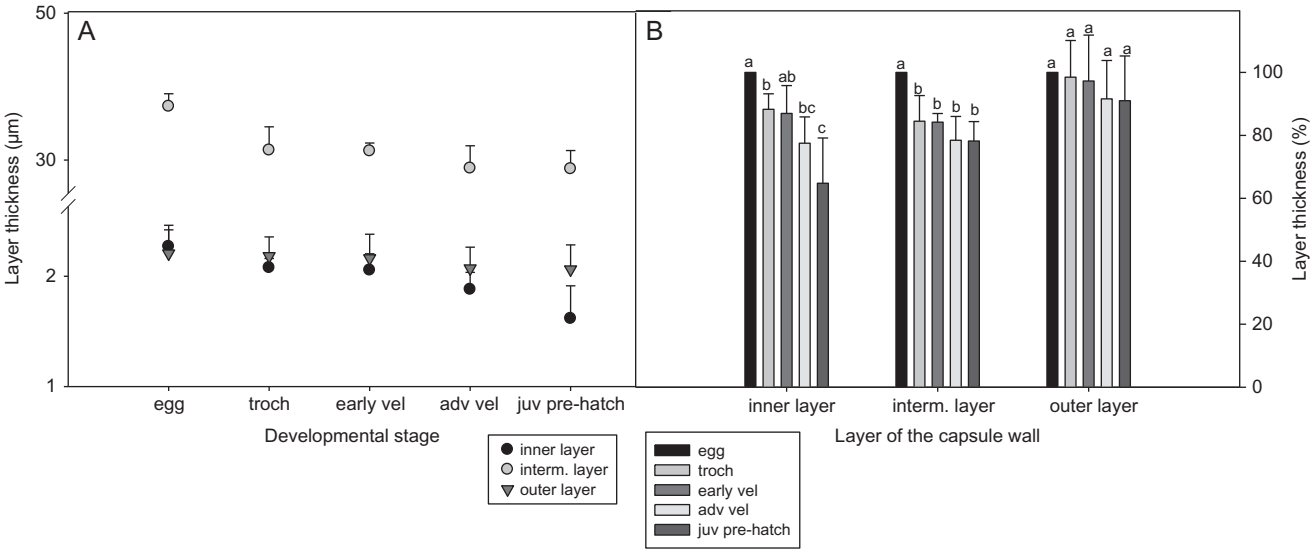

Figure 4. *Acanthina monodon.* **A.** Thickness (mean ± SD) of each layer of capsule wall in longitudinal section at different stages of development. **B.** Percentage changes in thickness for each layer in capsules at various stages of development. Group means with different letters are significantly different from each other. Total $n = 29$; $P < 0.05$.

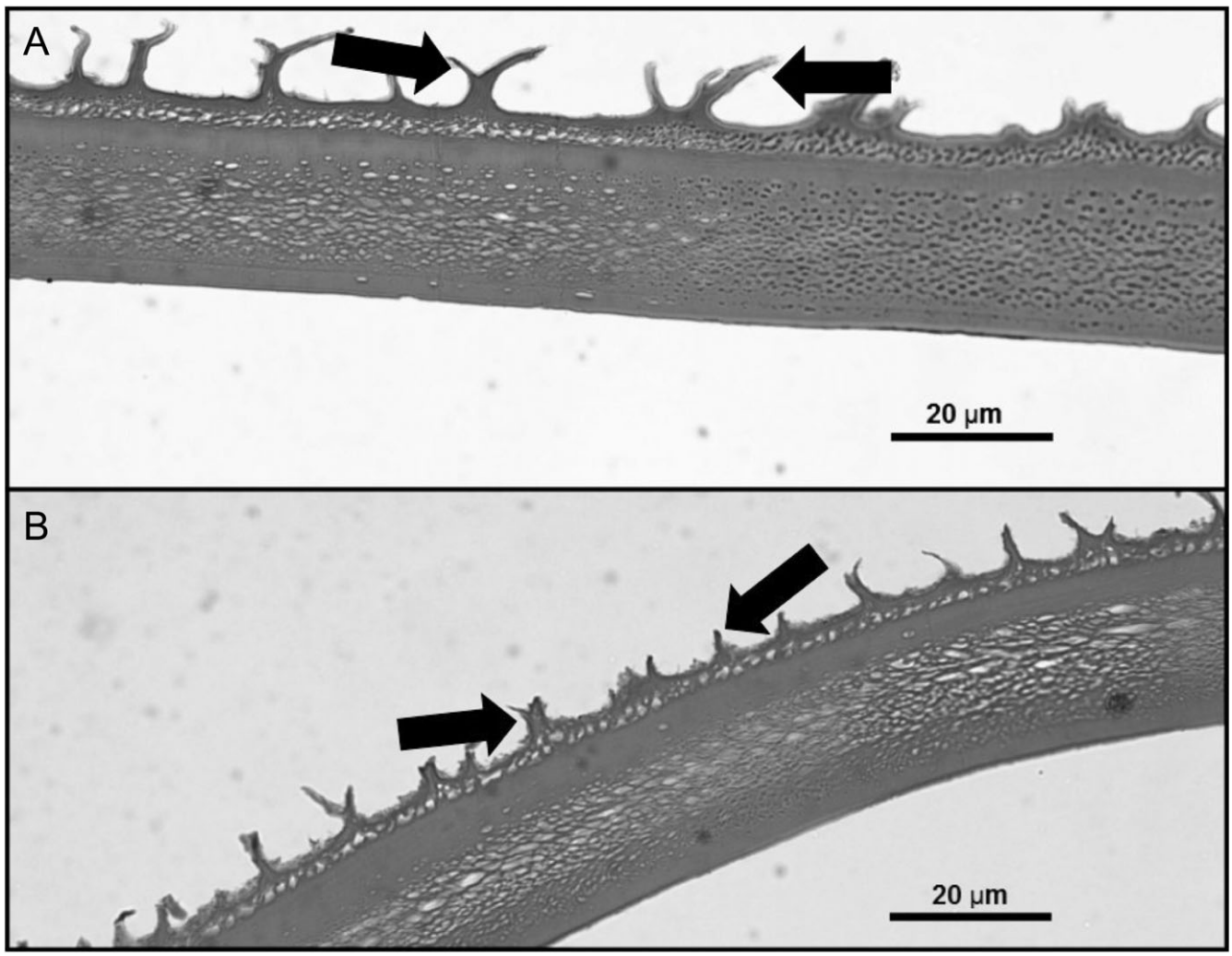

Figure 5. *Acanthina monodon.* Histological sections of capsules containing eggs (**A**) and prehatching juveniles (**B**). Arrows indicate projections of external layer of capsule wall.

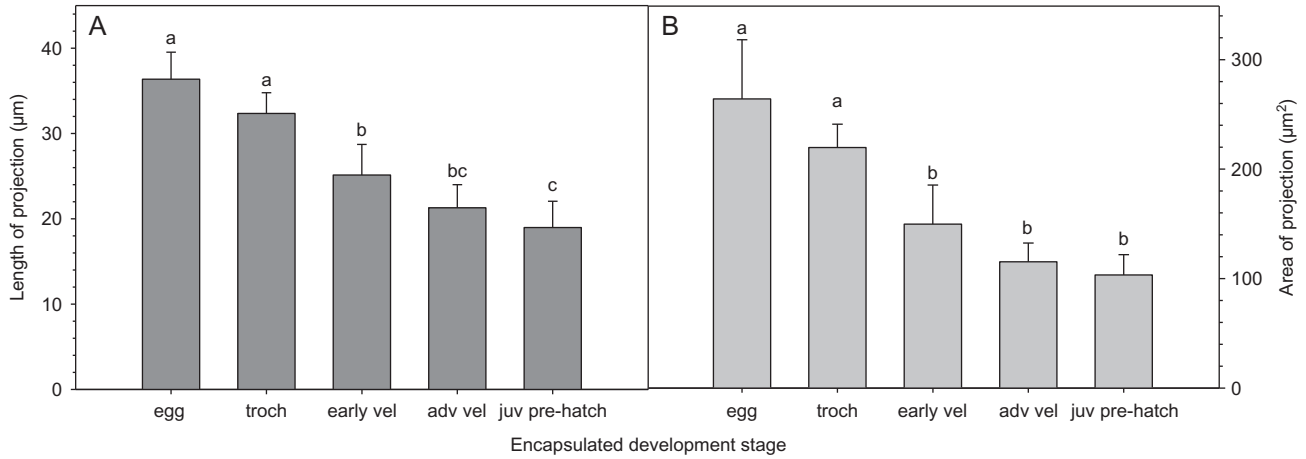

Figure 6. *Acanthina monodon*. Length (**A**) and area (**B**) of projections of outer layer of capsule wall during embryonic development. $n = 29$, $P < 0.0001$. Group means with different letters are significantly different from each other.

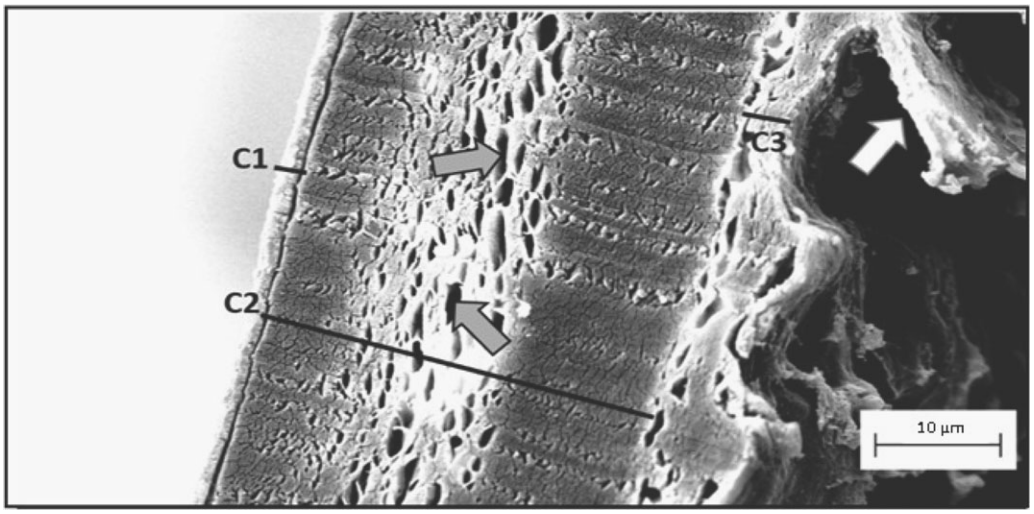

Figure 7. *Acanthina monodon*. Scanning electron micrograph of capsule wall at egg stage. Abbreviations: C1, internal layer; C2, intermediate layer; C3, external layer. Grey arrows indicate vacuoles in intermediate layer, white arrow a projection of external layer.

Protein concentration in the capsule walls, intracapsular fluid and embryos

The concentration of total proteins in the capsule wall decreased by 52.8% as development proceeded, from 5.639 ± 2.225 µg protein mg capsule^{-1} ($n = 7$) in the egg to 2.661 ± 1.024 µg protein mg capsule^{-1} ($n = 9$) for the prehatching juvenile (Fig. 10; $F_{4,32} = 2.781$, $P < 0.05$). Protein concentration in the intracapsular fluid was lower in the developmental stages with a shell (2.003 ± 0.914 mg ml^{-1}, $n = 10$) for the prehatching juvenile than in the egg (Fig. 11; 4.348 ± 0.795 mg ml^{-1}, $n = 10$; $F_{4,45} = 13.859$, $P < 0.0001$). At all stages of development protein levels in the intracapsular fluid were much higher than in the seawater at the location from which the specimens were collected (Fig. 11; $P < 0.0001$).

Although there was a significant difference in the protein content of the embryos among developmental stages (Fig. 12; $F_{4,32} = 3.477$, $P < 0.02$), Tukey's HSD test revealed only one significant paired comparison: the mean value for the advanced veliger 1.021 ± 0.576 µg protein embryo^{-1} ($n = 8$) was 60% greater than that of the egg (0.411 ± 0.211 µg protein embryo^{-1}, $n = 5$). There was no significant difference in protein content between embryonic eggs and nurse eggs (Fig. 12).

DISCUSSION

Gravimetric analysis of capsules and embryos

Intracapsular development of embryos in *Acanthina monodon* occurs over a period of 55 to 65 days (Gallardo, 1979), during which the capsules are exposed to changes in environmental conditions in the intertidal, and the embryos to changes in the internal milieu. This study examines the physical and chemical changes of the capsule wall that take place during intracapsular development in this species. We observed a dry weight loss of 27.2% in the capsule walls during embryonic development, associated with a decrease in thickness of the wall, and a reduction in size of the projections of the outer layer. The organic content of the capsule wall fell by 23.3% from the egg stage to the prehatching juvenile stage, consistent with previous reports (De Mahieu *et al.*, 1974; Ojeda & Chaparro, 2004). This loss of capsule wall material, particularly by erosion/dissolution of the inner layer into the intracapsular fluid that surrounds the embryos, represents a potential source of nutrition for them (Hendler & Franz, 1971; De Mahieu *et al.*, 1974; Ojeda & Chaparro, 2004; Brante *et al.*, 2009). We also recorded a reduction in the inorganic content of the capsule wall, which may have been attributable to the decrease in area and

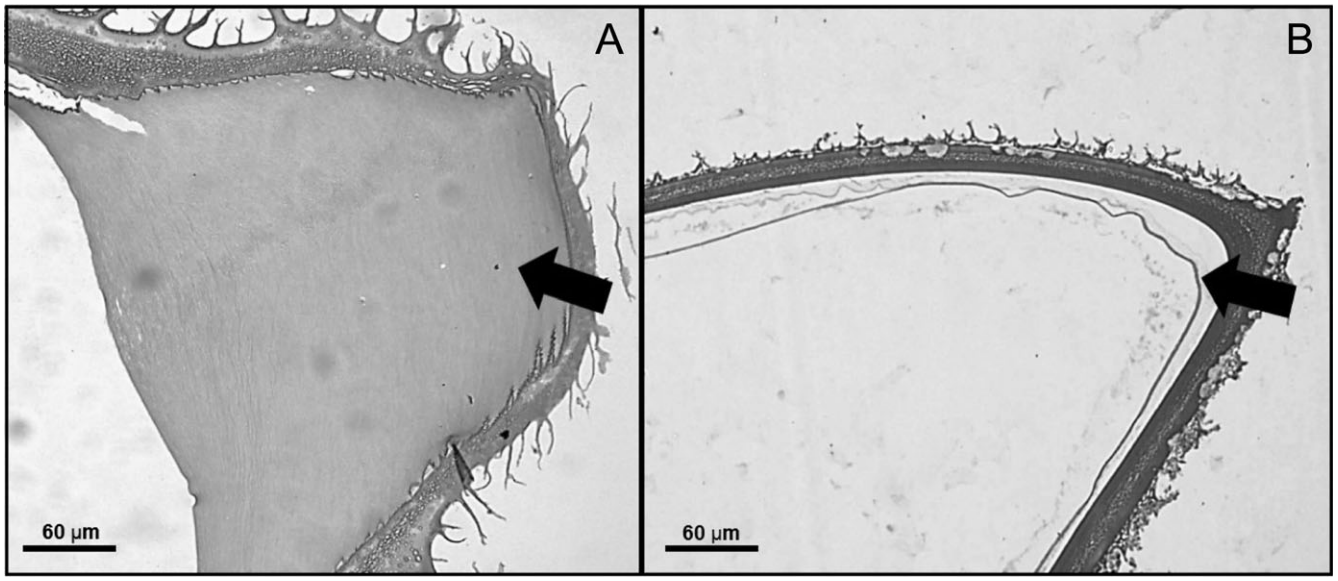

Figure 8. *Acanthina monodon*. Vertical sections through centre of hatching plug in capsules at egg stage (**A**) and prehatching juvenile stage (**B**). Arrows indicate hatching plug.

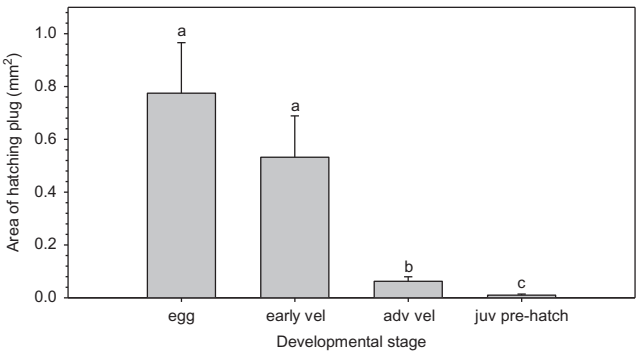

Figure 9. *Acanthina monodon*. Area of a vertical section through centre of hatching plug (mean ± SD) at various stages of intracapsular development. Group means with different letters are significantly different from each other. Total $n = 24$; $P < 0.0001$.

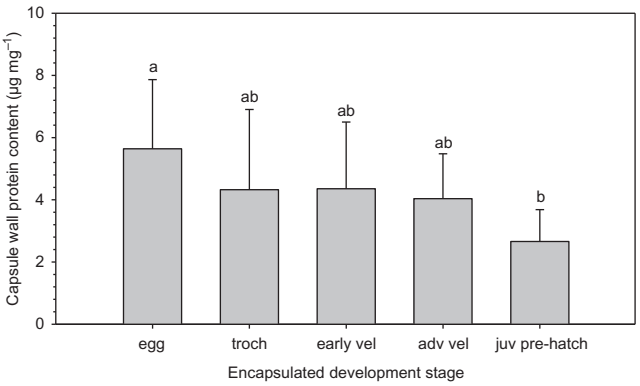

Figure 10. *Acanthina monodon*. Total protein content (mean ± SD) in capsule wall during embryonic development. Group means with different letters are significantly different from each other. Total $n = 37$; $P = 0.0433$.

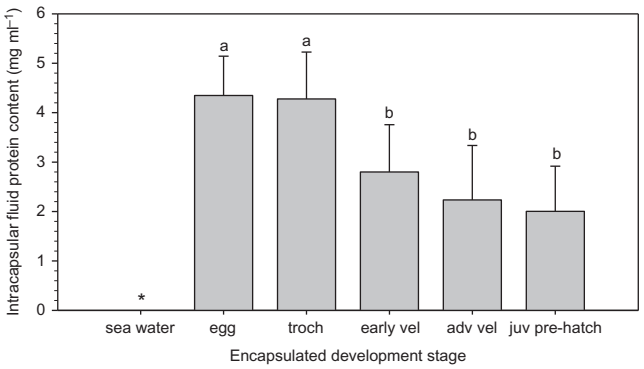

Figure 11. *Acanthina monodon*. Total protein content (mean ± SD) in intracapsular fluid of capsules with embryos at various developmental stages. Group means with different letters are significantly different from each other. Total $n = 50$; $P < 0.0001$.

length of the projections of the outer layer through embryonic development. The reasons for the gradual size reduction of the wall projections are not clear, but one factor may be wave action, since the capsules are deposited in a high-energy environment. Throughout intracapsular development of the volutid gastropod *Odontocymbiola magellanica* there is a continuous loss of the calcareous material covering the egg capsule (Bigatti *et al.*, 2010). Nevertheless, the loss of inorganic material from the capsule wall during development is not general for gastropod egg capsules, since in some species no such loss occurs (Ojeda & Chaparro, 2004). However, in these cases there is maternal care of the capsules, which provides partial protection from wave action, unlike the situation experienced by *A. monodon*.

The loss of material from the projections of the outer wall of the capsule during embryonic development in *A. monodon* may have implications for the encapsulated embryos, since the capsules are exposed to air, and hence to desiccation, during low tide (Gallardo, 1979). The function of the projections is not known, but they may allow retention of some water in the interstitial space during exposure of the capsule, thereby reducing the rate of evaporative loss and the risk of desiccation of embryos. The water retained may also provide a source of dissolved oxygen for the embryos during periods of exposure. The decrease in thickness of the capsule wall that occurs as development proceeds may be a mechanism to increase diffusion of oxygen into the capsules to meet the increasing metabolic requirements of growing embryos,

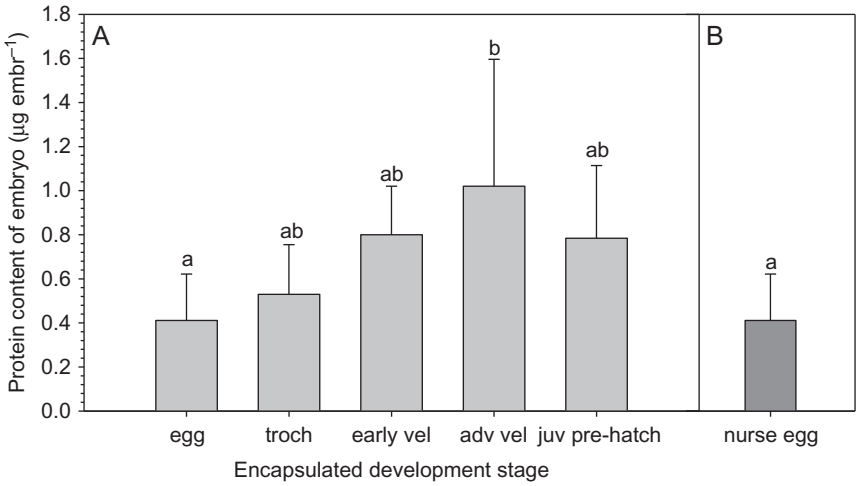

Figure 12. *Acanthina monodon*. **A.** Total protein content (mean ± SD) per embryo at various stages of development. **B.** Total protein content (mean ± SD) in nurse eggs. Group means with different letters are significantly different from each other. Total $n = 37$; $P = 0.0182$.

as has been shown for some encapsulating species (e.g. *Fusitriton oregonensis*, Brante, 2006; *Crepipatella dilatata*, Segura *et al.*, 2010). The dynamics of oxygen transport across the capsule wall merits further study, since an adequate oxygen supply is essential to embryonic development (Brante *et al.*, 2009).

The total dry weight of the embryos of *A. monodon* approximately doubles during development, which is consistent with published data for other gastropod species with encapsulated embryos (Chaparro & Paschke, 1990; Ojeda & Chaparro, 2004). The weight increase we recorded during the initial phase of development resulted from the ingestion of nurse eggs by the trochophore, as also occurs in *Nucella crassilabrum* (Gallardo, 1979). The subsequent increase in total dry weight of the veligers was attributable to the growth fuelled by energy derived from dissolved organics in the intracapsular fluid, as well as to calcification of the shell (as also described by Brante *et al.*, 2009, for *Crepidula fornicata*). Nurse eggs represent an important exogenous source of nutrition for encapsulated embryos in many gastropod species (Spight, 1976; Gallardo, 1977, 1979, 1980, 1981; Pechenik *et al.*, 1984). In *Pleuroploca aurantiaca* barely 1% of the eggs initially present survive to the hatching stage, the rest being ingested by the developing embryos (Meirelles & Matthews-Cascon, 2005). The shell is an important contributor to the increase in dry weight of the more advanced developmental stages in marine gastropods (Bayne, 1968; De Mahieu *et al.*, 1974; Clark & Jensen, 1981). Free-living bivalve larvae are capable of taking up dissolved amino acids (Manahan & Crisp, 1982) and several studies have demonstrated the importance of dissolved organics to developing embryos (including encapsulated embryos) in various caenogastropods (Creek, 1951; Demian & Yousif, 1973; Rivest, 1986; Bigatti *et al.*, 2014).

In *A. monodon* the mouth, oesophagus and stomach of the trochophore expand to permit the ingestion of an entire nurse egg, which is approximately 204–293 µm in diameter (Gallardo, 1979). However, Lesoway, Abouheif, and Collin (2014) have shown in *Crepidula navicella* that, as development proceeds to the shelled veliger stage, changes in the shape of the mouth impede this process, which accounts in this species for the presence of some noningested nurse eggs within the capsule at this time. Ingestion of nurse eggs by trochophores of *A. monodon* is probably an extremely important extraembryonic source of nutrition, since the number consumed determines the size of the juvenile at hatching in several neogastropods with intracapsular development (Spight, 1976; Rivest, 1983) and influences the subsequent survival of the posthatching juvenile (Gallardo, 1979, 1980). In *Nucella ostrina* the largest individual juveniles at the hatching stage are those that subsequently exhibit the highest rates of growth and survival in the posthatching period (Moran & Emlet, 2001).

Histology and SEM

As in other gastropods, the capsule wall of *A. monodon* is composed of three layers (Perron & Corpuz, 1982; D'Asaro, 1988; Rawlings, 1995; Matthews-Cascon *et al.*, 2010). Typically, there is a protective outer layer, a thick intermediate layer that serves as the internal skeleton of the capsule wall, and one or two internal layers that line the cavity containing the developing embryos and the intracapsular fluid (D'Asaro, 1988; Rawlings, 1995). In *A. monodon* the thickness of the single internal layer decreases during embryonic development, and dissolution of the internal layer into the intracapsular fluid may contribute nutrients to the embryos, especially proteins and amino acids, as has been demonstrated in other capsule-producing species (De Mahieu *et al.*, 1974; Pechenik *et al.*, 1984; Chaparro & Paschke, 1990; Moran, 1999; Ojeda & Chaparro, 2004). Considerable decreases in the thickness of the capsule wall (up to 50% of the initial value) during embryonic development have been recorded in *F. oregonensis* and may be a mechanism to increase the rate of oxygen supply to the embryos and/or a means of providing them with dissolved nutrients (Brante, 2006).

The strongest evidence for dissolution of the capsule wall in *A. monodon* comes from the fate of the hatching plug, which is an extension of the internal capsule wall. The plug disappears almost completely during the prehatching juvenile stage of development; although the mechanism is not clear, evidence from other gastropod species suggests that the plug is dissolved by enzymatic action immediately before release of the juveniles (Chaparro *et al.*, 2012). Dissolution of the plug into the intracapsular fluid proceeds throughout development, so it is likely that organic material from the hatching plug is available to support growth of the embryos during all developmental stages.

Total protein in the capsules

Total protein in the capsule walls of *A. monodon* decreased by 53% during embryonic development, representing an energy contribution to the intracapsular fluid. Similar observations have been made in the calyptraeids *Crepipatella fecunda* (Ojeda & Chaparro, 2004; Chaparro *et al.*, 2012) and *C. dilatata* (Chaparro *et al.*, 2012). Our observations on *A. monodon* demonstrate that the nurse eggs are the principal source of protein for embryos during development (preliminary estimate 5.7 µg protein embryo^{-1}). Complementing the

above, the presence of dissolved proteins and their possible significance as a nutritional source for developing embryos have been described in many gastropod and bivalves species (De Mahieu *et al.*, 1974; Manahan & Crisp, 1983; Jaeckle & Manahan, 1989; Stöckmann-Bosbach & Althoff, 1989; Chaparro & Paschke, 1990; Welborn & Manahan, 1990; Moran, 1999; Marin *et al.* 2003).

In *A. monodon* the protein concentration in the intracapsular fluid decreases as development of the embryos proceeds, despite the influx of protein from the inner layer of the capsule wall. This decrease is most evident in the early veliger stage, after the trochophores have ingested all the nurse eggs, strongly suggesting that the embryos are absorbing dissolved protein as it is released into the intracapsular fluid from the inner layer of the capsule wall (see also Stöckmann-Bosbach & Althoff, 1989; Shilling, Hoegh-Guldberg & Manahan, 1996; Vavra & Manahan, 1999; Bigatti *et al.*, 2014). Bigatti *et al.* (2014) have shown that growth of encapsulated embryos of *O. magellanica* is accompanied by a large increase in their protein content and a concomitant decrease in dissolved protein levels in the intracapsular fluid. The concentrations of dissolved protein we recorded in the intracapsular fluid of *A. monodon* (2.0–4.3 mg ml^{-1}) are similar to those reported for some other gastropod species (e.g. 2.1–7.4 mg ml^{-1} in *Nucella lapillus*, Stöckmann-Bosbach & Althoff, 1989), but lower than for others (e.g. 22.5–82.9 mg ml^{-1} in *O. magellanica*, Bigatti *et al.*, 2014). The nutritional value of the intracapsular fluid to encapsulated embryos is well known (Bayne, 1968; Perron & Corpuz, 1982; Stöckmann-Bosbach & Althoff, 1989; Penchaszadeh & Rincón, 1996; Penchaszadeh & Miloslavich, 2001; Ojeda & Chaparro, 2004; Brante *et al.*, 2009; Chaparro *et al.*, 2012; Bigatti *et al.*, 2014). Leroy *et al.* (2012) have demonstrated uptake of a labelled amino acid (alanine) from solution by encapsulated embryos of *C. fornicata*. In *N. lapillus* a fraction of the organic component of the intracapsular fluid is incorporated by pinocytosis into the integument cells of the cephalic region of the embryo (Fioroni, 1985), and in *N. lapillus* (Fioroni, 1985), *Buccinum undatum* (Fioroni, 1985) and *Searlesia dira* (Rivest, 1980) some of the components of the fluid are taken up by 'ectodermal larval kidneys'. Absorptive cells associated with the larval kidney complex have been identified in encapsulated embryos of *C. navicella* (Lesoway *et al.*, 2014) and are in direct contact with the intracapsular fluid (Rivest, 1992). Embryos of *S. dira* exposed to a solution of ferritin and albumin-fluorescein isothiocyanate conjugate (FITC) rapidly take up dissolved protein through pinocytosis by these absorptive cells and store it in heterophagosomes (Rivest, 1992). The absorptive cells increase in volume during the trochophore stage, reaching a maximum in the early veliger, then undergo resorption during the late veliger and prehatching stages. This process has also been observed in other gastropods e.g. *Thais savignyi* (Eisawy & Sorial, 1974) and *T. haemastoma* (Belisle & Byrd, 1980). Thus there is substantial evidence from several capsule-producing gastropod species that dissolved organic material from the intracapsular fluid serves as a nutritional source for the developing embryos, although this has yet to be demonstrated unequivocally in *A. monodon*.

In conclusion, our data show that during embryonic development the capsule walls of *A. monodon* are dynamic in terms of thickness, dry weight and protein concentration, and that proteins are transferred to the intracapsular fluid by erosion/dissolution of the inner layer of the capsule wall. Observed decreases in protein concentration in the intracapsular fluid after the completion of nurse egg ingestion by the trochophore are associated with a subsequent increase in the dry weight of the embryo and high protein levels in the advanced veliger stage. We conclude that some organic compounds, specifically proteins, dissolved in the intracapsular fluid originate in part from the capsule wall and contribute to the nutrition of the embryo, complementing the nurse eggs. Future studies with labelled organic compounds, especially amino acids, should be undertaken to demonstrate unequivocally that material originating in the capsule wall is incorporated into the tissue of developing embryos.

ACKNOWLEDGEMENTS

This work was supported by the Fondo Nacional de Investigación Científica y Tecnológica (FONDECYT Chile, Grant 1180643) to OC.

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