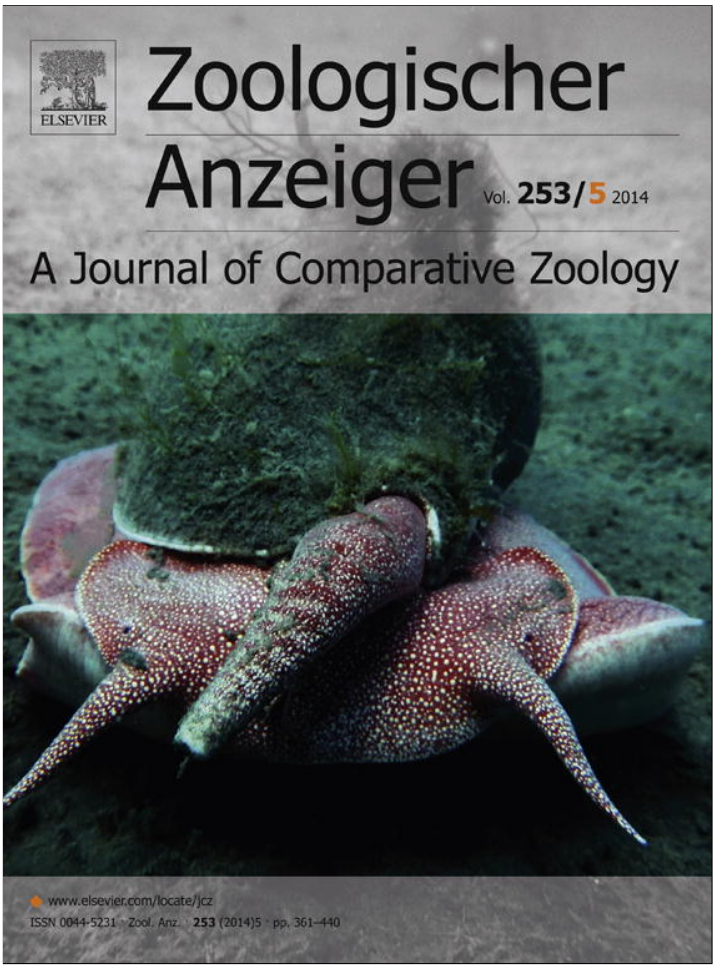

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Embryonic development in the Patagonian red snail *Odontocymbiola magellanica* (Neogastropoda: Volutidae): Morphology and biochemistry

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ABSTRACT

Embryo morphology, feeding mechanism and changes in composition of the egg capsule content during development (intracapsular fluids and embryos) were studied in *Odontocymbiola magellanica* from newly spawned egg capsules to the pre-hatching juvenile stage. Changes in embryo morphology and behavior are presented, based on observations and micrographs of living specimens and scanning electron microscopy. The arrangement of velar cilia and athrocytes and shell gland location and development differed markedly from other studied caenogastropods. Embryo ingestion of intracapsular fluid was promoted by velar ciliary currents at least from the early veliger stage, while feeding by grazing on the inner membranous layer of the egg capsule was rarely observed until juveniles were about to hatch. The main growth of embryos occurred during the veliger stages. A significant nutritional investment in egg capsules, as compared with other South American volutids was observed. Nutrition from proteins seemed to predominate at the expense of a high molecular weight fraction (>220 kDa). Calcium concentration in the intracapsular fluid remained constant during development, but notably, the total intracapsular content (i.e., the amount contained in both fluid and embryos) increased 3-fold, which may be explained by extraction from the egg capsule magnesium-rich calcite cover, or alternatively, by uptake of calcium from the surrounding sea water. Ammonia, a major end-product of nitrogen metabolism in marine invertebrates, was present in both embryos and intracapsular fluid, from which it may easily diffuse to the surrounding sea water through the egg capsule wall. Our results on embryo morphology, development and biochemical changes provide useful comparative data for evolutionary and developmental studies in the Volutidae as well as in other caenogastropods.

1. Introduction

The family Volutidae (Caenogastropoda, Neogastropoda) is widely distributed in the oceans and includes twelve genera represented in the coasts of the Caribbean sea, Brazil, Uruguay, Argentina and Chile (*Adelomelon*, *Enaeta*, *Minicymbiola*, *Miomelon*, *Nanomelon*, *Odontocymbiola*, *Pachycymbiola*, *Plicoliva*, *Provocator*, *Tractolira*, *Voluta* and *Zidona*) and more than twenty species (e.g., Bail and Poppe, 2001). Interest in their biology has increased recently as a consequence of the ecological and economic significance of several of the included species (e.g., Bigatti and Penchaszadeh, 2005; Cledón et al., 2005a,b, 2006; Giménez et al., 2004, 2005; Bigatti and Carranza, 2007; Bigatti and Ciocco, 2008; Penchaszadeh et al., 2009; Márquez et al., 2011; Roche et al., 2011; Zabala et al., 2014).

As far as it is known, embryonic development of South American volutids is completed within closed egg capsules, with no free-living larval stages (Ponder, 1973; Pechenik, 1986; Miloslavich, 1996a,b). Among them, *Odontocymbiola magellanica* (Gmelin 1791) is the only known species depositing calcareous egg capsules (Penchaszadeh and De Mahieu, 1976; Penchaszadeh et al., 1999; Bigatti et al., 2010), a condition which it only shares (among the Sorbeoconcha) with the New Zealand volutid *Alcithoe arabica* (Ponder, 1970).

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Reproductive and developmental biology provides valuable information for comparative purposes. With this in mind, we have been involved in a program on South American neogastropods, in which the reproductive structures and cycle, reproductive and feeding behavior, age, growth and imposex have been studied in representatives of five genera of Volutidae – *Adelomelon*, *Odontocymbiola*, *Provocator*, *Voluta* and *Zidona* – two genera of Muricidae – *Trophon* and *Coronium* – and one genus of Nassariidae (*Buccinanops*) (Penchaszadeh and Miloslavich, 2001; Bigatti, 2005; Bigatti et al., 2007, 2009, 2010; Bigatti and Penchaszadeh, 2005; Cledón et al., 2005a; Pastorino et al., 2007; Giménez et al., 2008; Pastorino and Penchaszadeh, 2009; Averbuj et al., 2010; Cumplido et al., 2010; Matthews-Cascon et al., 2010; Cumplido et al., 2011). In this context, the current paper reports morphological and biochemical information on the development of *O. magellanica*.

2. Material and methods

2.1. Sampling

Egg capsules of *Odontocymbiola magellanica* in different stages of development were collected during the oviposition season (June to December) by SCUBA diving, at depths between 5 and 20 m during low tides, in Golfo Nuevo, Argentine Patagonia (42° 43′ S; 65° 01′ W). Duration of intracapsular development was determined for egg capsules taken from egg-laying females and put in 30 L aerated aquaria, at a constant temperature of 13 °C and with a photoperiod of 12 h light/12 h darkness (approximating spring conditions in Golfo Nuevo).

2.2. Embryonic morphology and feeding behavior

Living embryos from egg capsules of different developmental stages were observed, measured and photographed under a stereoscopic microscope, while kept in Petri dishes containing intracapsular fluid and/or filtered sea water. Also, feeding by swimming embryos was observed with a conventional light microscope under a coverslip in an excavated glass slide. As the white calcareous egg capsules progressively became translucent, feeding by crawling embryos (late pediveliger to pre-hatching juveniles, see Section 3) were mostly observed by transparency; also, some crawling embryos were observed in sea water when attached to the inner side of fragments of experimentally opened capsules.

Embryos in both the late veliger and early pediveliger stages were fixed for 3 h in 2.5% glutaraldehyde in 1 M phosphate buffer (pH 7.4), critical point dried and metal coated, and their velar structures were observed under a scanning electron microscope.

2.3. Correlation between morphological and biochemical changes during development

Egg capsules of different developmental stages were collected and taken to the laboratory, where they were opened in ice-cold Petri dishes, and the pH of the intracapsular fluid content was estimated with a pH indicator paper (64271 Darmstadt, Germany).

The fluid contained in each capsule was separated on ice from the eggs or embryos (which were counted and homogenized in distilled water) and then the intracapsular fluid was measured to the nearest 0.1 mL and homogenized. All samples were preserved at −80 °C until determinations of protein, glucose, calcium and ammonia were made. Results for each substance were expressed as mean ± SEM of either mg per embryo or mg per mL of intracapsular fluid.

For protein determinations, 500 μL aliquots of each homogenate were added to an equal volume of 0.75 M sodium hydroxide solution and left overnight before being homogenized again. Then the Bradford's (1976) method was applied to duplicate 25 μL aliquots; bovine serum albumin was used as standard (method sensitivity was 10 μg per tube).

For glucose determinations, samples were treated with glucose oxidase and the amount of oxygen peroxide formed was quantified by the peroxidase catalyzed reaction with 4-aminophenazone and chlorophenol, which produces a colored quinoneimine product (Trinder, 1969); method sensitivity was 0.25 μg per tube.

Total calcium was measured in 1 M HCl-treated, 500 μl aliquots (which ensured the dissolution of calcium salts' crystals) and were then exposed to *o*-cresolftalein in alkaline solution (Schwarzenbach, 1955); the reaction product is a complexone that was measured spectrophotometrically; method sensitivity was 0.2 μg per tube.

Ammonia was quantified through the production of indophenol blue, after reacting with phenol and sodium hypochlorite in an alkaline medium (Berthelot's reaction, Fawcett and Scott, 1960); method sensitivity was 0.5 μg per tube.

Proteins in the fluid contents of individual capsules were separated by SDS/PAGE according to Laemmli (1970) using gels of 5, 7.5, 10 and 12% polyacrylamide. Prior to gel electrophoresis, samples of each extract were diluted with sample buffer to the following final concentrations: Tris 62.5 mM (pH 6.8), 6.25% glycerol, 3% sodium dodecyl sulfate, 5% mercaptoethanol, and 0.0125% bromophenol blue. The samples were heated at 100 °C for 10 min and then centrifuged (10,000 × *g* for 1 min). Fifteen μg of protein were loaded per lane; molecular weight of the obtained bands was estimated by using protein molecular weight markers (in kDa: myosin, 220; phosphorilase B, 97; bovine serum albumin, 66; ovoalbumin, 45; carbonic anhydrase, 30). Then, the gels were stained with a Coomassie Brilliant Blue R250 solution and the excess dye was washed using a methanol:acetic acid:water solution (45:45:10). Digital image densitometry of the photographed gels was made using the 1.6/ppc Freeware program.

2.4. Statistical analysis

Multigroup comparisons were made by Kruskal–Wallis one-way analysis of variance followed by the Dunn's test as post hoc analysis; significance level was fixed at $P < 0.05$.

3. Results

3.1. Egg capsules and contents

Embryos hatched after 60–95 days from 9 newly laid capsules taken to the laboratory from the field and maintained in aquaria. The external calcareous cover usually deteriorated during development allowing the embryos to be seen through the remaining membranous layer by the end of the process (Fig. 1).

The intracapsular fluid of recently laid capsules had two macroscopical components: (1) white sticky clumps where either the eggs or the early embryos were included and (2) a translucent liquid surrounding it.

When recently laid egg capsules were opened in the laboratory, eggs and embryos were found included in the white sticky capsule content, from which they could only be laboriously separated. However, from the pediveliger stage, only the translucent fluid remained, and the embryos were found mostly at the bottom of the capsule cavity. Later, signs of radular scrapping of the translucent egg capsule (the calcareous layer had usually disappeared by that time) were only observed to be associated with pre-hatching juveniles crawling at the inner membranous wall. Each capsule contained 9.6 ± 0.4 eggs or embryos (mean ± SEM; range was 4–18 eggs or embryos; $N = 34$) indicating that adelphophagy did not occur.

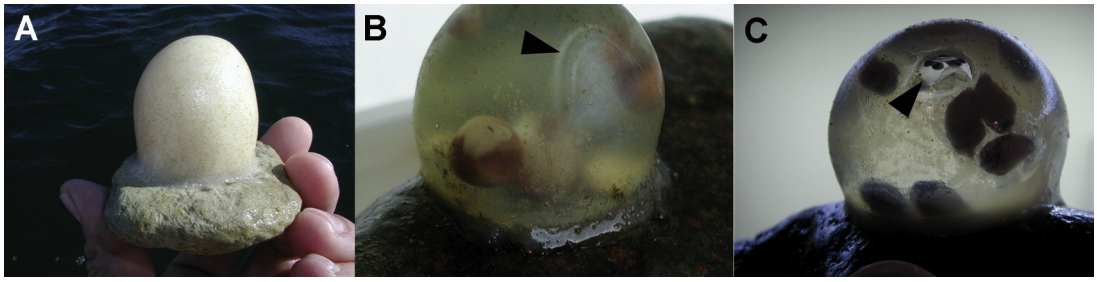

Fig. 1. Egg capsules of *Odontocymbiola magellanica*. (A) A capsule with its intact white calcareous cover. (B) A capsule in which the calcium layer has deteriorated and pediveliger embryos are seen by transparency; a crescent-shaped seam (arrowhead) is seen on the membranous layer. (C) A capsule in which the hatching process has begun; the seam in the membranous layer is being eroded by the pre-hatching juveniles.

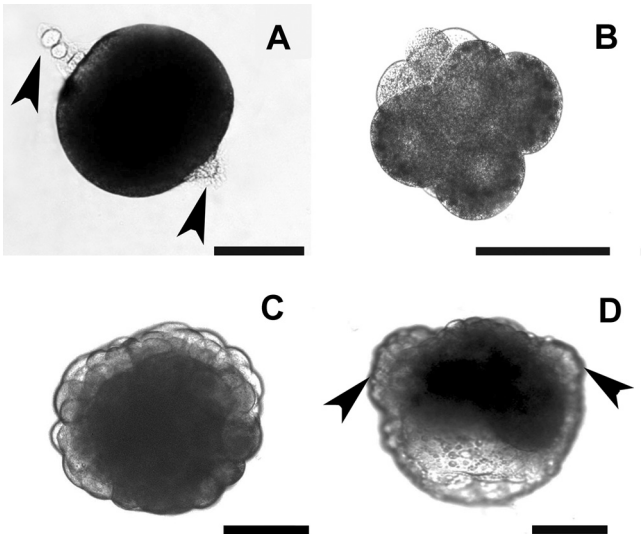

Fig. 2. Early development of *Odontocymbiola magellanica*. (A) Fertilized egg, showing polar bodies (upper arrowhead) and micropyle (lower arrowhead. (B) Eight-blastomere stage. (C) More advanced embryo (probably a gastrula). (D) Trochophore embryo; arrowheads indicate the groups of presumptive provelar cells. Scale bars: (A) = 100 μm; (B) = 100 μm; (C) = 150 μm; (D) = 200 μm.

Embryos of different sizes were mainly observed at the mid and late veliger stage.

3.2. Embryo morphological development

In recently fertilized eggs, polar bodies showed the animal pole and a sperm cluster at the vegetal pole indicated the micropyle (Fig. 2A). The early morula at the 8-blastomere stage presented a slightly asymmetric segmentation (Fig. 2B), developing to the gastrula stage (Fig. 2C). Trochophore embryos were round, actively rotating embryos which showed an opaque subcentral zone which may correspond to the archenteron, as well as two ectodermal bulging groups of large cells (Fig. 2D). The latter cells apparently give rise to the primitive velum of the early veliger stage, a peculiar globe-shaped and translucent structure, with a uniformly ciliated surface. Yet, it was not obvious that ciliary currents were introducing particles into the stomodaeum in trochophore embryos. Also, it was likely that the stomodaeum/archenteron connection were not yet permeated at this stage, since the archenteron did not contain any of the whitish material which will fill it later.

Embryos were active at the veliger stages, showing both rotational and translative movements. Early veliger embryos were characterized by a globe-shaped velum (Figs. 3A–C and 4). At the beginning of this stage the embryonic body appeared as an inverted cone, whose blunt vertex was slightly bent to the prospective front. The globe-like velum was wholly covered by short cilia, which gave

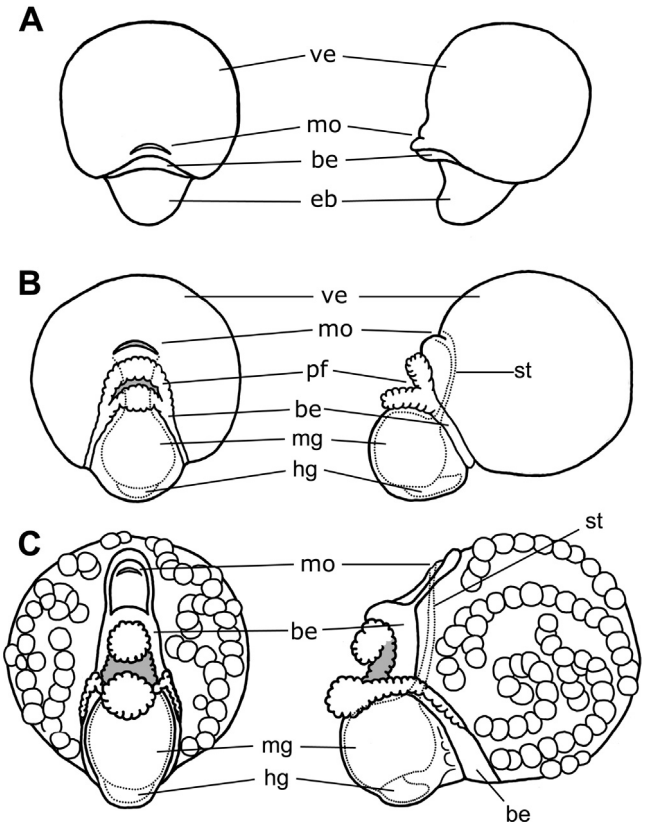

Fig. 3. Diagrams of early veliger embryos at three stages of development (left, frontal views; right, lateral views). (A) Embryo with a velvety, globe-like velum and no athrocytes. (B) More developed embryo in which small athrocytes cover the borders of the early foot and spread over part of the belt. (C) An even more developed embryo in which larger athrocytes are distributed over the velum. Abbreviations: be, belt; hg, hindgut; mg, midgut; mo, mouth; pf, early foot; st, stomodaeum; ve, velum.

it a velvety appearance, and small clumps of the coarse material of the intracapsular fluid appeared formed by ciliary movements (particularly clear on Fig. 4C). The embryonic body and the velum were connected through a thick tissue belt that was slightly wider at the prospective front. This widening of the tissue belt (and the transparent vacuolar cells which will cover it), just below the primitive mouth, i.e., the entrance to the stomodaeum, is the zone where the foot will develop. Also, threads of the white intracapsular fluid extended from the primitive mouth to the archenteron, filling the stomodaeum. The archenteron was evident by transparency at this stage as a white, approximately spherical structure, located at the base of the inverted blunt cone.

At the midst of the early veliger stage (Figs. 3B and 4D), the velum remained as a velvety globe, while the early foot had begun to develop and its borders were becoming covered by transparent

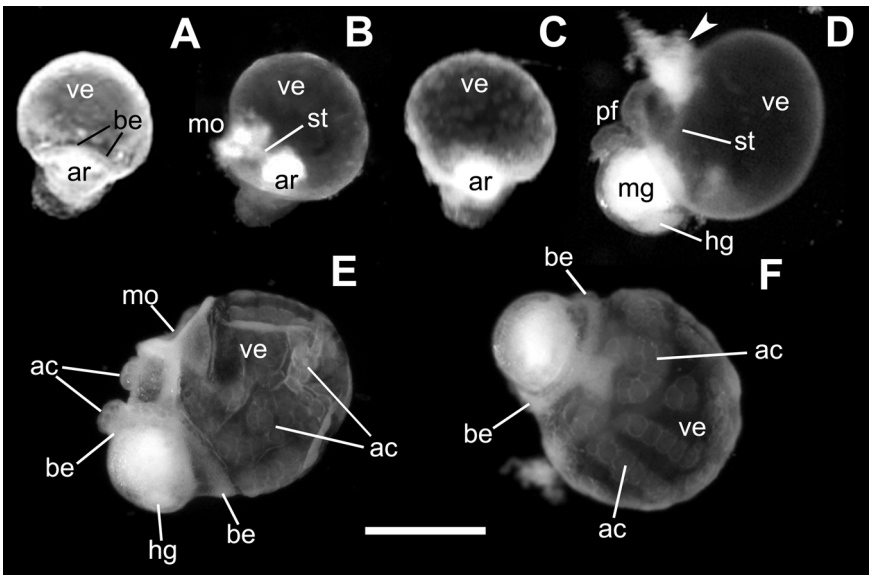

Fig. 4. Micrographs of live early veliger embryos supporting the diagrams on Fig. 3 (corresponding diagrams are referred to in parenthesis). (A) Early veliger (Fig. 3A), frontal view. (B) Early veliger (Fig. 3A), lateral view showing clumps of white albumen entering through the mouth and indicating the stomodaeum and the archenteron. (C) Early veliger (Fig. 3A), posterior view showing small clumps of white albumen on the velar surface. (D) A more advanced early veliger (Fig. 3B), lateral view showing clumps of white albumen entering through the mouth and stomodaeum and filling the midgut (the hindgut has already differentiated from the midgut). (E) An even more advanced early veliger in which the velum was accidentally blown-up (Fig. 3C). F. A similar early veliger (Fig. 3C), inferior view. Abbreviations: (other abbreviations as in Fig. 3) ac, athrocytes; ar, archenteron; be, belt; hg, hindgut; mg, midgut; mo, mouth; pf, early foot; st, stomodaeum; ve, velum. Scale bars: 500 μm.

vacuolar cells of different sizes (='athrocytes', sensu Hyman, 1967; see Section 4). The embryonic body had become a white round structure, mostly occupied by the now distinct mid and hindgut. Athrocyte number reached its maximum by the end of the early veliger stage (Figs. 3C and 4E and F), when they extended to the center of the early foot and to the tissue belt joining the velum and the visceral mass. Also, conspicuous rows and groups of much larger and flattened athrocytes were found over the velar surface. Their arrangement suggests they may originate the postero-ventral part of the tissue belt (Fig. 4F).

Embryos were bottle-shaped at the midveliger stage, in which an elongated anterior oral tube had developed and the velum had become a dorsoventrally flattened and laterally extended structure (Fig. 5A and B) which was able to produce fluid currents in the surrounding intracapsular fluid that conveyed it into the mouth. Each side of the velum was composed of two thin separate tissue layers, the upper one reminds somehow an umbrella, while the lower one was flat and showed undulating borders. The space between both velar layers connected the former velar cavity with the surrounding intracapsular fluid (Figs. 5A and B and 6A). During this stage the early foot was just a thin and transparent membrane, and the number of its athrocytes had sharply diminished to just a small group on the prospective anterior border. The large athrocytes on the velar surface had also disappeared. A preoral fossa of rhomboidal shape developed in front of the mouth slit and above the early foot. Translucent bands of tissue were located at both sides of the oral tube and extended around its base, and seemed to be the primordium of the shell gland, which will be clearly recognized at this place in pediveliger embryos (see below).

At the late veliger stage (Figs. 5C, 6C and D and 7A–C) embryos were balloon- or pear-shaped in which the visceral mass had grown much larger than the cephalopodial structures and the velum. The foot had grown thicker and both velar layers on each side apparently merged by the end of the late veliger stage, when the velum appeared as a wide bilateral structure, wholly covered by very long cilia, which gave it a ruffled appearance (Figs. 5C and 7A–C). Interestingly, ball-like aggregates of different sizes appeared attached on or around the trochs and to the base of the cephalopodium of both late veliger and pediveliger embryos (Fig. 7A–B and G). These aggregates appeared composed of an orderly arrangement of much smaller particles (about 0.6 μm, Fig. 7G). They may correspond to proteins present in the intracapsular fluid.

Pediveliger embryos showed a reduced motility. The membranous early foot changed to a thick, muscular structure, while velar structures still remained at both sides of the mouth, but the extensive ciliary development seen in late veliger embryos had markedly receded while two distinct bilateral trochs were seen (Figs. 5D and 7E and F). The trochal borders showed the expected prototroch with long cilia, the adoral ciliary band of extremely short cilia and the metatroch of short cilia. Intriguingly, however, instead of the expected outer position of the prototroch (Nielsen, 2004), the prototrochal long cilia of these embryos formed the inner trochal row (Fig. 7E and F).

The mantle cavity first appeared in early pediveliger embryos as a slit to the right of the foot (Figs. 5E and 6E) and will later widen and surround the cephalopodial structures (Figs. 5F and 6F and G). The shell gland was distinct in early pediveliger embryos at the base of the cephalopodial structures (Fig. 7D) and it later grew surrounding the pallial cavity border in late pediveliger embryos (Figs. 5E and F and 6F and G). Then the eyes and the tentacle buds appeared and the siphon were clearly seen to the left of the shell's aperture (Figs. 5F and 6F and G).

The midgut wall appeared made of large cells in early pediveliger embryos (Fig. 6E). Even though the visceral mass is being relatively reduced at this time, it remained large enough to prevent crawling in early pediveliger embryos. Crawling and grasping of the membranous egg capsule will begin in the late pediveliger stage.

Afterwards (Fig. 6H), the embryos became coiled, the siphon grew and the shell extended from around the aperture toward the tip of the protoconch, which then becomes progressively hardened. Two small velar structures still remained in these coiling embryos, which were crawling and feeding on the inner membranous egg capsule.

The velum finally disappeared when the apical protoconch whorl was completed in pre-hatching juveniles (Fig. 6I), the siphon

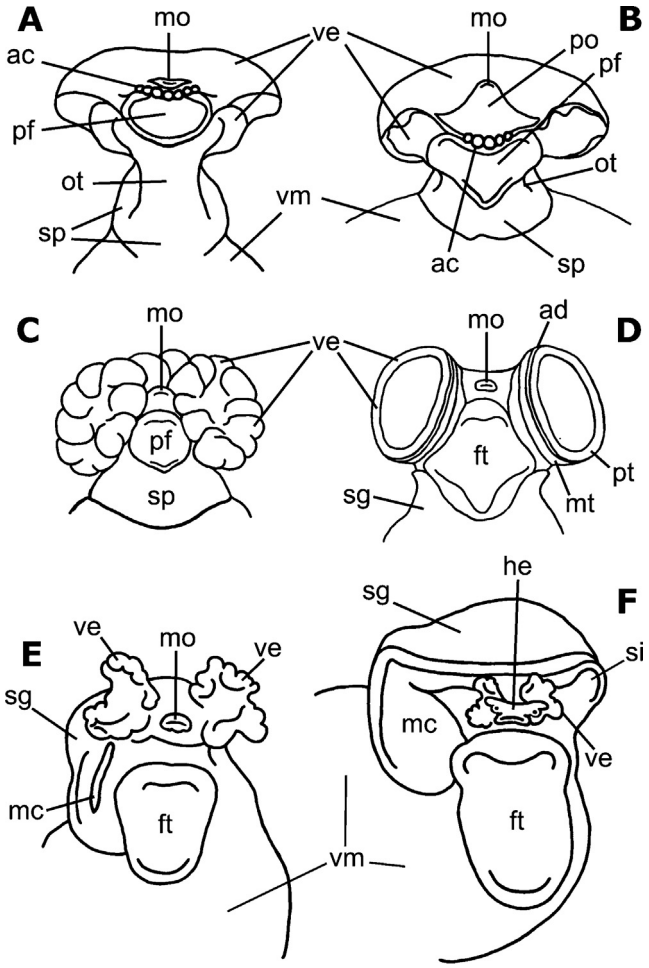

Fig. 5. Diagrams of cephalopodium development from midveliger to pediveliger stages in ventral views. (A) Mid veliger in which the formerly globe-like velum has communicated to the exterior and two velar lamina are formed on each side; some athrocytes remain on the early foot. (B) Further developed mid veliger in which the oral tube has shortened, the preoral fossa has been formed and both velar laminae are tending to merge on each side; some athrocytes remain on the anterior part of the early foot. (C) Late veliger in which the velum has being wholly covered by long cilia. (D) Early pediveliger in which the foot has become thicker and the velum has been reduced to two approximately round lateral trochs where three distinct ciliary bands are seen (prototroch, adoral ciliary band and metatroch). (E) Mid pediveliger in which the shell gland has extended from dorsal to the right and the slit of the mantle cavity has appeared to the right side of the foot, and the cephalic vesicle is differentiating around the mouth. (F) Late pediveliger in which the head (with mouth, eyes and tentacles) has differentiated and the mantle cavity is surrounding the head and the velar remnants; also, the siphon is seen to the left of the head and the anterior part of the shell gland is surrounding the mantle cavity border. Abbreviations; ad, adoral ciliary band; ac, athrocytes; ft, foot; mc, mantle cavity; mo, mouth; mt, metatroch; ot, oral tube; pf, early foot; po, preoral fossa; pt, prototroch; sg, shell gland; sp, shell gland primordium; ve, velum; vm, visceral mass. Diagrams are not to scale.

became fully developed and the foot showed small white spots on a reddish background, as it will be in adult snails.

Thus, except for the shape of the shell, pre-hatching juveniles appeared externally as miniatures of the adult, with differentiated head and foot regions, eyes, siphon and gill.

3.3. Changes in embryonic length as a function of developmental stage

A total of 206 eggs or embryos were measured to construct a growth curve. This approximated a sigmoidal one (Fig. 8) in which the growth rate was minimal up to the trochophore stage; it later showed a steep increase during the veliger stages and became slower from the pediveliger up to the pre-hatching juvenile stage.

3.4. The hatching process

Hatching was registered 12 times in the laboratory. It began when the hatching juveniles started forming the second protoconch whorl. First, a crescent-shaped seam could be recognized on an upper/lateral region of the membranous egg capsule (Fig. 1B). This region was later opened and eroded by the pre-hatching juveniles (Fig. 1C) that were able to emerge from the egg capsule. However, those juveniles usually remained for several days within the egg capsule, sheltered and feeding on its membranous component.

3.5. Correlation between morphological embryo stages and biochemical changes during development

Another set of observations was made in egg capsules collected and processed for biochemical determinations during different stages of development. The mean number of eggs/embryos per capsule (±SEM) did not show significant changes in the studied egg capsules: (1) eggs to early veliger embryos: 9.3 ± 1.5, $N = 11$; (2) mid to late veliger embryos: 10.3 ± 1.0; $N = 9$; (3) pediveliger embryos: 10.0 ± 1.7; $N = 7$; (4) pre-hatching juveniles: 10.4 ± 0.5; $N = 7$.

Results of the biochemical determinations are presented in Table 1. Growth was accompanied by a significant increase in embryo protein and by a concomitant decrease of total protein in the intracapsular fluid. Glucose changes, however, were not statistically significant in either embryos or intracapsular fluid.

Embryonic calcium, expressed on a per embryo basis, showed a seven hundred-fold increase by the end of development (when protoconch calcification is completed), while the concentration of calcium in the intracapsular fluid remained constant (Table 1). However, if the total intracapsular content was calculated from the same capsules (i.e., the content in embryos plus fluid, data not included in Table 1), a 3-fold calcium gain by the capsules was observed during development (eggs to early veliger embryos, 3.58 ± 0.40; mid to late veliger embryos, 3.88 ± 0.76; pediveliger embryos, 5.40 ± 1.20; pre-hatching juveniles, 12.13 ± 5.51).

Ammonia was consistently measured in samples, but the levels were low and did not show statistically significant changes in either embryos or intracapsular fluid.

The decrease in protein observed in the capsule fluid was further investigated by protein electrophoresis (SDS/PAGE). Determinations were made in the fluid from capsules in different stages of development: (1) eggs to trochophore embryos; (2) early veliger embryos; (3) mid to late veliger embryos, (4) pediveliger embryos; and (5) pre-hatching juveniles. The electrophoresis showed (Fig. 9A) at least 4 bands of apparent molecular weights ranking high (more than 220 kDa) and low (19.6 ± 0.7, 25.3 ± 0.6 and 32.6 ± 0.7 kDa; mean ± SE, $N = 4$). The band of highest molecular weight (ranking higher than the myosin standard of 220 kDa) could not be resolved either in 5% gels or even in presence of mercaptoethanol and SDS, both at 10% in the sample buffer.

The relative density of the four main protein bands changed markedly during development (Fig. 9B). High molecular weight proteins showed highest relative levels in capsules of the first two groups (up to the early veliger stage), started to fade away at the mid to late veliger stage, and had disappeared in pediveliger and pre-hatching juvenile stages; consequently, the relative levels of the other three protein bands tended to increase during development.

In most cases (27 out of 29 capsules) pH of the intracapsular fluid ranged from 7.5 to 7.8 (the pH of sea water was 7.8 in the studied area); in two cases (both early veliger embryos), however, pH values were 8.2 and 8.5, respectively.

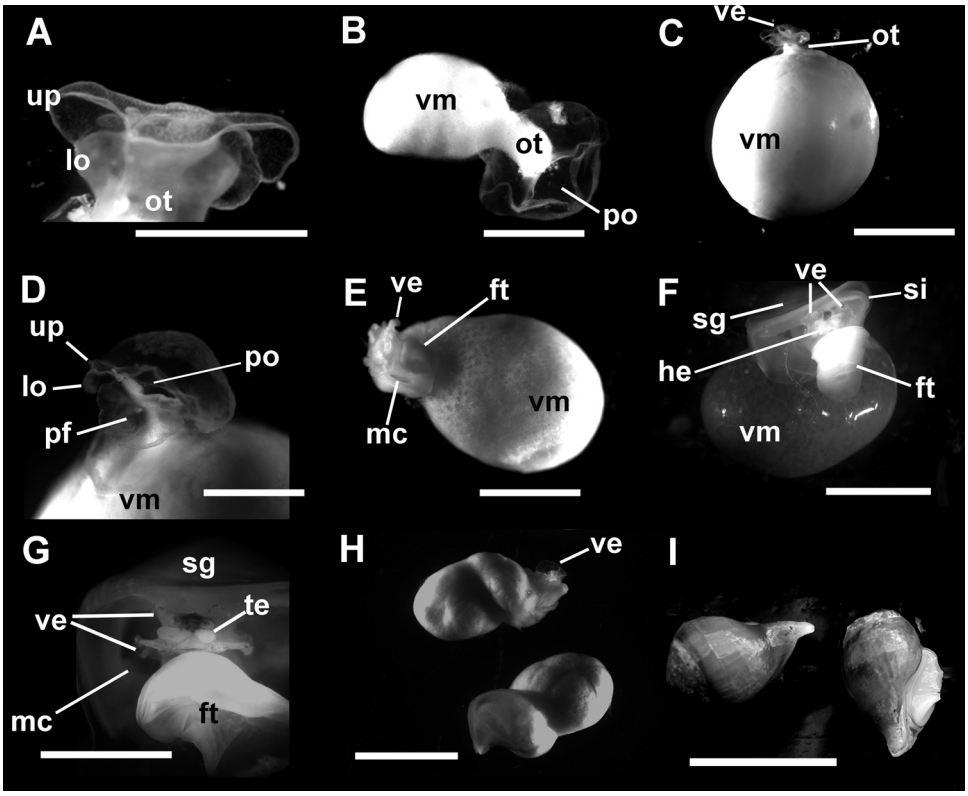

Fig. 6. Micrographs of live embryos (from the midveliger to the pre-hatching juvenile stage). (A) Frontal view of the dissected cephalopodium of a midveliger. (B) Dorsal view of a further developed midveliger. (C) Balloon-shaped late veliger. (D) Frontolateral view of the cephalopodium of a late veliger. (E) Frontolateral view of an early pediveliger. (F) Late pediveliger. (G) Further developed late pediveliger. (H) Coiling embryos in which the apex is yet uncalcified. (I) Pre-hatching juveniles. Abbreviations: ft, foot; he, head; lo, lower velar lamina; mc, mantle cavity; ot, oral tube; pf, early foot; po, preoral fossa; sg, shell gland; si, siphon; te, tentacle; up, upper velar lamina; ve, velum; vm, visceral mass. Scale bars: (A) and (B) = 1 mm; (C) = 3 mm; (D–G) = 2 mm; (H) = 5 mm; (I) = 10 mm.

4. Discussion

4.1. Embryonic stages, feeding behavior, feeding structures and growth rates

The different growth rates in *O. magellanica* embryos can be correlated with morphological and behavioral changes during development. The first phase of slow growth in *O. magellanica* (up to the trochophore stage, Fig. 8) probably reflects the nutrition resources used are mostly those invested by the female in oocyte production.

The second phase of rapid growth occurs during the early, mid and late veliger stages. This rapid phase appears associated to the intake of the white and sticky intracapsular material, and of the high molecular weight proteins (>220 kDa) found in SDS–PAGE (Fig. 9). The particle aggregations seen under the scanning electron microscope (Fig. 7B and G) may also correspond to this material.

The mid- and hind gut differentiate in early veliger embryos of *O. magellanica*, but most developmental changes in the mid and late veliger stages occur in the cephalopodium (Figs. 5 and 6) while intracapsular fluid is being incorporated in the gut. Therefore, most visceral organogenetic processes should occur from the pediveliger to the pre-hatching juvenile stages in this species, i.e., after the embryo has grown in size, and while a significant part of nutrients of the intracapsular fluid has already been ingested. This is not so in another South American volutid, the black snail *Adelomelon brasiliana* (De Mahieu et al., 1974; Luzzatto, 2006) in which the velum disappears early in development and has a reduced role in embryo feeding, and organ formation occurs while the endowment of intracapsular proteins is slowly being reduced (De Mahieu et al.,

Table 1
Correlation between morphological embryo stages and biochemical changes in embryos (mg per embryo) and intracapsular fluid (mg/mL) during development of *Odontocymbiola magellanica*.

Stage	Proteins		Glucose		Calcium		Ammonia	
	Embryos	Fluid	Embryos	Fluid	Embryos	Fluid	Embryos	Fluid
Eggs to early veliger	0.8 ± 0.2^{a}	82.9 ± 5.5^{a}	0.01 ± 0.00	1.62 ± 0.24	0.01 ± 0.00^{a}	0.24 ± 0.01	0.003 ± 0.000	0.014 ± 0.002
	$N = 3$	$N = 11$	$N = 3$	$N = 11$	$N = 3$	$N = 11$	$N = 2$	$N = 11$
Mid- to late veliger	18.3 ± 4.4	36.1 ± 6.6	2.82 ± 0.57	0.60 ± 0.32	0.07 ± 0.01^{b}	0.22 ± 0.02	0.026 ± 0.005	0.007 ± 0.001
	$N = 8$	$N = 9$	$N = 8$	$N = 9$	$N = 8$	$N = 9$	$N = 8$	$N = 9$
Pediveliger	31.9 ± 8.0	27.9 ± 8.2^{a}	2.46 ± 0.47	0.89 ± 0.61	0.67 ± 0.32	0.27 ± 0.04	0.036 ± 0.008	0.010 ± 0.002
	$N = 7$	$N = 7$	$N = 7$	$N = 7$	$N = 7$	$N = 7$	$N = 7$	$N = 6$
Pre-hatching juveniles	32.7 ± 5.3^{a}	22.5 ± 8.4	1.50 ± 0.21	0.26 ± 0.09	$7.42 \pm 2.01^{a,b}$	0.32 ± 0.02	0.037 ± 0.005	0.004 ± 0.000
	$N = 7$	$N = 2$	$N = 7$	$N = 2$	$N = 7$	$N = 2$	$N = 7$	$N = 2$

Values are means ± SEM; N = number of capsules. Superscripts a and b indicate statistically significant differences between the labeled groups ($p < 0.05$; Kruskal–Wallis test, followed by the Dunn's test).

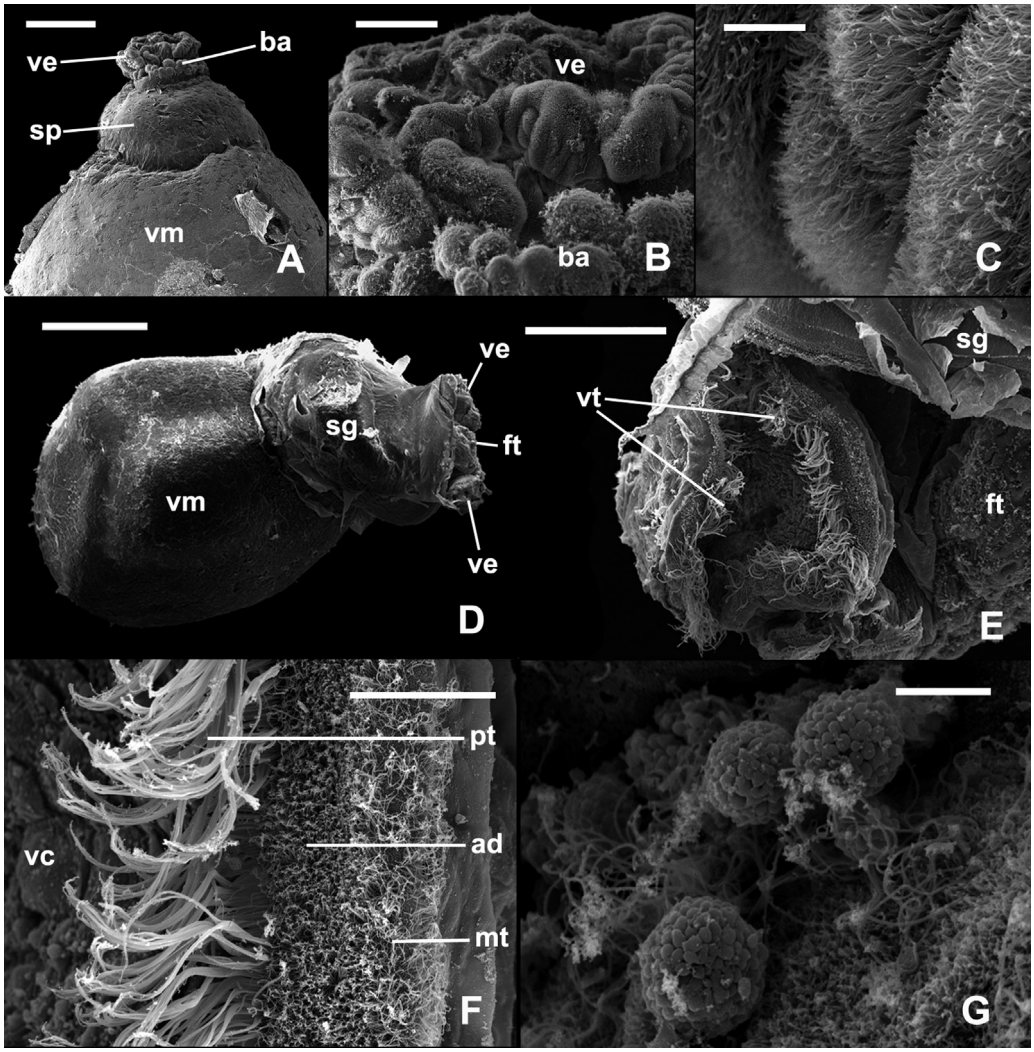

Fig. 7. Scanning electron microscopy of a late veliger and a pediveliger embryo. (A) Cephalopodium and primordium of the shell gland in a late veliger. (B) Higher magnification of velar development in the embryo shown in panel A. (C) Detail of the long velar cilia of the same embryo. (D) Dorsolateral view of an early pediveliger embryo. (E) Detail of the left troch of the embryo shown in panel D. (F) The left troch of the embryo shown in the preceding panels at even higher magnification and showing the unusual disposition of the ciliary bands. (G). Detail of ball-like aggregations intermingled with cilia in the embryo shown in (D). Abbreviations: ba, ball-like aggregations; ft, foot; sg, shell gland; ve, velum; vm, visceral mass. (A) = 500 μm; (B) = 100 μm; (C) = 10 μm; (D) = 1 μm; (E) = 200 μm; (F) = 40 μm; (G) = 5 μm.

1974). On its part, *Voluta musica*, another South American volutid, a large velum grows rapidly in the veliger stage and starts to be resorbed much later, at the pediveliger stage, when the protoconch is becoming calcified (Penchaszadeh and Miloslavich, 2001).

The final phase of a lower growth rate occurs from the pediveliger up to the pre-hatching juvenile stage. Early pediveliger embryos undergo a reduction of velar size which precedes the functional competency of the foot for crawling, and of the radula for grasping, which may explain the reduced growth at this stage. An even more reduced velum still remains in the coiling embryos of *O. magellanica*, which are already capable of radular feeding.

It should be noted that the velar ciliary bands of pediveliger embryos are made by three distinct ciliary rows which remind those found in species with planktotrophic larvae (i.e., prototroch, adoral ciliary band and metatroch; Nielsen, 2004). However, it is intriguing that the band bearing long cilia (the prototroch) form the inner trochal ciliary band instead of the outer one, as it has been reported in most gastropods (e.g., Werner, 1955; Cañete and Ambler, 1992; Collin, 2000; Romero et al., 2004; Collin et al., 2007; Cumplido et al., 2010; Lahbib et al., 2010). A trochal arrangement similar to that of *O. magellanica* has also been found in free living larvae of some littorinimorphs (*Serpulorbis variabilis*, Vermetidae, and *Littorina keenae* and *Littorina scutulata*, Littorinidae, Hadfield and Iaea, 1989; Hofstee and Pernet, 2011) but this peculiarity was not commented by these authors. Genotypic or phenotypic changes in velar size and structures have been described associated to feeding habits or the availability of food (Klinzing and Pechenik, 2000; Hofstee and Pernet, 2011; Phillips, 2011) and the presence of velum and ciliary bands in nonfeeding larvae has been interpreted as retained ancestral characters (Hofstee and Pernet, 2011). A similar interpretation can be made of the presence of velar structures in intracapsularly developing embryos, as those of *O. magellanica*; however, we cannot advance any hypothesis on the functional or phylogenetic significance of an inner prototroch in this species. Further studies are needed to establish whether a similar arrangement occurs in other gastropod species.

4.2. Comparative aspects of athrocyte development and distribution

We have identified as athrocytes (sensu Hyman, 1967) the numerous clear, vesicular cells occurring mostly in early veliger embryos of *O. magellanica* that spread mainly over the early foot and velum. Similar cells have been found in many caenogastropod

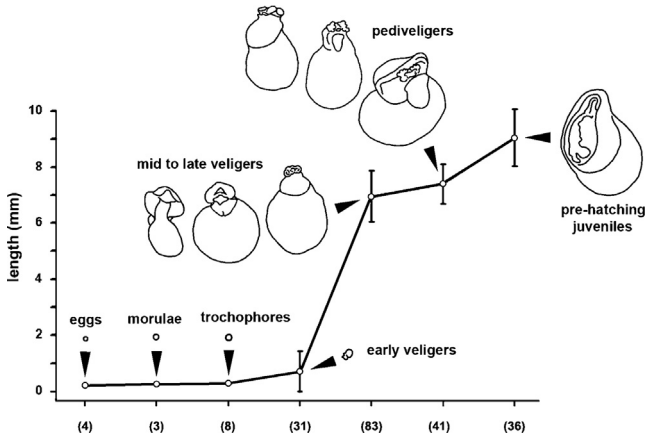

Fig. 8. Changes in embryonic length (mean ± SE) during development. Embryo outlines are approximately to scale.

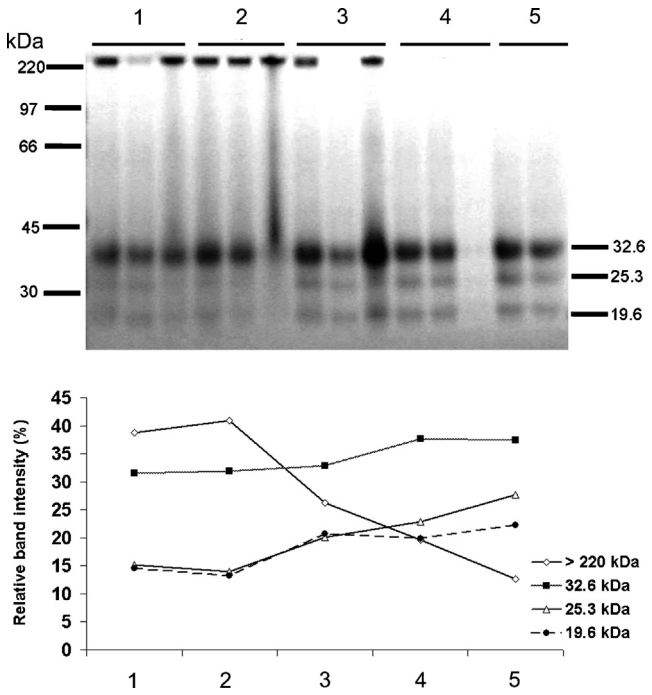

Fig. 9. (A) Electrophoresis (SDS–PAGE) of intracapsular fluid proteins in different stages of development. (B) Changes in mean relative band intensity in the same stages. Lanes: 1, eggs to trochophore embryos; 2, early veliger embryos; 3, mid- to late veliger embryos; 4, pediveliger embryos; 5, pre-hatching juveniles.

species and Ruthensteiner and Schaeffer (1991) have speculated they may be a synapomorphy of the Caenogastropoda.

These cells (and the larval organs they may form) have been interpreted as larval kidneys by early authors, but Rivest (1992) presented evidence for their role in endocytosing and storing proteins from intracapsular fluid prior to the functional differentiation of the gut, and he called them 'absorptive cells'. Indeed, athrocytes in general may be accomplishing functions similar to those of the giant cells in the midgut of other caenogastropods in which they endocytose and store the ingested perivitellin fluid (Koch et al., 2009). Both athrocytes and midgut cells may sequentially serve the role of endocytosis and intracellular digestion before intracapsular fluid materials are actually absorbed by the embryo, so that the designation of the former as 'absorptive' may be misleading, and the term 'athrocyte' seems more accurate (from Greek *athroos*, accumulation, heap, pile; Hyman, 1967).

These cells are arranged in bilateral 'organs' made of large single cells or of multiple smaller cells (e.g., Collin, 2000). In *O. magellanica*, numerous athrocytes of different sizes cover the early foot, the tissue belt and the velum of early veligers, when the stomodaeum-archenteron connection has just been permeated. Also, athrocytes have become much reduced in number at the mid-veliger stage when the visceral mass has developed further. This differential timing of athrocyte and gut development may be in agreement with Rivest's (1992) prediction.

4.3. Comparative aspects of protoconch development

The shell gland (and hence, the start of protoconch formation) is clearly evident in early pediveliger embryos of *O. magellanica*, but the primordium of this gland can be identified at the base of the cephalopodium from the mid veliger stage. To our knowledge, this anterior position of the shell gland has not been reported in other gastropods. Indeed, a posterior and cap-like shell gland has been found, among others, in representatives of the Vetigastropoda (Gaume et al., 2011), Patellogastropoda (Patten, 1886), Architaenioglossa (Hylton Scott, 1958; Demian and Yousif, 1973; Koch et al., 2009), Littorinimorpha (Cañete and Ambler, 1992; Collin, 2000) and Neogastropoda (D'Asaro, 1966; Romero et al., 2004), in which protoconch formation proceeds from the apex to the aperture. Contrariwise, the protoconch growth in *O. magellanica* proceeds from the aperture toward the prospective apex, which also is the last part of the protoconch in becoming calcified. Pre-hatching juveniles of *O. magellanica* (Fig. 6I) show a protoconch similar to some other volutid species in the Southwestern Atlantic (Clench and Turner, 1970) which suggest that shell gland development and protoconch formation may also occur in these species in a way similar to that observed in *O. magellanica*. Even though the development of the shell gland was not explicitly registered in preceding studies on *A. brasiliana*, the drawing of stage I by De Mahieu et al., 1974 and Fig. 2(E–G) on Luzzatto (2006) would suggest that a shell gland extending posterodorsally from the base of the cephalopodium would also occur in the latter species.

4.4. Capsule endowment of nutritive substances and embryo excretion

The recently spawned egg capsules of *O. magellanica* are endowed with a large amount of protein in the intracapsular fluid (~1200 mg per capsule) which may be compared with ~1500 mg in *Adelomelon brasiliana* (De Mahieu et al., 1974), 160–240 mg in *A. ancilla* (Miloslavich et al., 2003) and 30–90 mg in *Voluta musica* (Penchaszadeh and Miloslavich, 2001). Notwithstanding, the protein amounts that can be inferred from Fig. 7A of Luzzatto (2006) are at variance with the previously reported findings in *A. brasiliana*.

A significant decrease of protein in the intracapsular fluid protein (Table 1) occurred mainly at the expense of the high molecular weight fraction (>220 kDa, Fig. 9). It is possible that catabolism of aminoacids derived from protein digestion were contributing to energy needs, since glucose concentration in capsules only showed a statistically non-significant trend to decrease during development.

The importance of ammonia production as a nitrogen excretory product in aquatic animals is related to its high solubility and rapid diffusion in water, which prevents the toxic effects of its accumulation (Schmidt-Nielsen, 1997).

4.5. Comparative aspects of the calcareous capsule

The calcareous cover of the egg capsule laid by *O. magellanica* is unusual among the Volutidae, and this kind of egg cover has only been reported for another member of the family (Ponder, 1970;

Bigatti et al., 2010). The egg capsules of neritimorph gastropods may incorporate calcareous particles of varying origins into their walls (Andrews, 1936; Berry, 1965; Smith et al., 1989; Tan and Lee, 2009) but otherwise, calcareous egg covers are found in some terrestrial (Tompa, 2005) and freshwater gastropods (Hayes et al., 2009). The calcareous egg cover of *O. magellanica* is also peculiar in that it is made of magnesian calcite (Bigatti et al., 2010), a calcium carbonate polymorph in which calcium is partially and randomly substituted by magnesium in the calcite lattice, which is thought to confer a greater crack resistance to the mineral (Magdans and Hermann, 2004) than that of the frequently found aragonite or calcite (Bandel, 1990). This mineral external cover may prevent predation by sea urchins (Bigatti et al., 2010). Also, this cover may serve as a calcium reservoir for the developing embryos, as it has been proposed for some architaenioglossan (Turner and McCabe, 1990) and pulmonate gastropods (Tompa, 1975, 1980, 2005). In the current study, the calcium concentration in the intracapsular fluid remained constant during development, but the amount of calcium per embryo increased several hundred fold by the end of intracapsular development, indicating deposition in the protoconch (Table 1).

4.6. The hatching process

Opercular openings occur in many neogastropod forms (e.g., in the Muricidae, Knudsen, 1994, 2000; Pastorino et al., 2007; Cumplido et al., 2010), but in others, the emergence of hatchlings may occur through seams in the capsule walls or simply by deterioration of the wall (Rawlings, 1999).

In part, the latter seems to be the case of *O. magellanica*, since the calcareous egg cover has deteriorated sufficiently, in most cases, to expose the membranous wall by the end of intracapsular development (Bigatti et al., 2010). However, the membranous wall of *O. magellanica* may be providing an escape way through a crescent-shaped seam on its upper/lateral side which may be similar to the 'hyaline zone' observed in the egg capsules of *A. brasiliana* near hatching (Penchaszadeh and De Mahieu, 1976). This seam is later opened, but juveniles may remain in the capsule for some days, while they continue feeding on the membranous wall.

5. Conclusions

The current study has disclosed several developmental features which show remarkable differences with gastropod species that have been studied previously. It would be interesting to know whether these features also occur in other caenogastropod species. In particular, the velar arrangement of long and short cilia, the number and distribution of athrocytes and the location of the shell gland, as well as the direction of protoconch development, should be recorded in future studies.

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