Development beyond the gastrula stage and digestive organogenesis in the apple-snail *Pomacea canaliculata* (Architaenioglossa, Ampullariidae)

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ABSTRACT: Development of *Pomacea canaliculata* from the gastrula stage until the first day after hatching is described. Trochophore embryos are developed after gastrulation, showing the prototroch as a crown of ciliated orange-brownish cells. However, no true veliger embryos are formed, since the prototroch does not fully develop into a velum. Afterward, the connection between the fore- and midgut is permeated and the midgut becomes full of the pink-reddish albumen, which is stored into a central archenteron's lake, from where it is accumulated into the large cells forming the midgut wall ("giant cells"). Electron microscopy of giant cells in late embryos showed that albumen is engulfed by large endocytic vesicles formed between the irregular microvilli at the top of these cells. By the end of intracapsular development, giant cells become gradually replaced by two new epithelial cell types which are similar to those found in the adult midgut gland: the pre-columnar and the pre-pyramidal cells. Pre-columnar cells have inconspicuous basal nuclei and are crowned by stereocilia, between which small endocytic vesicles are formed. Pre-pyramidal cells have large nuclei with 2-3 nucleoli and show a striking development of the rough endoplasmic reticulum. The genesis of the three cell lineages (giant, pre-columnar and pre-pyramidal cells) is hypothetically attributed to epithelial streaks that occur at both sides of the midgut since early stages of development.

Introduction

The influential monograph of Frantz Leydig (1850) on the embryology, anatomy and histology of *Viviparus viviparus* (L. 1758) (syn. *Paludina vivipara*) (Ampullarioidea, Viviparidae) turned ampullarioids' biology into a focus of scientific interest for more than a century after him. In particular, the embryology of *Viviparus* was investigated by several authors, includ-

ing Bütchli (1877), Erlänger (1891), Drummond (1903) and Otto and Tönniges (1906). These early studies were followed (within the family Ampullariidae) by those of Hylton Scott (1934; 1958) on *Pomacea canaliculata* Lamarck 1822, of Ranjah (1942) on *Pila globosa* (Swainson 1822), and of Demian and Yousif (1973a; 1973b; 1973c; 1973d; 1975) on *Marisa cornuarietis* (L. 1758). All these authors have addressed several aspects of digestive organogenesis. Later on, however, the subject seems to have fallen into oblivion, as most descriptive embryological studies.

The current report is part of a program dealing with the biology of a putative endosymbiont (Vega *et al.*, 2006) which is contained within the epithelial cells of

the midgut gland of adult *P. canaliculata*. In this context, we have revisited digestive organogenesis in this snail to extend our knowledge of the *scenario* where this putative endosymbiosis is established.

References to the adult structures are made according to the works of Hylton Scott (1958), Andrews (1965a, 1965b), Koch *et al*. (2006) and Gamarra-Luques *et al*. (2006).

Material and Methods

Embryos

The embryos observed in the current study derive from a cultured strain of *Pomacea canaliculata*, whose original stock was collected at the Rosedal Lake (Palermo, Buenos Aires, Argentina), at several times between 1993 and 1996. Voucher (ethanol preserved) specimens of the original population and of the cultured strain were deposited at the collection of Museo Argentino de Ciencias Naturales (Buenos Aires, Argentina; lots MACN-In 35707 and MACN-In 36046, respectively). Both water and ambient temperatures were regulated at 23-25ºC and artificial lighting was provided between 7 am and 9 pm. The pink reddish egg masses were removed from the aquaria and were incubated in dry Petri dishes in the aquarium room. Embryos were collected daily, between 9 am and 6 pm, from day 1 (the day of spawning) to day 11, as described in the next paragraph. The collection time (either am or pm) was only recorded for day 1 (the day of spawning). The day of hatching was defined as that in which the first juveniles were leaving capsules (usually, most eggs within a clutch were also hatching on that day). Juveniles on the day of hatching and on day 1 after hatching were also collected.

Observations and micrographs of fresh material

The egg shells were cracked with small forceps and the embryos were removed and observed in Petri dishes containing NaCl 0.1%. Digital micrographs (24 bits colour format, 640 x 480 pixels) were obtained with a color video camera (Sony, model DXC-151A, Japan) mounted on either a conventional stereoscopic microscope or a bright field microscope. The size of scale bars was determined with Image Pro-Plus 4.5® (Media Cybernetics, Silver Spring, MA, USA). Digital images obtained with the 10x and 40x objectives were cut from its original background and pasted on a uniform background, thus eliminating albumen remnants and other debris in the background. In some cases (as indicated in Results), composites of 2-5 micrographs obtained from the same embryo at different focus planes were used. To facilitate interpretation, a line drawing of the relevant structures was pasted over an accompanying copy of the original micrograph (Figs. 1-8).

Observations on fixed material (light microscopy and transmission electron microscopy)

Juveniles that were about to hatch were removed from both their egg capsules and their shells, and each visceral hump was excised with fine forceps and fixed for transmission electron microscopy in a 3% glutaraldehyde solution buffered with 0.1 M phosphate buffer pH 7.40 for 5 h at room temperature, and then postfixed in 1% osmium tetroxide in the same buffer overnight. Tissues were later treated with an aqueous solution of 2% uranyl acetate for 45 min. Afterwards, they were serially dehydrated in ethanol, passed through acetone and embedded in Spurr's resin, and sectioned in Leica Ultracut R ultramicrotome; 0.8-1 µm sections were obtained for topographical orientation and stained with toluidine blue. Ultrathin silver gray sections were stained with uranyl acetate and lead citrate and examined with a Zeiss 900 transmission electron microscope.

Results

Observations on living embryos

The aerial eggs of *P. canaliculata* are of a bright reddish color due to its albumen (Fig. 1A). In the laboratory they are laid in the walls of aquaria, while in their natural environments these snails prefer the emerging stems of water plants (Fig. 1A). Intracapsular development lasted 12-14 days under present study conditions, with a mean of 13.8 days (N=230) and a median of 14 days.

No developmental abnormalities were recorded, except for occasional capsules bearing two embryos. However, even in the controlled laboratory conditions, the developmental stage of embryos within the same spawn was considerably variable, and was even more variable between different spawns of the same age. Also, apparently normal embryos at the same developmental stage could be of very different sizes.

On day 1, the embryos collected were between the morula and the gastrula stage, both am and pm. A trans-

lucent zone containing sperm surrounds most embryos on this day (Fig. 1B).

Later on, trochophore-like embryos as those of Fig. 1C show an uncolored and not distended archenteron which appears connected to the exterior by the rudiment of the hindgut. This rudiment ends more or less at the center of an anal plate of large colored cells. The anus is presumably opened there, as a remnant of the blastopore. At the anterior end, a funnel-like ectodermal depression appears located below another group of large colored cells (the oral plate) and is the stomodaeum, the rudiment of the foregut. Two other "plates" of large cells are seen at the upper and the lower parts of the embryo (apical and pedal plates, respectively).

Cells at these four plates contain an orange-brownish pigment. The apical and pedal plates are joined bilaterally by the prototroch, a crowning band of large and ciliated colored cells (not in focus in Fig. 1C; better seen on Fig. 2A). All these embryos have lost the translucent zone containing sperm, and show active rotary movements produced by the prototroch cells' cilia. In Fig. 2A, the embryo shows a pink, round and fully distended archenteron, which indirectly indicates that the stomodaeum has been permeated and that the embryo is feeding on the colored albumen. Indeed, a continuous flow of albumen particles going through the stomodaeum may be observed at high magnification in living embryos. A typical feature is the differentiation of very large, albumen-loaded cells which form the archenteron wall and which will be indentified as "giant cells". Fig. 2A also shows an uncolored epithelial ridge surrounding a midline depression, which is the rudiment of the embryonic shell gland, and which will secrete the protoconch (or embryonic shell). At this stage the primitive foot starts growing ventrally and slightly forwardly (neither the pedal plate nor the anal plate are seen on Fig. 2A).

P. canaliculata never shows true veliger embryos, since velar development does not go beyond the stage of a colored crown of ciliated cells (see below, Fig. 4). Fig. 2B shows an embryo in which the three major gut sections (fore-, mid- and hindgut) have become distinct. The stomodaeum (foregut) can be recognized only as a
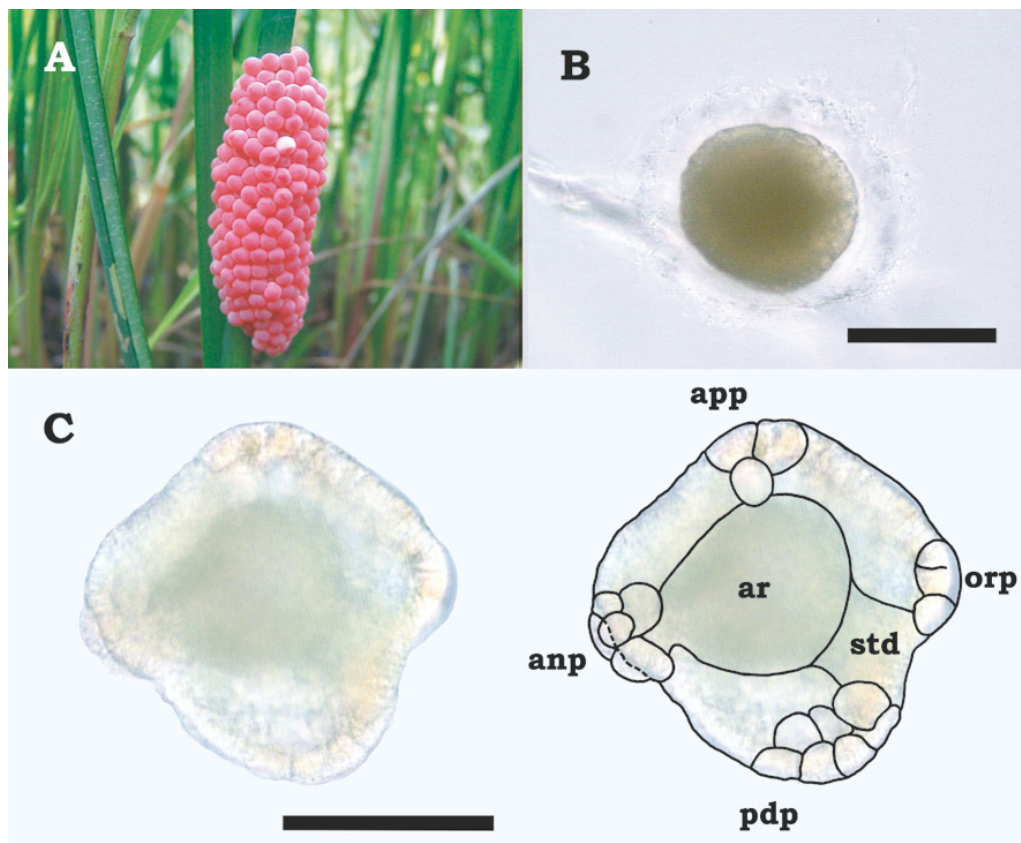

FIGURE 1. A. Typical spawn of *P. canaliculata* in a natural environment. **B.** A gastrula surrounded by a translucent zone containing sperm (collected on day 1 morning). C. Trochophore-like embryo (right view) which has lost the surrounding translucent zone (collected on day 2). Scale bars represent 100 μm. Structures in this figure are indicated as: *app*, apical plate; *anp*, anal plate; *ar*, archenteron; *orp*, oral plate; *pdp*, pedal plate; *std*, stomodaeum.

funnel which opens dorsally into the midgut, while the radular sac which will develop at its ventral side has not yet differentiated. The mouth is now covered by a cephalic ectodermal flap, whose anterior section will originate both eyes and tentacles, while its posterior section will originate the right and left nuchal lobes. Both the mid- and hindgut show the typical reddish color of the eggs of *P. canaliculata*, indicating that the albumen that flows through the foregut is concentrated in the midgut and it later passes to the hindgut (which shows no giant cells). The rudiment of the mantle cavity is being formed as an ectodermal depression which appears in the figure as a clear zone close to the anus. An outgrowth of this primitive cavity, to the right of the midgut, is the ureter (the rudiment of the adult anterior kidney) which joins the embryonic right kidney (the rudiment of the adult posterior kidney). The latter also communicates with the pericardial cavity (now located dorsally to the midgut) through a slit-like renopericardial pore.

At this stage, the midgut is mostly a round structure lined by its characteristic giant cells. For the first time, a superficial grayish band of very small cells is seen in Fig. 2B extending along the side of the midgut, and which are external to the layer of giant cells. These midgut streaks, as bilateral structures, extend from a cellular patch surrounding the connection with the stomodaeum to another similar patch near the connection with the hindgut.
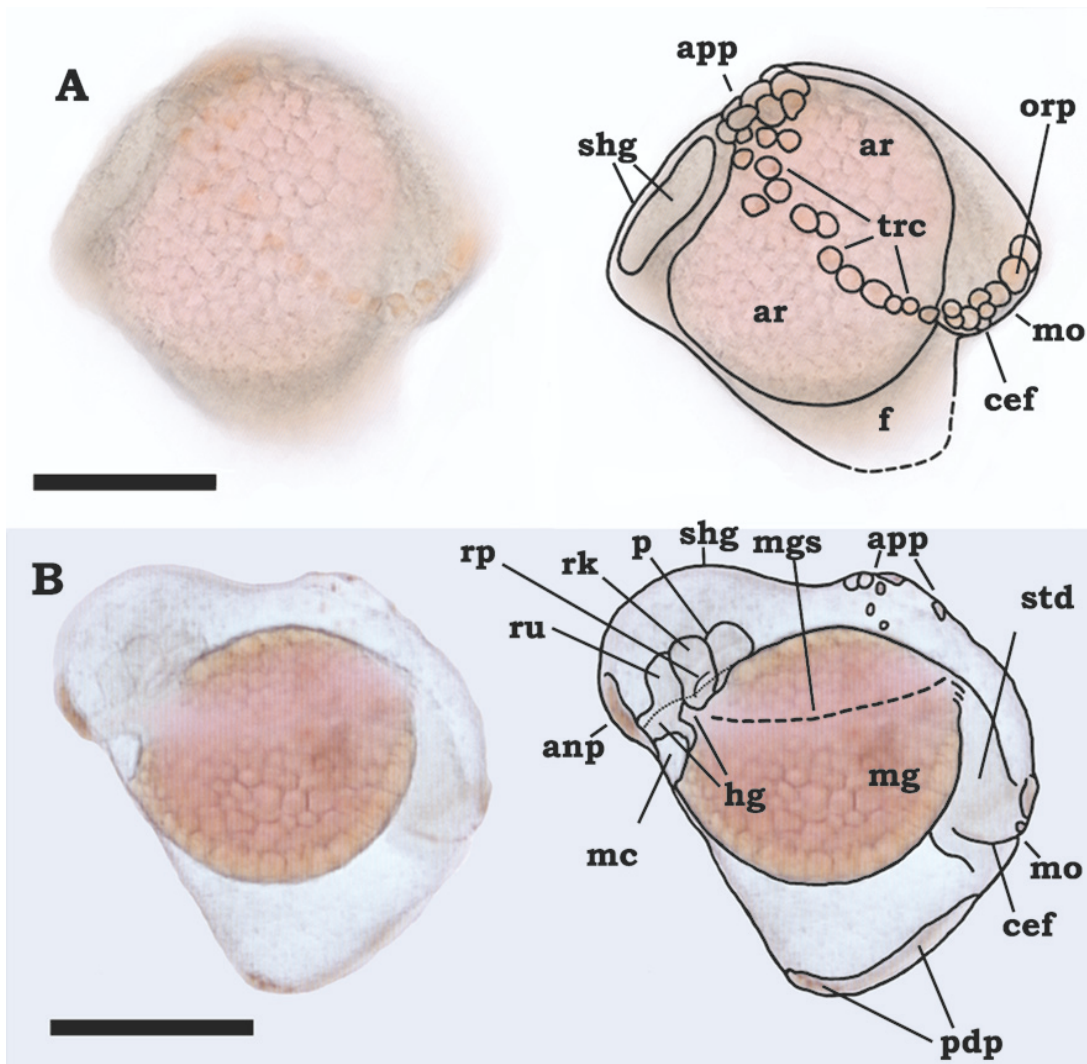

FIGURE 2. A. Embryo (right side) in which the archenteron has become full of the pink-reddish albumen and the shell gland has differentiated (collected on day 2). Scale bar represents 100 µm. **B.** Embryo (right side) where the primitive mantle cavity is differentiated as an ectodermal invagination dorsal to the anus; the bilateral midgut streaks, the pericardium, and the right kidney and ureter have appeared (collected on day 3). Scale bar represents 300 µm. Structures in this figure are indicated as: *anp*, anal plate; *app*, apical plate; *ar*, archenteron; *cef*, cephalic ectodermal flap; *f*, foot; *hg*, hindgut; *mc*, primitive mantle cavity; *mg*, midgut; *mgs*, midgut streak; *mo*, mouth; *orp*, oral plate; *p*, pericardial cavity; *pdp*, pedal plate; *rk*, right kidney; *rp*, renopericardial pore; *ru*, right ureter; *shg*, shell gland; *std*, stomodaeum; *trc*, trochophore cells.

In Fig. 3A, the embryo is shown from the left to allow viewing of the formerly dorsal and concave shell gland which has now been displaced to the left of the embryo and it is becoming a slightly convex structure. The vesicle superimposed at its center is the pericardial cavity, which shows both a slit-like cavity and a dorsal thickening, while the renal structures, which are located to the right of the hindgut, are not visible in Fig. 3A. In the foregut, the radular sac is now seen as a midsagittal ventral outgrowth of the stomodaeum. The cephalic ectodermal flap is demarcated by groups of pigmented cells. The midgut is elongating anteroposteriorly, and is continued by the hindgut which terminates between cells of the anal plate.

Fig. 3B is a composite of two micrographs of a slightly more developed embryo, which shows a constriction just behind the apical plate and the foot. This constriction will separate the posterior mass (the prospective visceral hump) from the anterior mass (the prospective head and foot). The shell gland (still on the left and being only slightly convex) has grown and its perimeter surrounds the whole posterior mass containing the cardio-renal organs and the primitive mantle cavity. The foregut is in a developmental stage similar to that of Fig. 3A. Also, an incipient sulcus is being formed at the front of the embryo, which will separate the head from the foot. The midgut is now pear-shaped and shows the albumen concentrating giant cells forming most of the wall of the midgut cavity, with no infoldings. However, a zone of smaller albumen-loaded cells appears at the posteroventral and right part of the midgut: this zone will originate the adult stomach. Also, the right midgut
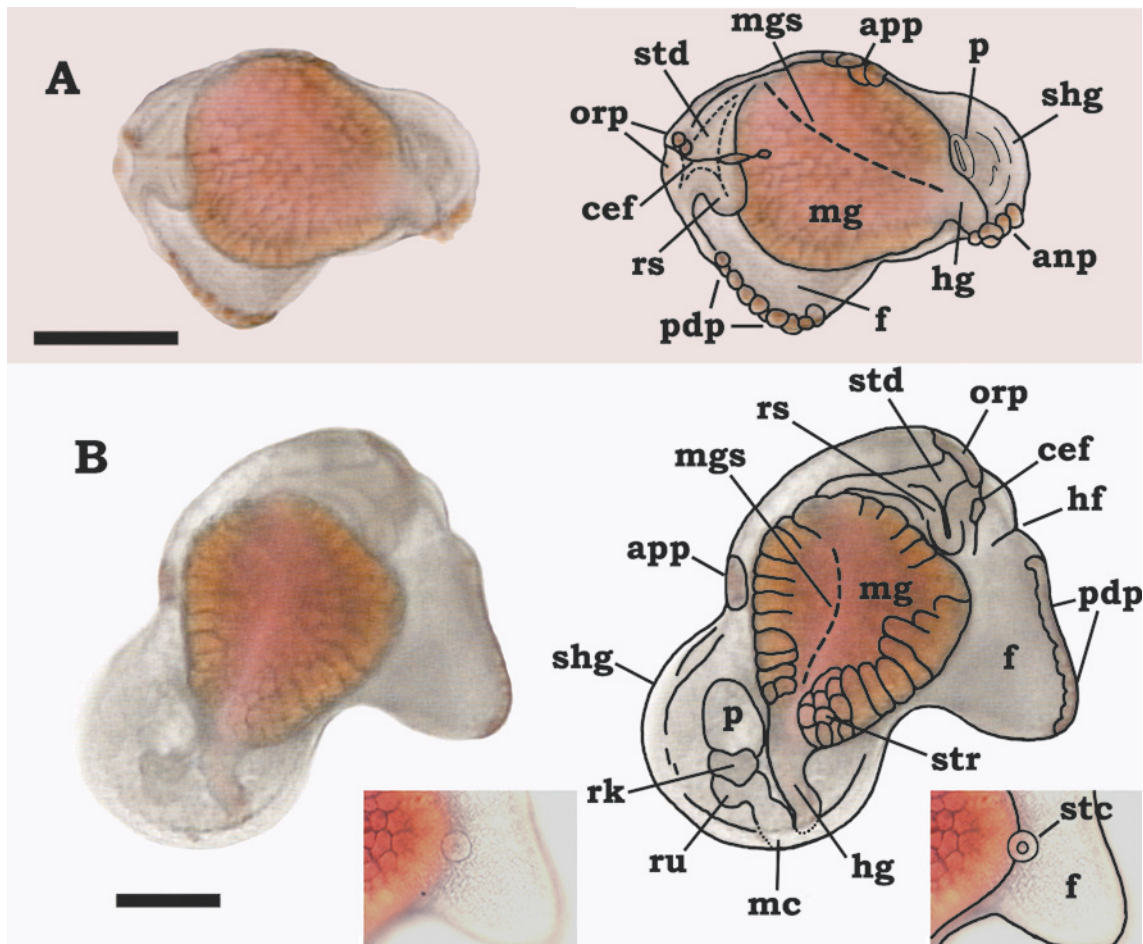

FIGURE 3. A. Embryo (left side) where the three major sections of the intestine (fore-, mid- and hindgut) can be recognized (collected on day 3). **B.** Embryo (right side) where a constriction posterior to the apical plate begins separating the head-foot region from the prospective "visceral hump". The stomach rudiment appears in the posterior part of the midgut. The inset shows the right statocyst (collected on day 4). Scale bars represent 200 μm. Structures in this figure are indicated as: *anp*, anal plate; *app*, apical plate; *cef*, cephalic ectodermal flap; *f*, foot; *hf*, head-foot sulcus; *hg*, hindgut; *mc*, primitive mantle cavity; *mg*, midgut; *mgs*, midgut streak; *orp*, oral plate; *p*, pericardial cavity; *pdp*, pedal plate; *rk*, right kidney; *rs*, radular sac; *ru*, right ureter; *shg*, shell gland; *stc*, statocyst; *std*, stomodaeum; *str*, stomach rudiment.

streak is seen. The anus is approaching the primitive mantle cavity. Also, the statocysts are now formed bilaterally at the space between the ventro-lateral wall of the anterior midgut lobe and the foot epithelium (the inset shows the right one).

Fig. 4A shows an embryo in a stage of development similar to that of Fig. 3B, in a left but slightly frontal view which allows viewing the development of the prototroch remnants and of the midgut streaks. The prototroch remnants are seen as a superficial band of colored cells which extends forward from the apical plate and which borders the cephalic ectodermal flap. The other colored plates are also seen: pedal plate cells extend as a midline narrow band, at the front of the primitive foot, while the anal plate cells extend lateral and dorsal to the anus. The apical and pedal cells are the likely precursors of the numerous orange-brownish spots which are spread over the head and the dorsal aspect of the foot in adult animals. Those orange-brownish spots are distinct from the numerous melanic spots that will
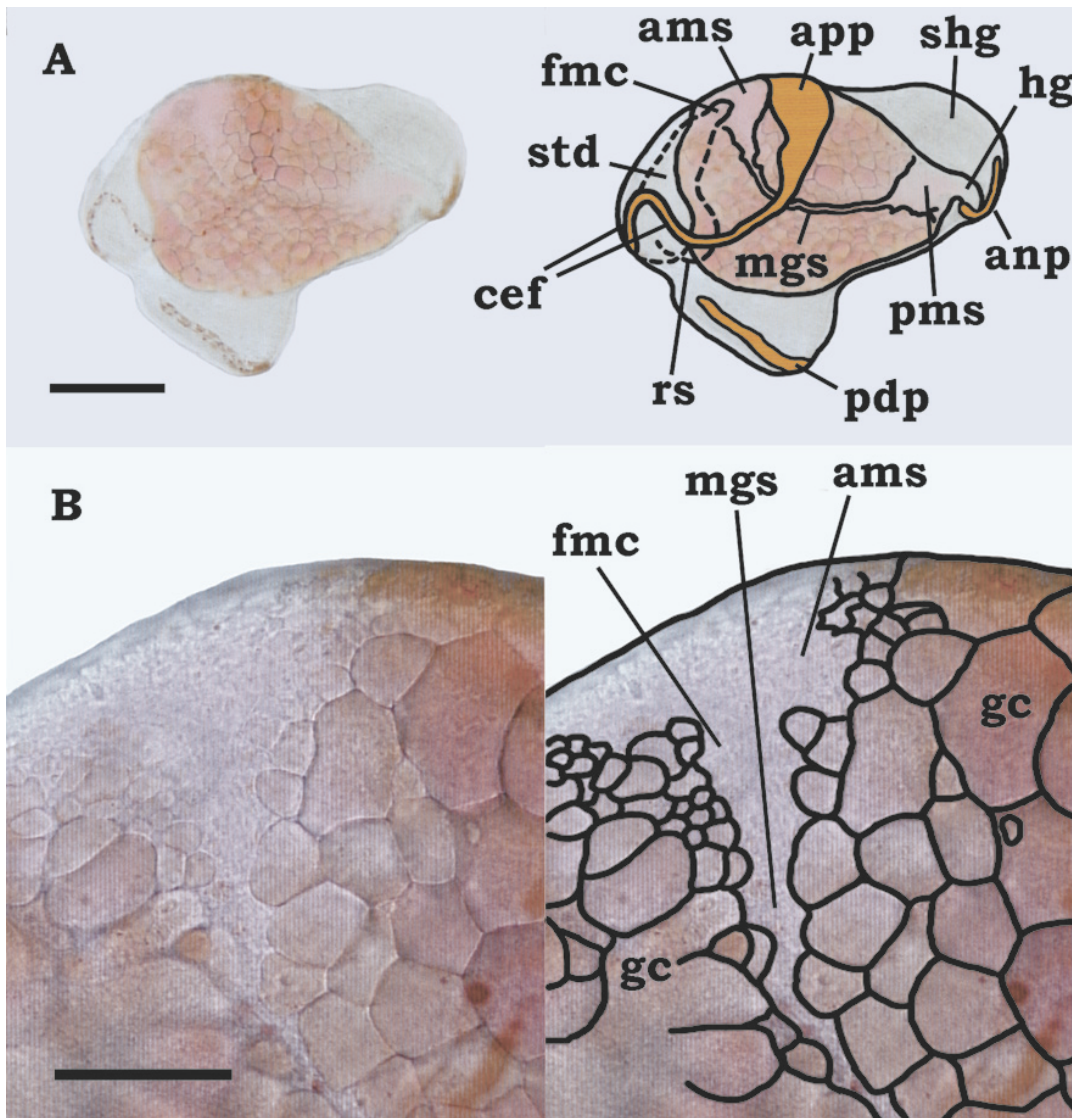

FIGURE 4. A. A slightly frontal and left view of an embryo in a stage similar to that of Fig. 3B. The micrograph shows the distribution of the ectodermal pigmented cells and of a grayish, apparently highly proliferative tissue, which constitutes the midgut streak and two cellular patches, at both the front and the rear of the midgut. The colored cells regions are indicated in light brown in the accompanying line drawing (embryo collected on day 7). Scale bar represents 300 μm. **B.** A close up of the same embryo shown above: the micrograph is focused in the anterior cellular patch and in the midgut streak which continues it. Only the approximate location of the foregut/midgut connection is indicated (*fmc*) since it is not in the micrograph's focus. Scale bar represents 75 μm. Structures in panels A and B are indicated as: *ams*, anterior cellular patch of the midgut streak; *anp*, anal plate; *app*, apical plate; *cef*, cephalic ectodermal flap; *fmc*, foregut/midgut connection; *gc*, giant cells; *hg*, hindgut; *mgs*, midgut streak; *pdp*, pedal plate; *pms*, posterior cellular patch of the midgut streak; *rs*, radular sac; *shg*, shell gland; *std*, stomodaeum.

later develop over the head, foot and mantle (Fig. 8). The fate of those of the anal plate is less clear: because of their position close to the border of the primitive mantle cavity (Fig. 2B) one would look for their descendants in the adult mantle edge, and the only organ there with a similar pigmentation is the outer penial sheath in males.

Also in Fig. 4A, two grayish cellular patches are seen: one at the front, dorsal and lateral to the foregut/midgut connection, and the other at the rear, lateral to the midgut/hindgut connection. The left midgut streak is also seen extending between these two patches. Both patches and streaks are apparently composed of a same proliferative tissue from which giant cells differentiate.

Fig. 4 B shows a close up of both the anterior cellular patch and the anterior end of the left midgut streak: small albumen-loaded cells are developing close to this tissue, and apparently they become typical giant cells as they are displaced by the new differentiating cells.

Fig. 5A is a composite of two micrographs of an embryo in which dextral torsion is already evident. The external surface of the shell gland is now fully convex and it has elongated antero-posteriorly to some extent. Its border, which is covered by the primitive mantle edge, is now opening dorsally. The sulcus separating the head and the foot has deepened and the head is now a distinct set of structures, including the eye and tentacle buds have appeared. The midgut has differentiated into a large
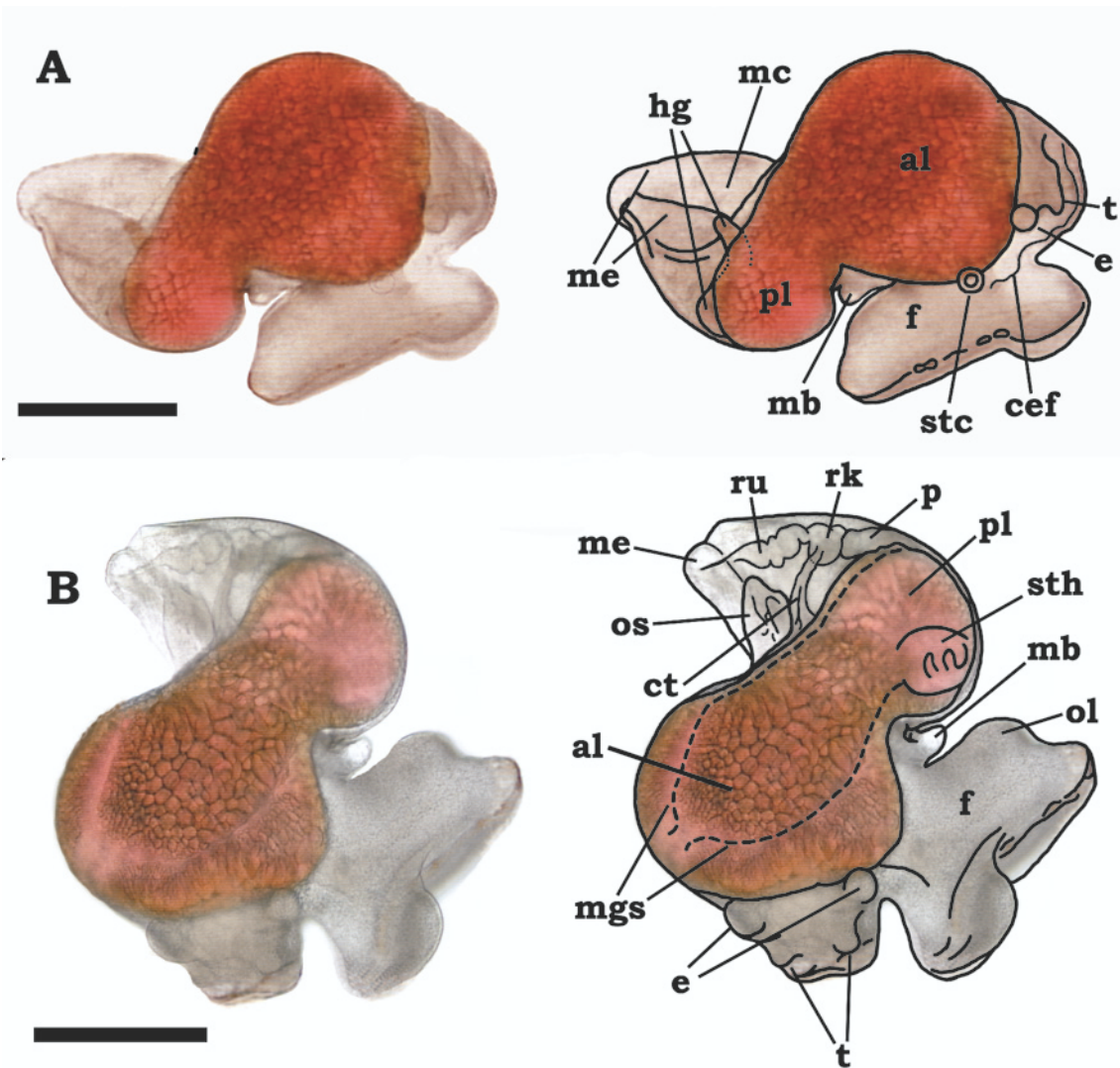

FIGURE 5. A. Embryo (right side) where embryonic torsion is already evident. The posterior midgut lobe is being formed and partially included within the shell (embryo collected on day 4). **B.** A slightly more developed embryo in which the anterior midgut streaks reach their maximal development (collected on day 4). Scale bars represent 500 μm. Structures in panels A and B are indicated as: *al*, anterior midgut lobe; *cef*, cephalic ectodermal flap; *ct*, ctenidium; *e*, eye bud; *f*, foot; *hg*, hindgut; *mb*, midline bud; *mc*, primitive mantle cavity; *me*, mantle edge; *mgs*, midgut streak; *ol*, operculiger lobe; *os*, osphradium; *p*, pericardial cavity; *pl*, posterior midgut lobe; *rk*, right kidney; *ru*, right ureter; *stc*, statocyst; *sth*, stomach; *t*, tentacle bud.

anterior lobe and a smaller posterior lobe which is being included into the growing shell. The foregut/midgut connection is now occurring at the anteroventral aspect of the midgut. The midgut streaks are fuzzily seen on Fig. 5A. The statocyst lies ventrolaterally to the anterior lobe. Altogether, the primitive mantle cavity with the ctenidium, and the osphradium, the cardio-renal structures and the posterior midgut lobe, are being included within the developing shell (structures not shown in Fig. 5A). The colored hindgut is bent and the anus is now opening into the mantle cavity. A midsagittal teatlike bud has appeared near the constriction that separates the head-foot mass from the visceral hump. This "midline bud" (apparently a mesodermal outgrowth covered by ectoderm) is not a part of the shell gland's

ridge and of the developing mantle edge, but it is located below them. It will remain approximately midsagittal and it will originate the adductor muscle. A thickening on the posterior part of the foot is the rudiment of the operculiger lobe. Spots of colored cells, probably derived from the pedal plate, spread on the foot's dorsal aspect.

An upper and left view of a slightly more developed embryo is shown in the composite picture (five micrographs) of Fig. 5B. The mantle edge is now turned slightly to the right of the embryo. The bilateral midgut streaks are seen as they are merging at the antero-dorsal aspect of the midgut anterior lobe (i.e., they have separated from the foregut/midgut connection which is now at the ventral aspect of the anterior midgut lobe). It
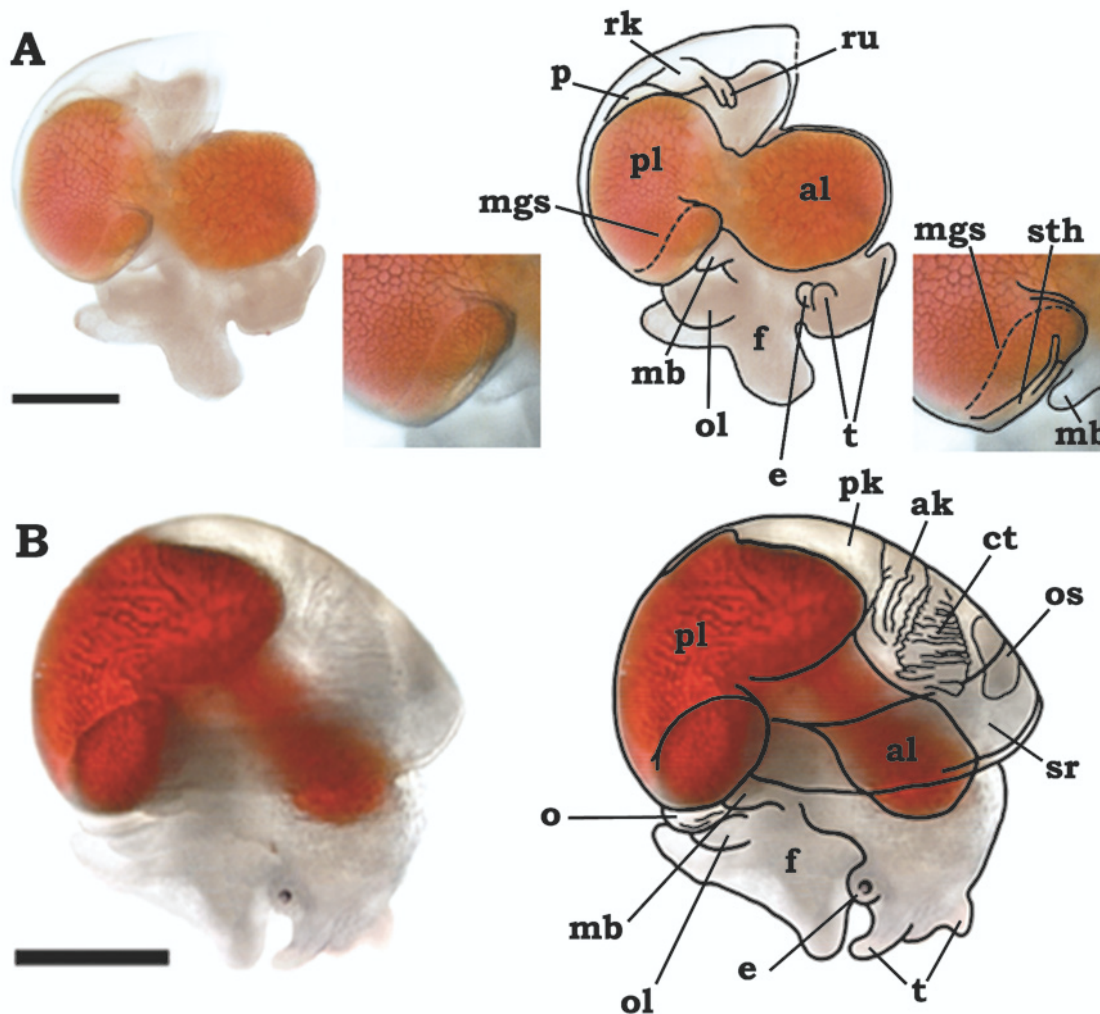

FIGURE 6. A. Embryo in which both midgut lobe are of approximately the same size. The inset shows the remaining midgut streak on the posterior lobe only, and the stomach as a bent tube on the right of the posterior midgut lobe (collected on day 6). **B.** Embryo with a snail like appearance in which the anterior midgut lobe is being reduced to a club-like anterior projection. The eyes are becoming pigmented (embryo collected on day 6). Scale bars represent 500 µm. Structures in this figure are indicated as: *ak*, anterior kidney; *al*, anterior midgut lobe; *ct*, ctenidium; *e*, eye bud or eye; *f*, foot; *mb*, midline bud; *mgs*, midgut streak; *o*, operculum; *ol*, operculiger lobe; *os*, osphradium; *p*, pericardial cavity; *pk*, posterior kidney; *pl*, posterior midgut lobe; *rk*, right kidney; *ru*, right ureter; *sr*, supramarginal ridge; *sth*, stomach; *t*, tentacle bud or tentacle.

also appears here that the giant cells originate from these streaks, and that these cells become loaded with albumen as far as they get apart from the streaks, contributing to the mass of giant cells.

Also in Fig. 5B, the silhouette of the stomach is seen on the rear part of the midgut posterior lobe, and it is located to the right of the midsagittal plane. The right ureter opens at the front of the mantle cavity, and is continued proximally by the right kidney and the pericardium. The constrictions in the wall of the right ureter seem to be the origin of the cristae which will be found in the adult organ (the anterior kidney). Also within the mantle cavity, the rudiments of the osphradium and the ctenidium are clearly seen. The lung has not yet appeared. The head and foot structures show a development similar to that of the embryo in Fig. 5A, except that the operculiger lobe has become conspicuous.

Fig. 6A (a composite of 4 micrographs) shows the single embryo we found at this stage, which seems to be a very transient one. It is characterized by an "eight-shaped" midgut, inasmuch as the anterior midgut lobe is being reduced while the posterior grows up. The operculum and its operculiger lobe are notable on the posterior part of the foot. Interestingly, the epithelial streaks have disappeared from the surface of the anterior midgut lobe, and are reduced to short ones on the posterior lobe, joining behind the stomach and close to the midline (inset). The mantle edge has been separated from the shell's aperture during experimental

handling, and consequently, the mantle cavity has collapsed, even though the now shorter ureter reminds better the shape of the adult anterior kidney. Backward, the ureter is continued by the translucent right kidney (the adult's posterior kidney) and by the pericardium. Collapse of the mantle cavity has brought the osphradium and the ctenidium close together and their shades cannot be distinguished.

As development proceeds, the embryo acquires a definite snail-like appearance (Fig. 6B, a composite of two micrographs). The cephalic ectodermal flap which was covering the mouth in earlier stages (Figs. 2A, 2B, 3A and 3B), has given origin to the right nuchal lobe, backward, and to the eyes and tentacles, forward. The eyes are now cup-like structures which are becoming pigmented. The operculum has clearly differentiated from the operculiger lobe. The opaque mass of the adductor muscle extends into the shell from the "midline bud", but the bud itself still remains outside the shell. An also opaque band is seen surrounding the shell's aperture: it is the supramarginal glandular ridge which is now secreting the teloconch, much before hatching occurs (contrariwise to many caenogastropod forms). The left part of the mantle cavity shows the osphradium, the ctenidium, and the anterior kidney. The lung will later develop at the narrow space between the osphradium and the ctenidium, displacing the former to the extreme right of the mantle cavity while the latter will be displaced to the left.
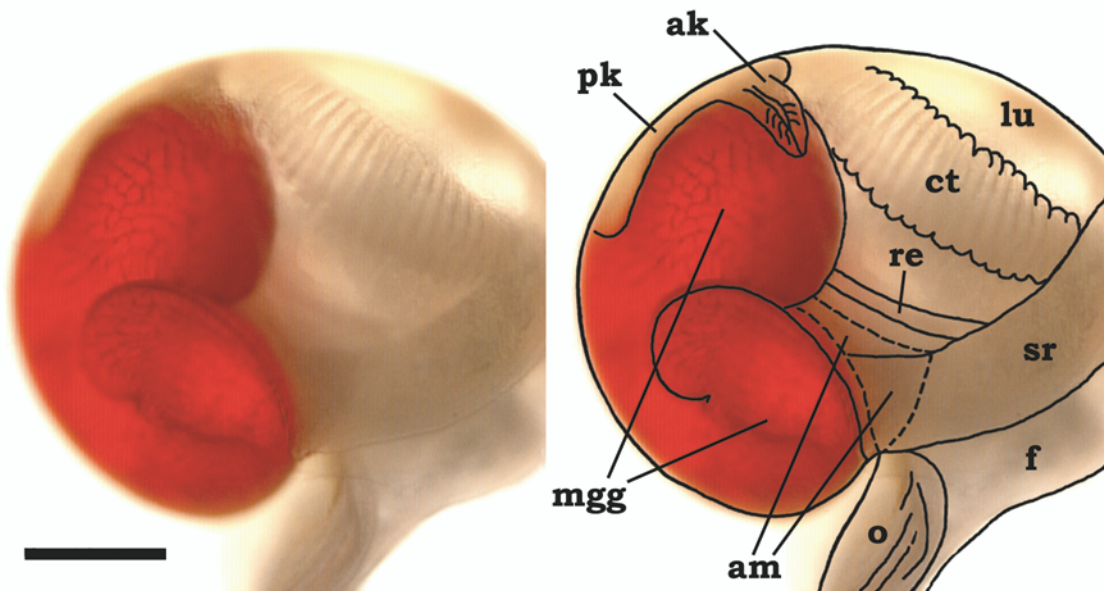

FIGURE 7. Embryo in which the anterior midgut lobe has disappeared and the lung is displacing the ctenidium to the right of the mantle cavity (collected on day 8). Scale bar represents 500 μm. Structures are indicated as: *ak*, anterior kidney; *am*, adductor muscle; *ct*, ctenidium; *f*, foot; *lu*, lung; *mgg*, midgut gland; *o*, operculum; *pk*, posterior kidney; *re*, rectum; *sr*, supramarginal ridge.

Important in the context of digestive organogenesis, the anterior lobe of the midgut has been reduced in this embryo to a narrow club-like structure (Fig. 6B). At this stage, the posterior midgut lobe is still a hollow structure lined by giant cells, and it is dividing into two communicating lobes which will originate the midgut gland: (1) the right one is occupying the shell apex, (2) the left one is extending dorsally to the right of the posterior kidney, and it is also extending ventrally to cover the visceral aspect of the stomach and its surroundings (this latter part is not seen in Fig. 6B). The closed space delimited by the posterior kidney (dorsally), the posterior wall of the mantle cavity (forward) and the two parts of the left midgut lobe (ventrally and to the right), constitutes the posterior renal chamber, where the coiled part of the intestine is developing.

Fig. 7 shows the right side of an embryo in which both lobes of the midgut gland are still lined by the albumen giant cells. Both the anterior and posterior kidneys are seen dorsal to the left lobe. The rectum appears as a narrow tube parallel to the adductor muscle (in the clear zone between both structures the distal part of gonoduct will develop). The lung is now developing and it is displacing the ctenidium to the right of the mantle cavity. The supramarginal ridge extends as an opaque band of tissue bordering the shell aperture.

Fig. 8A shows the lower left side of a more developed embryo. The micrograph is centered in the stomach, showing the esophagus entrance to the embryo's right and the thin gut to the left. The pericardial cavity has been migrating from its initial position on the mid-sagittal plane, and it is now closer to its final place, attached to the adductor muscle and near the shell's umbilicum. Both parts of the left midgut lobe appear on the micrograph: one is superficially seen surrounding the stomach, while the other is seen in the background (dotted lines). Between both of them, and covered by the posterior kidney, are the posterior renal chamber and the developing coiled intestine. The stomach shows a tongue-like extension at the place where both midgut epithelial streaks had united, and around this place, the differentiation of the midgut alveoli begins, slowly replacing the giant cells that were covering the surface of the midgut gland. This "tongue" will later extend as a translucent band of tissue (Fig. 8B) which will traverse the whole whorl, and it will be most conspicuous during the first days after hatching (Fig. 8E). A dark melanic spot start to develop at the midline, on the posterior end of the foot.

The ventral view of the embryo in Fig. 8B shows both the pericardium and the stomach at their final positions. The visceral artery (one of the two aortal branchs) is running parallel to the pericardium. The midgut gland alveoli continue to develop and extend to most of the surface of the midgut gland. Melanic spots appear throughout the mantle, and on the head-foot mass (particularly, the midline spot at the posterior end of the foot elongates and becomes darker). To the right of the pericardium, the esophagus is covered by a foamy tissue which is the first indication of the development of the adult urate tissue (Giraud-Billoud *et al.*, 2008). Notably, the "midline bud" has finally been included within the growing shell, near the umbilicum (Fig. 8C).

From this stage, the embryo practically will not grow in size within the egg, and it is a miniature of the adult, having the complete set of organs, with the exception of the reproductive ones, which are still at a very rudimentary stage (see Discussion). From now on (1-3 days before hatching) the embryo will continue to feed on the remaining albumen and it will consume that stored in the giant cells, which are progressively reduced in number. Also, the whitish threads and spots of urate tissue will increase on the surface of the visceral hump (Fig. 8E). If artificially they are taken out of the shell and laid in water, they will survive and be able to eat lettuce, though they will also eat the albumen of crashed eggs, if offered. Probably, normal hatching occurs when there is not any albumen left, and the embryos start to erode the calcareous egg shell.

Observations on fixed material (light microscopy and transmission electron microscopy)

These observations were limited to embryos in the last days of intracapsular development and to newly hatched juveniles, since we were unable to obtain useful preparations from embryos which were still showing a large albumen load in their midgut.

At the time of our first observations, however, the midgut gland was still an epithelial sac surrounding a large albumen lake. In some of these preparations, the walls of the midgut gland were composed of giant cells only, which were about 100 μm high. The apical region of giant cells showed some disorderly arranged microvilli and they were endocytosing large albumen vesicles as shown in Fig. 9B and C. In these cases, the giant cells' cytoplasm was almost replaced by a large vesicle full of endocytosed albumen. Albumen both in the central lake and in the large endocytic vesicles of the giant cells lining typically contain 20 nm granules which were intermingled with a microgranular material of lesser electron density. The albumen's microscopical
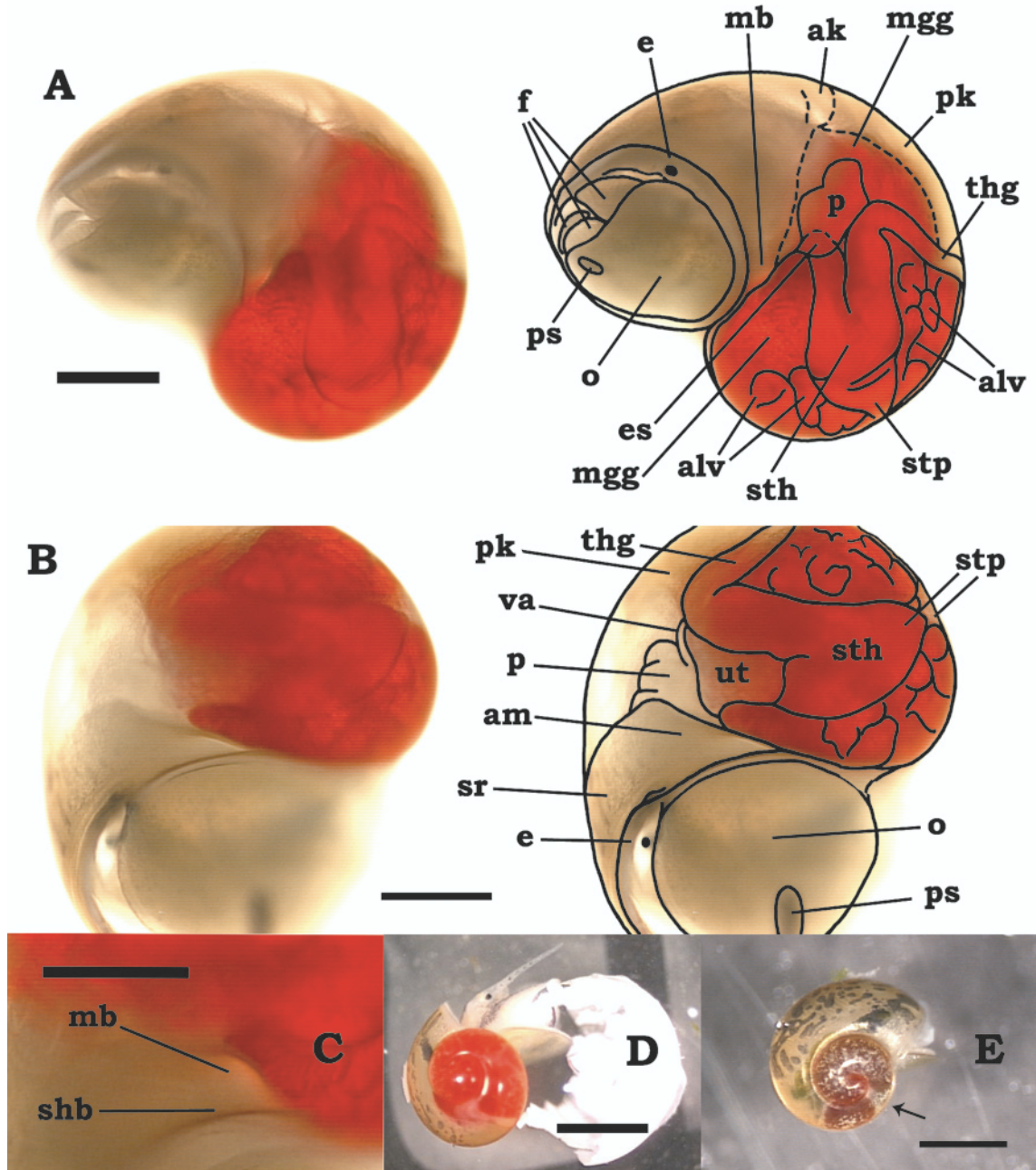
FIGURE 8. A. Embryo in which both the pericardium and the stomach are close to their final position. The midgut cristae and alveoli start to differentiate on the gland surface, close to the stomach's tongue-like projection (collected on day 8). Scale bar represents 500 µm. **B.** Embryo in which both the pericardium and the stomach are at their final position. Almost all the surface of the midgut gland is covered by cristae and/or alveoli. The urate tissue has appeared over the esophagus and the nearby stomach (embryo collected on day 9). Scale bar represents 500 µm. **C.** Embryo similar to that of panel B., showing the midline bud included within the shell and close to the developing columella (collected on day 9). Scale bar represents 500 µm. **D.** The hatchling is going out of the egg capsule and extending its tentacles in the air. Some dark spots are seen on the head and tentacles and on the mantle surface which is seen through the transparent shell. Scale bar represents 1000 µm. **E.** A day 1 juvenile, showing the whitish dots and threads of urate tissue on the surface of the midgut gland, and the clear zone of the stomach's extension traversing the second whorl (arrow). Numerous melanic spots are seen on the mantle surface. Scale bar represents 1000 µm. Structures in this figure are indicated as: *ak*, anterior kidney; *alv*, cristae and alveoli of the midgut gland; *am*, adductor muscle; *e*, eye; *es*, esophagus; *f*, foot; *mb*, midline bud; *mgg*, midgut gland; *o*, operculum; *p*, pericardial cavity; *pk*, posterior kidney; *ps*, melanic posteropedal streak; *shb*, shell border; *sr*, supramarginal ridge; *sth*, stomach; *stp*, stomach projection; *thg*, thin gut; *ut*, urate tissue; *va*, visceral artery.

appearance was essentially similar outside the cell and within the endocytic vesicles.

As we have already mentioned, development is asynchronous in embryos of the same age. So, in some other cases of similar age, two new epithelial cell types were differentiating in the midgut gland wall, while the relative number of giant cells was diminishing. These cells appeared either intermingled with giant cells or forming patches, cristae and alveoli (Fig. 9A) all of them attached to the external wall of the midgut gland. Eventually, giant cells disappear in more developed individuals and the whole wall of the midgut gland becomes
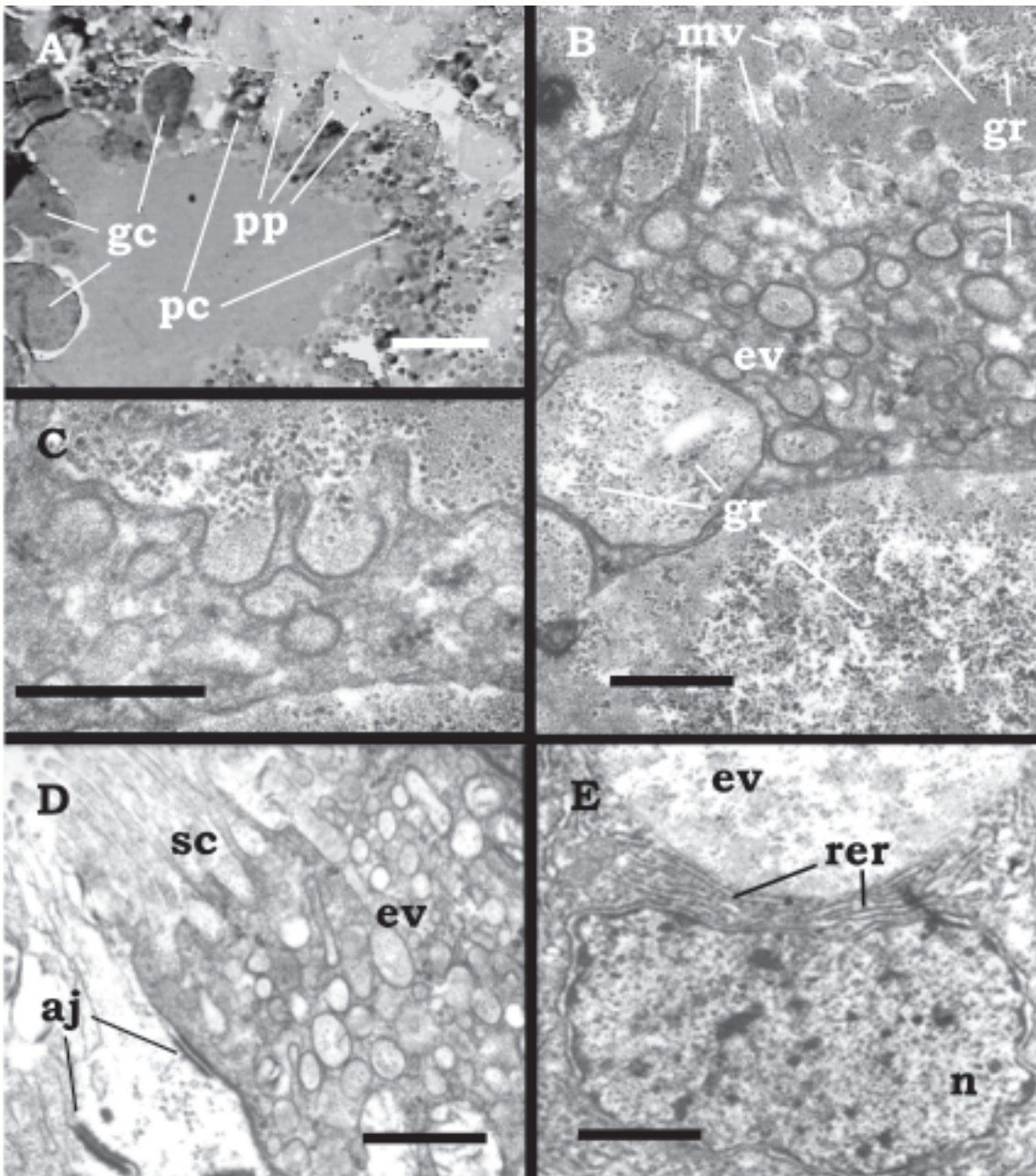

FIGURE 9. The midgut gland of pre-hatching juveniles. **A.** Midgut gland alveolus showing regressing giant cells (*gc*), pre-pyramidal cells with a clear cytoplasm, a large nucleus and 2-3 nucleoli (*pp*), and pre-columnar cells with the cytoplasm loaded of albumen vesicles (*pc*). Toluidine blue stain. Scale bar represents 100 μm. **B.** The apical region of a giant cell showing microvilli (*mv*) and large endocytic vesicles (*ev*) which merge into even larger ones; a material containing 20 nm granules (*gr*) is seen both outside the cells and within the vesicles. Scale bar represents 0.2 μm. **C.** Higher magnification of a similar region; the 20 nm granules are being included into large endocytic vesicles. Scale bar represents 0.2 μm. **D.** Apical region of a pre-columnar cell showing stereocilia (*sc*), small endocytic vesicles (*ev*) and adherent junctions (*aj*). Scale bar represents 2 μm. **E.** Nucleus (*n*) of a pre-columnar cell, located close to a large endocytic vesicle (*ev*) containing irregular granules, which are smaller than the typical 20 nm ones, which probably indicate digestion of the ingested albumen. The nucleus is surrounded by rough endoplasmic reticulum (*rer*). Scale bar represents 3 μm.

composed by the two new epithelial cell types (see below). No giant cells were found on day 1 after hatching, when the midgut gland appeared as a large sac lined by a continuous layer of both new epithelial cell types; this epithelial layer many times ply into cristae and alveoli. No indication of the excretory ducts found in the adult midgut gland had yet appeared. Presumably, the excretory ducts are to be formed as outgrowths of the stomach, which at the time appeared as a sac lined by tall ciliated cells.

One of the new epithelial cell types in the midgut gland was columnar in shape, had long stereocilia in their apex (Fig. 9D), and it also showed endocytosis of small albumen vesicles, that later joined in larger ones filled with partly digested albumen (Fig. 9E) which occupied most of the cytoplasm. Nuclei were unconspicuous and usually located at the cell base. Since these cells appeared similar to the columnar cells of the adult *P. canaliculata* (Koch *et al*., 2006) we will identify them as pre-columnar cells. These cells differ from the adult columnar cells by their lack of pigmented C corpuscles contained within them (as those described by Koch *et al*., 2006) and also, by the different content of their endocytic vesicles, a fact probably related to the different diet of adult animals. Pleomorphic membrane-bound inclusions are seen throughout their cytoplasm, and at least most of them should be interpreted as profiles of intracellular digestion.
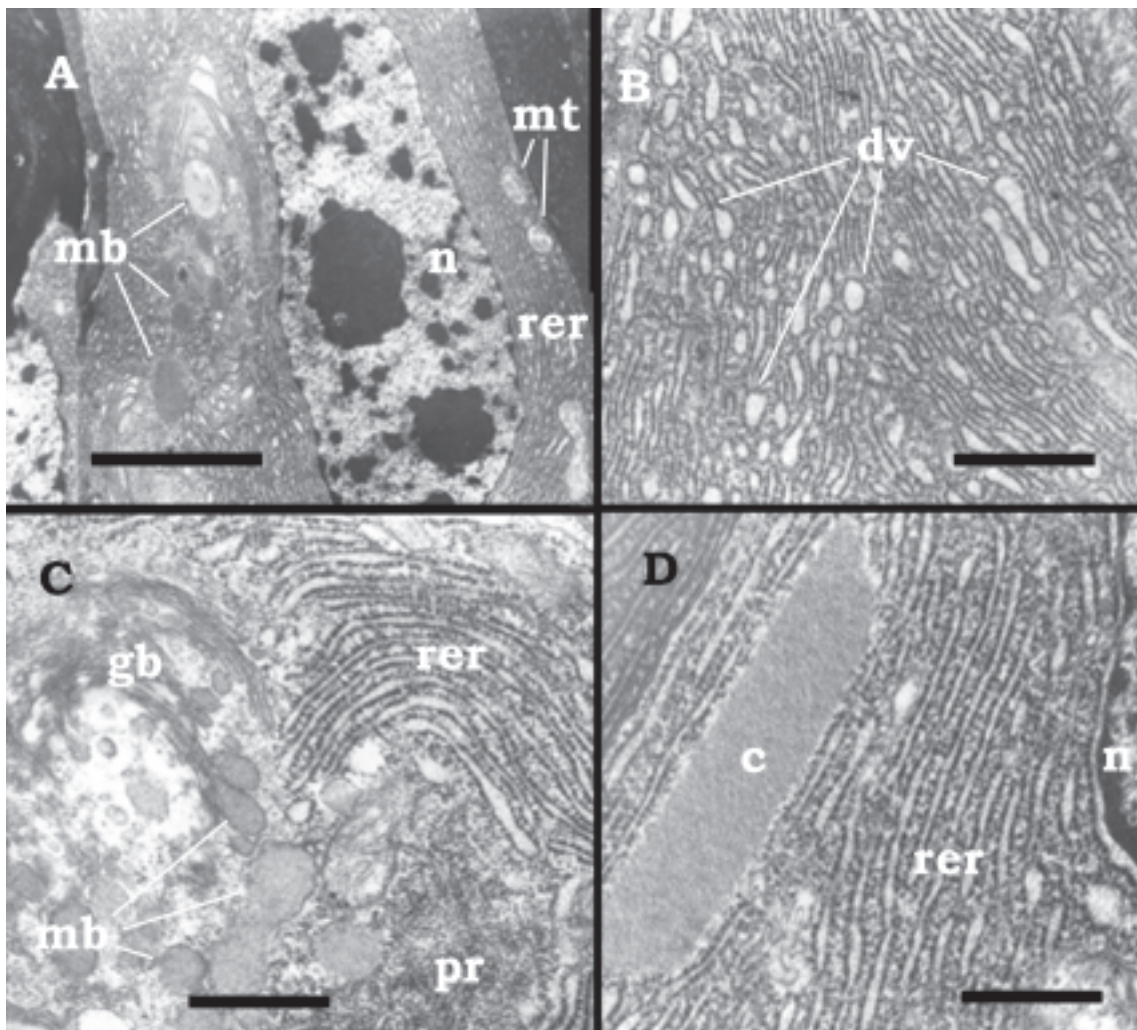

FIGURE 10. The midgut gland of pre-hatching juveniles. **A.** A pre-pyramidal cell showing a large nucleus (*n*) with heavy heterochromatic clumps and three nucleoli. The cytoplasm is full of rough endoplasmic reticulum (*rer*), and also shows some mitochondria (*mt*) and membrane bound bodies (*mb*) of different electron density. Scale bar represents 1 μm. **B.** Cytoplasm of a pre-pyramidal cell showing dilated vesicles (*dv*) of the rough endoplasmic reticulum. Scale bar represents 0.5 μm. **C.** Cytoplasm of a pre-pyramidal cell showing a Golgi body (*gb*), membrane bound bodies (*mb*) being formed out of it, profiles of the rough endoplasmic reticulum (*rer*) and polyribosomes (*pr*). Scale bar represents 0.5 μm. **D.** The nucleus (*n*) of a pre-pyramidal cell is surrounded by rough endoplasmic reticulum (*rer*) containing a crystalloid microgranular arrangement of moderate electron density (*c*). Scale bar represents 0.5 μm.

The other new epithelial cell type was characterized by very large nuclei which were bearing two to three nucleoli (Fig. 10A), and by a remarkable development of the rough endoplasmic reticulum. These cells will be identified as pre-pyramidal cells, since they were similar in all respects to the adult's pyramidal cells, except for their lack of the pigmented K corpuscles typical of adult animals (Koch *et al*., 2006). The rough endoplasmic reticulum showed zones with dilated vesicles (Fig. 10B) and it was also associated to fairly developed Golgi cisternae which were continued by large vesicles formed at the end of the dictyosomes. Those vesicles mostly contained a microgranular material of moderate electron density (Fig. 10C). A similar material also appeared condensed in crystalloid bodies of an apparently protein nature which were contained within the cytoplasm (Fig. 10D). Other pleomorphic and membrane-bound cytoplasmic inclusions are also seen but they are less frequent than in pre-columnar cells.

Finally, Fig. 11 shows the cytoplasm of a urate cell, or urocyte, containing large confluent vesicles corresponding to an early stage III of adult urocytes (Giraud-Billoud *et al*., 2008). Urocytes are large cells where urate crystalloids are being formed and they are the cellular component of the urate tissue which was first noticed as a foamy tissue covering the entrance of the esophagus into the stomach (Fig. 8B). This particular kind of tissue will later extend as numerous whitish dots and threads over the midgut gland (Fig. 8E).
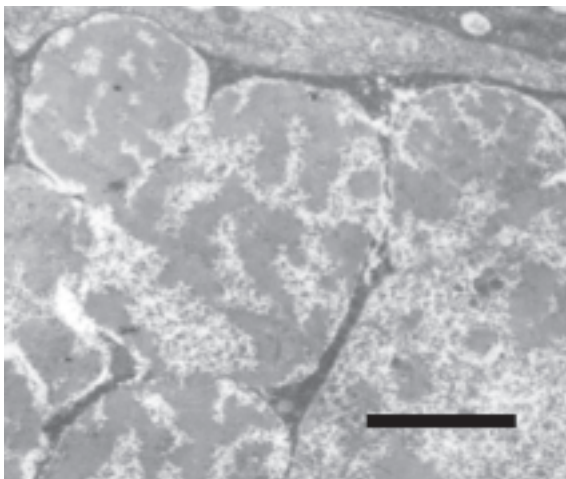

FIGURE 11. Urocyte in the midgut gland of a prehatching juvenile close to a midgut gland alveolus (corresponding to an early stage III of adult urocytes, Giraud-Billoud *et al*., 2008). Scale bar represents 2 μm.

Discussion

P. canaliculata shows a direct form of development, with crawling juveniles going out the individual egg capsules when hatching occurs (Fig. 8D). In general, our results confirm and extend those of Hylton Scott (1934; 1958) in the same species. However, the duration of intracapsular development she reported was about double the one we are reporting here, probably because her material was incubated in uncontrolled room conditions during the fall (Buenos Aires province) while ours was incubated at a higher controlled temperature (23-25ºC), approximating that of spring in Central Argentina. In fact, we have incidentally observed that if incubation is made at higher temperatures (27-28ºC) hatching may occur even earlier, on day 11. So, the duration of intracapsular development in *P. canaliculata* seem to be modulated by temperature in a rather wide range.

This species lays its eggs above water level (Fig. 1A), on either emerging vegetation or on harder surfaces (e.g., stones) where they may be exposed to extremely varying temperatures during the day, as a consequence of direct sunlight, warming of the surface where they have been laid, or cooling during the night hours. They may also be submerged as a consequence of rains or floods, but they show a remarkable ability to survive those harsh conditions (Pizani *et al*., 2005). This may be due, at least in part, to a multifunctional set of three perivitellin proteins contained in their albumen (Heras *et al*., 2007). Of this set, the glycolipoprotein ovorubin is quantitatively the most important, and it gives the eggs their typical bright color. Also, the embryos rely mostly on albumen as the energy source for development (Heras *et al*., 1998), since their yolk load is very restricted. These facts have affected our study in three mundane ways: (1) the oligolecithic oocytes resulted in mostly unpigmented, translucent embryos, which were easy to observe; (2) the ingested pink albumen served as a kind of specific marker to "stain" their alimentary canal; and (3) the thick albumen became both very hard and fragile after fixation, which precluded obtaining any useful microscopic section until hatching was near to occur.

The archenteron of *P. canaliculata* does not show any infoldings as those reported for *Pila globosa* (Ranjah, 1942) and this may be possibly related to the apparent role of giant cells in the midgut of *P. canaliculata*, since they would have assumed the role of endocytosing and concentrating the albumen contained in the midgut (Fig. 9B and C). Both the albumen

in the central lake and within the vesicles of giant cells contain 20 nm granules of moderate electron density, which are interpreted as being identical to the galactogen-containing granules found in the albumen secretory cells of the maternal albumen gland (Catalán *et al*., 2006).

The adult midgut gland in *P. canaliculata* is an organ in which any lobe can hardly be recognized. However the existence of two lobes has been traditionally held, based on several early embryological studies on Viviparidae (Bütchli, 1877; Erlänger, 1891; Drummond, 1903; Otto and Tönniges, 1906), as well as in other caenogastropod forms (Delsman, 1914; Crofts, 1938; Creek, 1951), and both Hylton Scott (1958) and Andrews (1965b) thought of their existence when they observed the two ducts (or two groups of ducts) which communicate the midgut gland with the stomach's vestibule. However, the midgut gland in *P. canaliculata* does not develop as a sprouting outgrowth of the gut, but as the appearance of groups of newly differentiated epithelial cells (the pre-columnar and the pre-pyramidal cells) within the wall of the primitive midgut gland itself, which at the beginning is nothing more than a sac lined by giant cells. The groups of pre-columnar and pre-pyramidal cells will later extend and merge, and will form cristae, and these cristae will soon develop into alveoli (Fig. 8A). These alveoli will extend to the whole of the organ's surface (Fig. 8B), and as giant cells recede and the central albumen lake is absorbed, they occupy the whole gland. The excretory ducts are presumably formed later, as stomach outgrowths.

One may wonder about the origin of the precolumnar and the prepyramidal cells. We are tempted to speculate that these cells derive from those of the tongue-like extension of the stomach (Fig. 8A and B), which develops at the posterior place where both midgut epithelial streaks merge and which is continued by the band of grayish tissue that becomes most conspicuous in early juveniles (day 1 after hatching), when the underlying midgut gland becomes darker (Fig. 8E). In fact, the crests and alveoli of the midgut gland first appear in the vicinity of this tongue-like projection (Fig. 8A). If this interpretation were right, the cells of the embryonic midgut streaks would be somehow committed to give rise to three distinct lineages: that of giant cells first and those of pre-columnar and pre-pyramidal cells later. Also on the same line of thinking, one may speculate that the rather drastic reduction of the anterior midgut lobe in *P. canaliculata* may be related to the disappearance of the corresponding anterior parts of the midgut streaks in this species.

Prepyramidal cells are different from precolumnar cells in many respects, but particularly because of their large nuclei, each one bearing 2-3 nucleoli (Fig. 10A). In fact, the pyramidal cells of the adult *P. canaliculata* are polyploid cells (Cueto *et al*., 2006), an observation which may be correlated with the observation of polyploid metaphases in embryos of another *Pomacea* species (Kawano *et al*., 1990). These cells also (Fig. 9B, C and D), show a conspicuous development of the rough endoplasmic reticulum, which sometimes shows dilated profiles (Fig. 9B). The Golgi apparatus is also fairly developed and large vesicles containing a microgranular material of moderate electron density are formed at the end of the dictyosomes. A similar material appears also condensed in crystalloid bodies of an apparently protein nature. We have no hypothesis for the functional role of this material.

It is clear from this study that no pigmented corpuscles as those of adult snails (Koch *et al*., 2006) are found in cells of the embryonic midgut gland. In fact, this is confirmative of earlier observations in which pigmented corpuscles appeared somewhat later (3-5 days after hatching for C corpuscles, and 1-2 weeks later for K corpuscles (Koch *et al*., 2003). We have paid special attention to any vesicular structure within pre-columnar and pre-pyramidal cells in this study: although there are many, and of a much varying appearance, we have not observed anyone which would permit to ascertain that they were precursors of either C or K corpuscles. However, we should say that the opposite is also true: we cannot ascertain that none of these vesicles are related to either C or K lineages.

An interesting finding that passed unnoticed to earlier authors is the occurrence of the "midline bud" shown in Figs. 5A and B, 6A and B and 8A and C, which is a structure distinct from the mantle edge. In fact it is located below the latter, and remains approximately in the midline until it is finally included within the shell, close to the columella, where the adductor muscle will be inserted.

Finally, we will refer to the development of the genital system, a subject which was not addressed by Demian and Yousif, and was only introduced by Ranjah (1942). In fact, we have referred only briefly to the development of the pericardium, and of the right renal structures (because our focus was digestive organogenesis) but we did not mention at all the development of the left pericardial cavity, the left kidney and the left ureter, which are related to the genital system origin. According to Ranjah (1942), based on the study of microscopic sections, both left and right pericardial cavities merge

into a single one rather early (his Stage 5, about 60 h of development) and the heart develops as a thickening of the dorsal wall after merging. We have also observed such a thickening in *P. canaliculata* (Fig. 3A). However, we could not identify anything like left renal structures in our embryos, and Hylton Scott (1934) did not mention them either in her study of *P. canaliculata*. This is probably so because these structures remain rudimentary and are very small, as observed by Ranjah (1942) in *Pila globosa*, and by Demian and Yousif (1973c) in *Marisa cornuarietis*.

Ranjah (1942) proposed that the gonad arises from a thickening of the pericardium, while the remaining parts of the gonoduct arise from the left kidney and the left ureter; however, he did not follow their development until hatching. We have found (Gamarra-Luques *et al.*, 2006) that hatchlings are sexually undifferentiated and that the single gonoduct has two sections: a pallial one which is hollow, and a visceral one which is a solid cord, and extends beneath the mantle epithelium covering the columellar surface of the visceral hump. This solid cord will originate both the gonad and the visceral gonoduct. A branch of this cord may be seen running through the posterior wall of the mantle cavity and reaching the proximity of the pericardium; however, it does not develop further and disappears within a few days after hatching. A complete account of post-hatching reproductive organogenesis will appear (Gamarra-Luques, Vega and Castro-Vazquez, in preparation).

Acknowledgements

References

Andrews EB (1965a). The functional anatomy of the mantle cavity, kidney and blood system of some pilid gastropods (Prosobranchia). *Journal of Zoology* **146**: 70-94.

Andrews EB (1965b). The functional anatomy of the gut of the prosobranch gastropod *Pomacea canaliculata* and of some other pilids. *Proceedings of the Malacological Society of London* **145**: 19-36.

Bütchli O (1877). Entwicklungsgeschichtliche Beiträge. I. Zur Entwicklungsgeschichte von *Paludina vivipara* Müller. *Zeitschrift für wissenchaftliche Zoologie* **29**: 216-254.

Catalán M, Dreon MS, Heras H, Pollero RJ, Fernández SN, Winik, B (2006). Pallial oviduct of *Pomacea canaliculata* (Gastropoda): ultrastructural studies of the parenchymal cellular types involved in the metabolism of perivitellins. *Cell and Tissue Research* **324**: 523-533.

Creek GA (1951). The reproductive system and embryology of the snail *Pomatias elegans* (Müller). *Proceedings of the Zoological Society of London, B* **121**: 599-640.

Crofts DR (1938). The development of *Haliotis tuberculata*, with special reference to organogenesis during torsion. *Philosophical Transactions of the Royal Society of London* **288**: 219-268.

Cueto JA, Vega IA; Koch E, Castro-Vazquez A (2006). Polyploid cells containing an endocyanobiont in the midgut gland of the applesnail *Pomacea canaliculata*. *Biocell* **31**: 189 (abstract).

Delsman HC (1914). Entwicklungsgeschichte von *Littorina obtusata Tijdschrift der nederlandsche dierkundige Vereeniging* **13**: 170-340.

Demian ES, Yousif F (1973a). Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda, Ampullariidae). I. General outlines of development. *Malacologia* **12**: 123-150.

Demian ES, Yousif F (1973b). Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda, Ampullariidae). II. Development of the alimentary system. *Malacologia* **12**: 151-174.

Demian ES, Yousif F (1973c). Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda, Ampullariidae). III. Development of the circulatory and renal system. *Malacologia* **12**: 175-194.

Demian ES, Yousif F (1973d). Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda, Ampullariidae). IV. Development of the shell gland, mantle and respiratory organs. *Malacologia* **12**: 195-211.

Demian ES, Yousif F (1975). Embryonic development and organogenesis in the snail *Marisa cornuarietis* (Mesogastropoda, Ampullariidae). V. Development of the nervous system. *Malacologia* **15**: 29-42.

Drummond IM (1903). Notes on the development of *Paludina vivipara*, with special reference to the urino-genital organs and theories of gastropod torsion. *The Quarterly Journal of Microscopical Science* **46**: 97-143.

Erlänger R von (1891). Zur Entwicklung von *Paludina vivipara*. II. *Morphologische Jahrblatt* **17**: 636-680.

Gamarra-Luques C, Winik BC, Albrecht EA, Catalan NM, Castro-Vazquez A (2006). An integrative view to structure, function, ontogeny and phylogenetical significance of the male genital system in *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). *Biocell* **30**: 345-357.

Giraud-Billoud M, Koch E, Vega IA, Castro-Vazquez A (2008). Urate cells and tissues in the South American apple snail *Pomacea canaliculata*. *Journal of Molluscan Studies* **74**: 259-266.

Heras H, Dreon MS, Ituarte S, Pollero RJ (2007). Egg carotenoproteins in Neotropical Ampullariidae (Gastropoda: Architaenioglossa). *Comparative Biochemistry and Physiology C* **146**: 158-167.

Heras H, Garín CF, Pollero RJ (1998). Biochemical composition and energy sources during embryo development and in early juveniles of the snail *Pomacea canaliculata* (Mollusca: Gastropoda). *Journal of Experimental Zoology* **280**: 375-383.

Hylton Scott MI (1934). Sobre el desarrollo embrionario de *Ampullaria canaliculata*. *Revista del Museo de La Plata* **34**: 373-385.

Hylton Scott MI (1958). Estudio morfológico y taxonómico de los ampuláridos de la República Argentina. *Revista del Museo Argentino de Ciencias Naturales "Bernardino Rivadavia"* **3**: 233-333.

Kawano T, Gómez Simoes LC, Correa FMA (1990). Chromosomes of *Pomacea* sp. (Perry, 1811) (Mesogastropoda, Mollusca). *Revista Brasileira de Genética* **13**: 675- 685.

Koch E, Vega IA, Albrecht EA, Gamarra-Luques C, Castro-Vazquez A (2003). Evidence for direct mother-offpring transmission of a possible symbiont of *Pomacea canaliculata*. *Biocell* **27**: 70 (abstract).

Koch E, Vega IA, Albrecht EA, Ortega HH, Castro-Vazquez A (2006). A light and electron microcopic study of pigmented corpuscles in the midgut gland and feces of *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). *The Veliger* **48**: 17-25.

Leydig F (1850) Über *Paludina vivipara*. Ein Beitrag zur näheren Kenntniss dieses Thieres in embryologischer, anatomischer und histologischer Beziehung. *Zeitschrift für wissenschaftliche Zoologie* **2**: 125-197.

Otto H, Tönniges C (1906). Untersuchungen über die Entwicklung von *Paludina vivipara*. *Zeitschrift für wissenschaftliche Zoologie* **80**: 411-514.

Pizani NV, Esteben*et al*, Martin PR (2005). Effects of submersion and aerial exposure on clutches and hatchlings of *Pomacea canaliculata* (Gastropoda: Ampullariidae). *American Malacological Bulletin* **20**: 55-63.

Ranjah AR (1942). The embryology of the Indian apple-snail, *Pila globosa* (Swainson) (Mollusca, Gastropoda). *Records of the Indian Museum: A journal of Indian zoology* **40**: 217-322.

Vega IA, Damborenea CN, Gamarra-Luques C, Koch E, Castro-Vazquez A (2006). Facultative and obligate symbiotic association of *Pomacea canaliculata* (Caenogastropoda-Ampullariidae). *Biocell* **30**: 367-375.