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## Mating behavior, egg laying, and embryonic development in the South American apple snail *Asolene pulchella* (Ampullariidae, Caenogastropoda)

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*Asolene pulchella* is a dioecious freshwater snail from the La Plata basin, belonging to the Ampullariidae family. Our aim was to study the reproductive biology, including mating behavior, egg laying, and embryonic development under laboratory conditions. Copulations occurred underwater and lasted 2.66 h on average. The males produce fusiform paraspermatic cells (with seven to nine flagella) in addition to euspermatic cells with a corkscrew head, straight middle piece and long flagellum. Females stored sperm for up to 169 days. The egg masses (containing 98.1 eggs on average) are deposited underwater and are composed of many gelatinous packs of 1–4 eggs; the jelly matrix presented numerous calcite microcrystals. The spheroidal egg capsules measured 2.25 mm and contained a yellowish perivitellus of rubbery consistence. The embryonic development extends for 10.8 days on average. Hatchlings measured 1.4 mm and remained on the egg mass, feeding on the jelly. They resemble miniature adults but only began to feed on senescent plant material in their third week of age and begin aerial lung respiration after 8–10 weeks. The details of the structure of the egg masses and development constitute a rich source of traits for comparative and taxonomic studies in the Ampullariidae.

**Keywords:** copulation; sperm cells; oviposition; egg mass; hatching; cannibalism

### Introduction

*Asolene pulchella* (Anton 1838) is a freshwater snail whose range extends from Bolivia to the northeast of Buenos Aires province, Argentina (Cowie and Thiengo 2003). Most of the available information about *Asolene* concerns *A. platae* (Maton 1811), a species found in the La Plata, Paraná, and Paraguay rivers (Martín 1987). Martín (1980, 1984, 1987, 1988) studied the anatomy and histology of the penial complex and reproductive cycle of *A. platae*. The egg masses of *A. platae* were described by Martín (2002), but the characteristics of embryonic development, copulation and oviposition behavior are totally unknown for the genus. Michelson (1961) stressed the importance of reproductive traits for apple snail taxonomy. Recently, Hayes et al. (2009) noted the importance of studying the ecological and behavioral traits of the ampullariids, because they provide fundamental insights into the evolutionary process of the diversification in this group.

The majority of the ampullariids studied have an aerial oviposition strategy (*Pomacea*, *Pomella*, and *Pila*). Among the species with gelatinous and subaquatic egg masses the ramshorn, *Marisa cornuarietis* (L.) is the best known due to its importance as a model for ecotoxicological tests

(Sawasdee and Heinz 2010) and as biocontrol agent (Pointier and Jourdan 2000; Pointier and David 2004), but information on reproductive and developmental traits is scarce for the rest of the genera in this group, for example, *Asolene*, *Felipponea*, *Lanistes* (and probably *Saulea* and *Afropomus*; Berthold 1991).

Apple snails (especially *Pomacea* spp.) have been recognized globally as successful invaders, as voracious pests of aquatic crops and as promoters of ecosystemic changes in natural wetlands (Cowie et al. 2009; Horgan et al. 2012). *M. cornuarietis* is the only apple snail with aquatic egg masses that has become a widespread invader in freshwater ecosystems, mostly due to its use as a biological control agent of aquatic weeds and *Schistosoma*-bearing planorbids (Pointier and Jourdan 2000; Pointier and David 2004). Several apple snails are used in the aquarium pet trade (including *Asolene* spp., under the name of Zebra apple snails) and so there is a high probability of dispersal to other regions of America and other continents by this mean in the future (Smith 2006). Knowledge of the morphology of egg masses and development characteristics of different species of *Marisa* and *Asolene* would be useful for detecting and controlling incipient invasions of these apple snails. The aim of the present study was to investigate the reproductive biology,

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including the mating behavior, egg laying and embryonic development, of *A. pulchella* under laboratory conditions.

## Materials and methods

### *Origin and maintenance of snails*

The snails used in this study were obtained from Regatas pond (Ciudad Autónoma de Buenos Aires, Argentina) and their descendents hatched in the laboratory. They were maintained in 20-L collective aquaria with CaCO<sub>3</sub>-saturated tap water at 25 ± 2 °C, under a 14:10 h (light/dark) photoperiod and fed with lettuce *ad libitum*.

### *Copulatory and egg laying behavior*

Behavior during copulation and egg laying was observed in trials performed in two types of collective aquaria: 24-L glass aquaria (20 × 30 × 40 cm) with 10–20 snails and in 3-L plastic aquaria (cylindrical in shape, diameter: 32 cm, height: 6 cm) with 14–17 snails. The snails were previously numbered with a paint marker for identification.

Snails were sexed by observation of their reproductive behavior due to the apparent lack of external sexual dimorphism and, when possible, by direct observation of the genital organs. Males were recognized as such when they performed mounting on the right side of the shell of another snail (a putative female) and by observation of the penis sheath during that process in some cases (Burela and Martín 2009). Females were recognized when they were observed laying an egg mass in a collective aquaria or when an egg mass was found in an individual aquaria where a putative female was isolated after being mounted by a male. Observations were also made of isolated couples of previously sexed snails in 2.8-L cylindrical glass aquaria (diameter: 14 cm, height: 18 cm). The copulations and egg-laying events that were observed from the beginning were checked every 15 min to estimate their duration.

Nonsystematic observations were made on the morphology and behavior of sperm under a phase-contrast microscope. Spermatic fluid samples were obtained during observation of a few copulations in which a small amount of whitish fluid was observed to drop from the female pallial cavity, and from one egg mass in which seminal fluid was observed on the surface. The sperm present inside the eggs were also observed (see Results).

### *Egg masses and embryonic development*

Fresh egg masses deposited in collective or individual aquaria were gently detached from the aquarium walls and placed in individual 15-cm-diameter Petri dishes with water from the same aquarium. They were maintained in the rearing room at 25 ± 2 °C under a 14:10 h (light/dark) photoperiod.

Egg masses, eggs, and embryos were observed under stereoscopic and binocular microscopes provided with

digital cameras. The crystalline structure of some refringent elements observed in the egg masses was studied using direct and polarized light microscopy (petrographic Olympus B2-UMA) on fresh egg masses and through X-ray diffraction (diffractometer Rigaku D-Max III-C, with a Cu anode) on finely ground fresh egg masses (less than a day old), previously desiccated with the aid of an electric fan at room temperature.

The number of hatchlings that emerged each day in each mass was recorded in a set of 15 egg masses that were minimally manipulated, until five days after the last hatching, and the number of unhatched eggs was also counted. The hatchlings that emerged from another set of egg masses were isolated one week after hatching, and placed in plastic vials with 200 mL of tap water and a small piece of frozen lettuce to observe their behavior.

Seven newly laid egg masses were used to record the growth of the embryos until hatching. Every day a scaled digital photograph was taken of each egg mass. Ten eggs and 10 embryos were randomly chosen on each photograph and the diameter of eggs or the major axes of the embryos were measured.

## Results

### *Copulatory behavior*

All copulations of *A. pulchella* observed in the aquaria occurred underwater, although on two occasions males that were out of water during the weekly change of water mounted other snails and protruded the penis sheath, during either day or night.

The male mounts the shell of the female on its right side to initiate copulation; then, the penis sheath is protruded and inserted into the female pallial cavity (Figure 1(A)). During copulation the male remains with its head partially withdrawn and its foot firmly attached to the female shell; the female also remains with its head withdrawn, either with its foot adhered to the substratum or almost totally retracted within the shell except for the posterior end. Both partners remain motionless (they do not crawl, feed, and ventilate their lungs), with their cephalic tentacles tightly coiled.

Copulation duration was highly variable, ranging from 20 min to 8.17 h. The duration of copulations observed in collective aquaria ( $n=38$ ) was significantly shorter (Mann–Whitney test,  $p<0.001$ ) than that recorded in individual aquaria ( $n=42$ ). The medians were 75.0 and 187.5 min, respectively, and the grand mean (±SD) was 157.45 (±117.05) min; 22.5% of the copulations lasted for less than 1 h.

Homosexual pairings were common in the collective aquaria: in a set of 43 observed pairings, 30% of the male snails mounted and introduced the penis sheath into the mantle cavity of other males. The mounted or

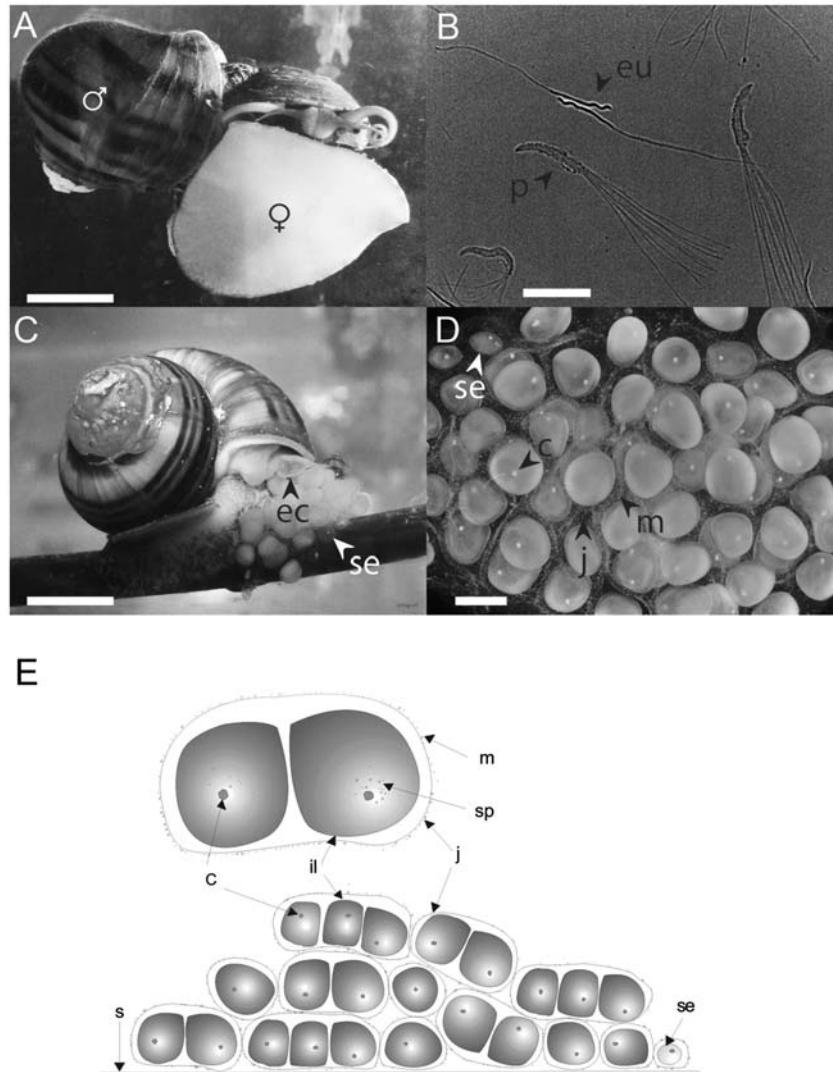


Figure 1. Reproductive behavior, egg masses, and sperm of *A. pulchella*: (A) Couple in copulation, scale bar= 1 cm. (B) Sperm cells, scale bar= 17  $\mu\text{m}$ . (C) Female ovipositing, scale bar= 1 cm. (D) Egg mass, scale bar= 2 mm. (E) Scheme of an egg mass (lateral view). (e) Eusperm, (p) parasperm, (ec) egg channel, (j) jelly matrix, (c) zygote, (il) egg capsule inner layer, (m) microcrystals, (sp) spherules, (s) substratum, (se) sterile egg.

passive snails were either snails already identified as males by their previous masculine mating behavior (probably adults, seven out of 13) or snails that only showed such behavior in subsequent trials (probably juveniles, six out of 13).

Observation of seminal fluid that was accidentally released during copulation or egg laying showed that it includes two types of spermatic cells (Figure 1(B)). The eusperm is uniflagellate, with a helicoidal head ( $4.92 \pm 0.56 \mu\text{m}$ ), a straight, rigid middle piece ( $8.84 \pm 0.59 \mu\text{m}$ ), and a long, mobile tail ( $31.29 \pm 3.98 \mu\text{m}$ ,  $n=11$ ). The other sperm type, the parasperm, has a fusiform, flexible body ( $16.93 \pm 1.37 \mu\text{m}$ ) with seven to nine flagella ( $35.61 \pm 3.07 \mu\text{m}$ ,  $n=10$ ). It was also noticed that, unlike the eusperm, the parasperm has translative movement caused by the propulsion of their flagella.

#### **Egg-laying behavior**

Egg masses were deposited underwater, mostly on the aquarium walls at highly variable depths but never on the bottom of the aquarium. Females frequently used other substrata (thermostat cables or air pump tubing) when available, to deposit egg masses; however, they never used the lettuce leaves provided as food, even though they were frequently attached to them for long periods.

During egg laying, the nuchal right lobe of the female forms an open channel through which gelatinous egg packs (with 1–4 eggs each) emerge (Figure 1(C)); the female's head remains almost totally withdrawn, and only the tightly coiled cephalic tentacles and the buccal mass are visible; it does not crawl, feed or ventilate its lung. At first, the female lays several egg packs without moving its foot and so they begin to accumulate and form a

clump, but later, it usually starts to move backwards or to turn its foot so the egg packs are deposited more loosely, although always in contact with the original clump.

Females never deposited egg masses without at least one previous copulation, although they were able to lay several consecutive egg masses without any intervening copulations. After being reared in collective aquaria with males, 8 out of 10 isolated females were able to lay an average 5.2 egg masses (range: 1–15) with a total production of 266.9 (36–597) embryonated eggs in a period of 138 days (73–169). Moreover, 3 females isolated after a single copulation laid four to nine egg masses during a period of 34–82 days; the total amount of embryonated eggs per female varied between 235 and 670. The total egg production was underestimated in both groups of females, since some of the egg masses were partially or totally eaten by the mother.

Predation of egg masses by snails present in the same collective aquarium was very common; it was necessary to protect them with plastic grid barriers or to isolate them in Petri dishes in order to allow the embryos to develop. Females readily attacked their own egg masses even when *ad libitum* lettuce was present. Egg masses laid by isolated females during the night were found partially or completely eaten early in the morning; in the latter case, only remains of jelly and egg capsules were found.

### **Egg mass structure**

Egg masses were attached to the substratum by a sticky secretion located in the place where the first eggs were laid. These eggs (3–5) were sterile, smaller than the normal ones and more opaque in appearance. The egg masses deposited on the aquarium walls were rounded or slightly oblong (average length  $26.03 \pm 4.58$  mm and width  $17.87 \pm 3.72$  mm,  $n=16$ ), and the surface opposite the substratum was irregularly convex (Figure 1(D) and (E)). The egg masses were composed of several egg packs and contained an average of  $98.10 \pm 28.90$  eggs ( $n=46$ ). The egg packs overlap irregularly and up to three ill-defined layers of eggs have been observed. Eggs from the same pack were frequently located on different planes relative to the substratum (Figure 1(D) and (E)). The egg packs were held together by a transparent jelly that presents very numerous microscopic refringent elements or microcrystals. The microcrystals (Figure 2(A) and (B)) were colorless on thin slides, generally anhedral and sometimes euhedral, with rhombohedral exfoliation, symmetric extinction and birefringence ranging from pearl-gray to white. On the other hand, the X-ray diffraction pattern of whole egg masses showed two distinct peaks at 29.56 and 39.61 (Figure 2(C)). These optic characteristics (Kerr 1965) and the X-ray analyses all indicate that the refringent elements were constituted of calcite.

During the first day after egg laying, eggs from the same pack can be recognized by their close contact and

because the contact surfaces were planar (Figure 2(F)). After egg laying, the jelly of the egg mass and the eggs swell, apparently by hydration and the eggs become spheroidal in shape.

Each egg is limited by an egg capsule with two thin layers, the outermost is thicker and in close contact with the jelly that binds the eggs of each pack together and the innermost contains the perivitellus and the embryo (Figure 2(D) and (F)). The perivitellus is translucent and yellowish, which gives the whole egg mass the same color. The peripheral zone of the perivitellus is finely layered and is of a rubber-like consistency, as can be seen when the egg is cut or punctured or when the embryos feed on it. The zygote is located in the innermost zone of the perivitellus, in an ill-defined cavity, generally eccentric, that contains yellowish viscous and turbid fluid, which becomes transparent several hours after egg laying. A chalazae-like thread composed of hundreds of euspermatic cells can be seen within this cavity. Spherules of  $\text{CaCO}_3$  have also been found in both the fluid and the rubber-like portions of the perivitellus (Figure 2(E)).

### **Embryonic development**

During development, the inner cavity of the perivitellus gradually increases in size and the rubbery portion decreases in thickness, apparently due to the feeding activity of the embryo. In the first stages of development, the embryo moves on the internal surface by ciliary action but later begins to crawl on it and the buccal mass is continuously moving. At the time of hatching, the perivitellus has been totally consumed by the embryo that crawls and grazes continuously on the inner layer of the egg capsule. Eventually, the embryo perforates the inner layer which suddenly collapses (Figure 3); the embryo crawls through both capsule layers with its foot and also through the jelly matrix of the egg pack, which has lost most of its original turgidity. The calcite elements present inside the egg disappeared soon after the start of embryo development, but those on the jelly matrix lasted up to the time of hatching and beyond.

The time elapsed up to hatching of the first egg in each egg mass (mean  $\pm$  SD) was  $10.80 \pm 2.19$  days and ranged from eight to 16 days (Figure 4(A),  $n=35$ ). Hatching within each egg mass was highly asynchronous: the time elapsed between the first and the last hatchings ranged from 4 to 15 days and for 87% of the 15 egg masses studied it was longer than six days. Considering all the eggs in the egg masses ( $n=1142$ ), more than 70% of the eggs hatched within five days of the first hatching of each egg mass (Figure 4(B)). The mean viability (percentage of hatched eggs per egg mass) was  $77.30 \pm 12.00$  ( $n=24$ ).

During development, a slow increase in 37.8% was observed in the diameter of the eggs, from  $2.25 \pm 0.28$  mm (mean  $\pm$  DS) for newly deposited egg masses

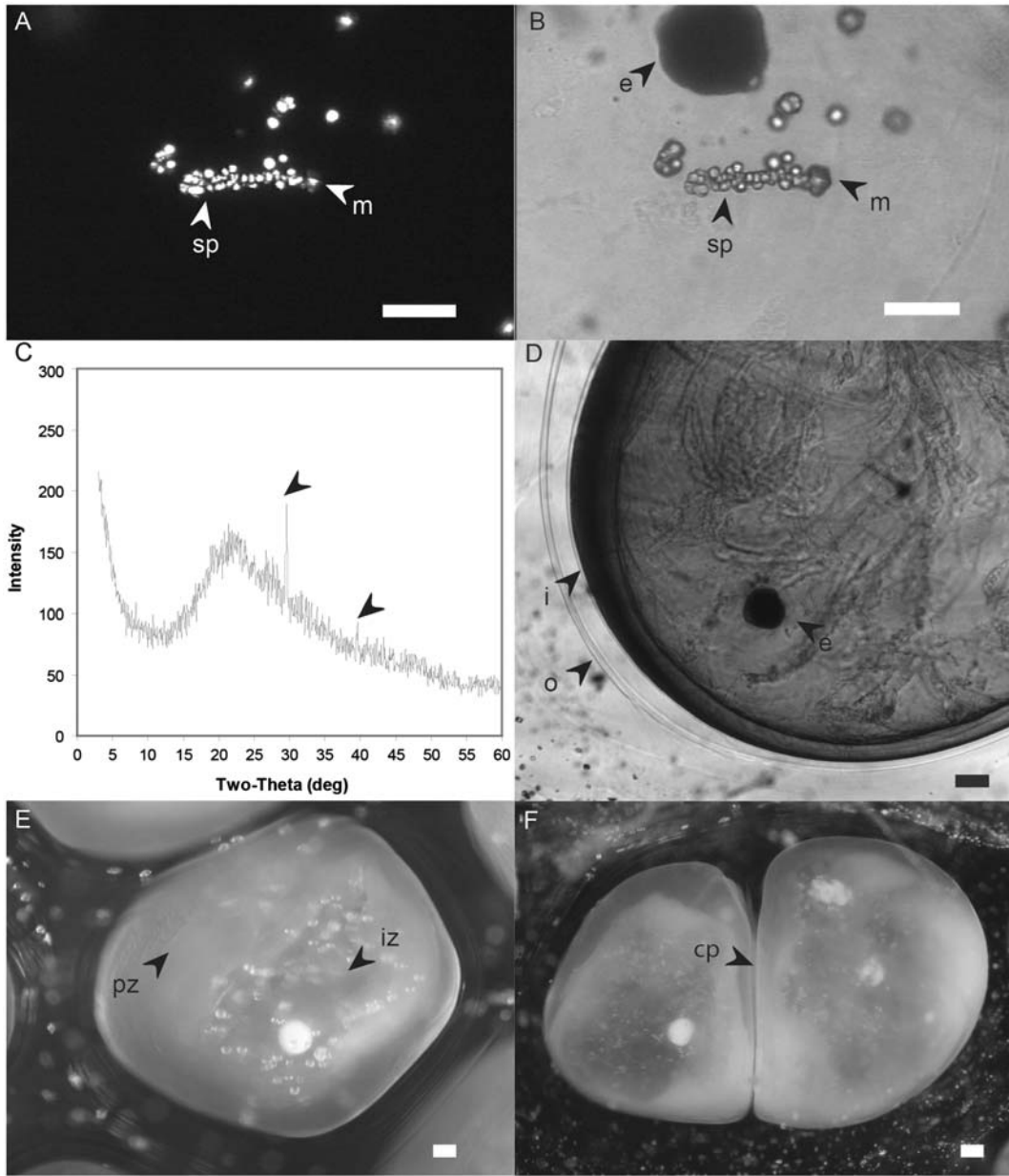


Figure 2. Details of the eggs of *A. pulchella*: (A) Partial view of an egg under polarized light. (B) The same section as in (A) in direct light. (C) X-ray diffractogram of ground whole egg masses; Arrows indicate two peaks corresponding to calcite. (D) Layers of the eggs capsule. (E) Newly deposited eggs. (F) Package with two eggs; (sp) spherules, (m) microcrystals, (o) outer layer, (i) inner layer, (e) embryo, (pz) peripheral zone of perivitellus, (iz) inner zone of perivitellus, (cp) planar contact surface, scale bars = 120  $\mu$ m.

to  $3.16 \pm 0.26$  mm at the beginning of hatching (Figure 5); on the other hand, the mean diameter of the zygote was  $0.16 \pm 0.014$  mm, and at the time of hatching, the length of the embryos was  $1.40 \pm 0.33$  mm.

The recently emerged hatchling (Figure 3(A) and (D)) has the appearance of a miniature snail with tentacles, eyes, ctenidium, osphradium, foot, operculum, and shell, but careful observation of the left side of the mantle cavity showed that they lack a lung. Under laboratory conditions, the jelly of the egg mass is the first food taken

by the hatchlings; they frequently perforate the capsules of other eggs provoking premature hatching of some embryos during emergence from the egg mass or during this initial feeding period. One-week-old hatchlings, isolated from the egg mass in plastic vials, did not start to eat the defrozen lettuce provided until another week had passed. At the third week of age, more than 70% of the hatchlings were feeding on defrozen lettuce, as noted by the presence of dark green fecal pellets in the intestine. 8–10 weeks after hatching an air bubble was observed in

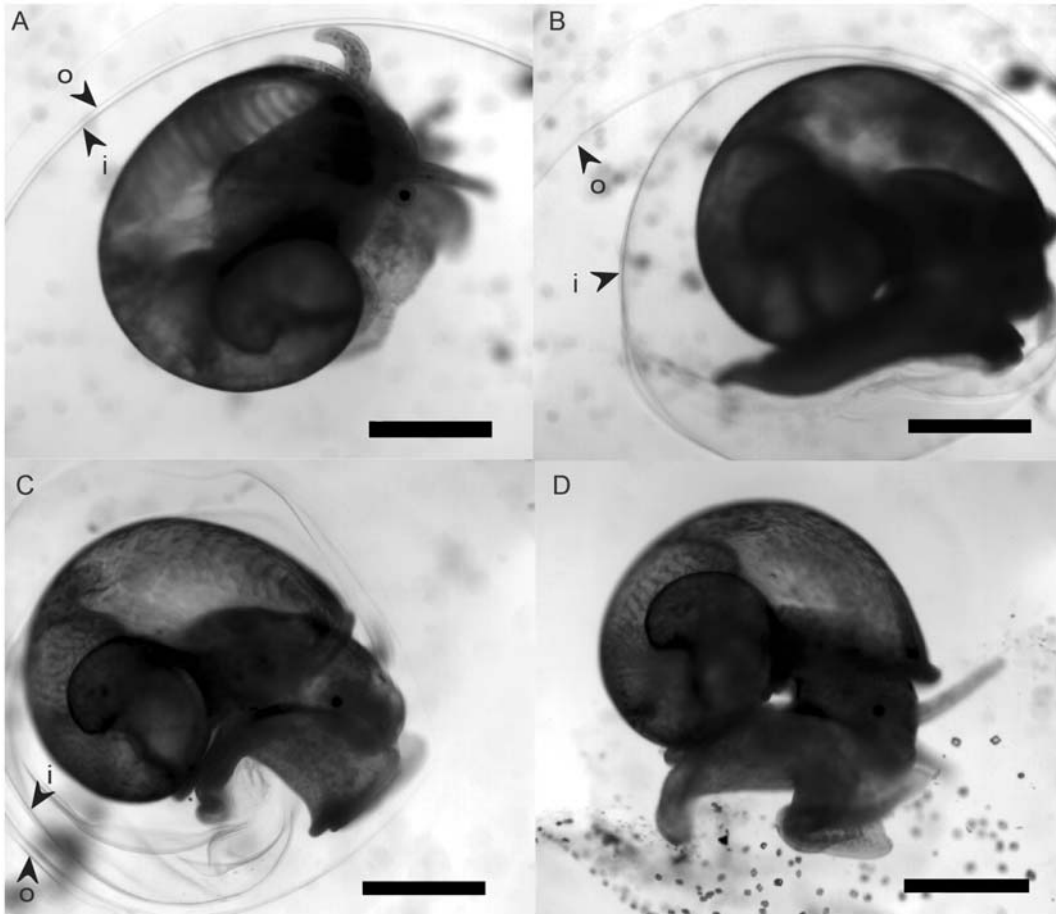


Figure 3. Details of hatching in *A. pulchella* (A) Fully developed embryo before hatching. (B) Embryo at the time of hatching, (C) Embryo escaping through the egg capsule. (D) Hatchling crawling over the jelly matrix, (i) inner layer, (o) outer layer, scale bar=0.5 mm.

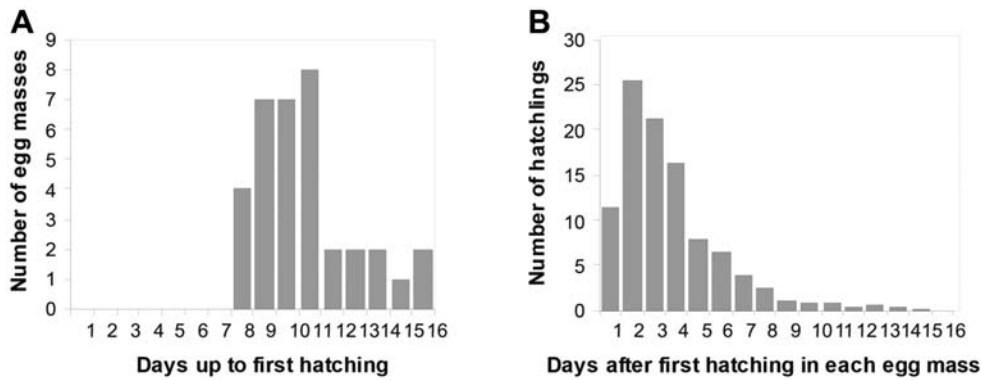


Figure 4. Duration of embryonic development in *A. pulchella*: (A) Time from oviposition until the first egg hatched. (B) Number of hatchlings per day, from the first to the last egg hatched.

the pallial cavity through the still transparent shell, indicating that the lung had become functional.

**Discussion**

Copulations in *A. pulchella* follow the general pattern described for other ampullariids (e.g. Burela and Martín

2011), occurring underwater and with the male firmly attached to the right side of the female shell, both with their cephalic tentacles tightly coiled. However, in *A. pulchella*, the female does not crawl, feed, or ventilate its lung as occurs in other ampullariids (e.g Bahl 1928; Demian and Ibrahim 1971; Albrecht et al. 1996; Burela

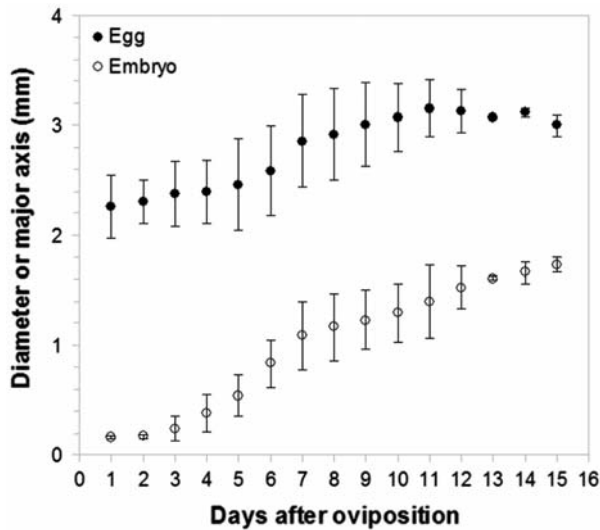


Figure 5. Embryonic development in *A. pulchella*: Diameter or major axis (mean  $\pm$  SD) of egg capsules and embryos of *A. pulchella* during embryonic development.

and Martín 2009). A firm substratum for female foothold is apparently a necessary requisite for copulation in *M. cornuarietis* (Demian and Ibrahim 1971), but in *A. pulchella*, the female often remains on the bottom during copulation with the cephalopodium partially withdrawn.

The mean duration of copulation in *A. pulchella* was less than 3 h (2.66 h) that falls near to the lower end of the range of mean copula duration in the Ampullariidae (38 min to 12.15 h; Burela and Martín 2011). As in other apple snails, copulation duration was highly variable, ranging from 20 min to 8.17 h. The duration was generally shorter in the pairs formed in collective aquaria, in which other males and females were present, perhaps due to the interference of prowler males that were sometimes observed crawling over copulating pairs and that could be competitors. Perhaps, some of the copulations of shorter duration were only failed attempts that ended without genital connection, as occurs in *Pomacea canaliculata* (Lamarck 1822) (Burela and Martín 2011), in which no sperm transference took place in copulations of less than 2 h. The high frequency of homosexual pairings in collective aquaria indicates that males are not able to recognize females until the advanced stages of the copulation process and probably they are not able to recognize receptive females either, which perhaps accounts for some of the very short copulations (failed attempts).

Females of *A. pulchella* are able to store viable sperm for periods of up to 169 days, during which they deposited up to 15 egg masses and 597 embryonated eggs. This capacity is shared with other apple snails and has been considered a key trait in their invasivity (Jerde et al. 2009). After a single copulation, *P. canaliculata* can lay thousands of eggs for a period of up to 140 days

(Estebenet and Martín 2002) and *M. cornuarietis* can lay several egg masses in a lapse of more than a month (Demian and Ibrahim 1971), the latter being comparable with our observations on *A. pulchella*.

The egg-laying process and oviposition site preferences in *A. pulchella* are quite similar to those described for *M. cornuarietis* by Demian and Ibrahim (1971), who suggested that the females require hard submersed substrata near the water surface for oviposition, but usually avoid those that are prone to predation or putrefaction (e. g. lettuce leaves or other palatable macrophytes). Our observations of *A. pulchella* indicate that egg cannibalism is not accidental, since it occurs frequently when the egg masses are deposited on inedible substrata. Intra- and inter-specific predation of apple snail egg masses has been suggested as an important selective pressure for the evolution of aerial oviposition of egg masses in genera like *Pomacea* (Turner 1998; Horn et al. 2008) and *Pila*.

*A. platae* uses the rootlets of submerged plants and floating logs for oviposition (Martín 2002). In the present study, no egg masses were found on the bottom of the aquaria (which probably helps to avoid siltation in natural waterbodies, a process that would impair oxygen interchange with the water and reduce the egg mass viability).

The structure of *A. pulchella* egg masses is also quite similar to that of *M. cornuarietis* described by Demian and Ibrahim (1971) and both differ from the gelatinous egg masses of *Lanistes ovum* (Peters 1845) (Heiler et al. 2007) in that eggs are not organized in a single layer. The distinctive characteristic of *A. pulchella* egg masses is the presence of numerous calcite microcrystals in the jelly matrix that surrounds each egg pack. These microcrystals had not been observed in *A. platae* egg masses collected in the field by Martín (2002). The first egg masses laid by field captured females also presented these crystals, so they were not an artifact due to the rearing conditions ( $\text{CaCO}_3$  saturated water).

The sources of calcium for embryo development are diverse in ampullariids with aerial egg masses, being both extracapsular and intracapsular in origin (Turner and McCabe 1990). In the aquatic egg masses of *M. cornuarietis*, there is direct intake of calcium from the water (Bartelt 1970), but the microcrystals and spherules inside the egg capsule of *A. pulchella* probably represent an extra source for the embryo's shell, since they disappeared rapidly during development. On the other hand, observations that hatchlings remain feeding on the egg mass during the first two weeks indicates that the microcrystals of the jelly matrix could perform the same function after hatching. Some terrestrial gastropods cover their eggs with a coat of fecal pellets to supply the embryos with calcium, representing a form of parental care (Baur 1994).

A noteworthy characteristic of the *A. pulchella* egg is the non-fluid, rubbery consistence of the peripheral zone of the perivitellus that surrounds a fluid inner core



containing the embryo. This condition has not been mentioned for other ampullariids with aquatic egg masses, but it is similar to that described for *Pila globosa* (Swainson 1821) by Bahl (1928) and Ranjah (1942). In this species there is a “sphere of solid albumen” below the calcareous egg shell, enclosing a “core of fluid albumen” that contains the embryo. In *A. pulchella* and *P. globosa*, the embryo develops in the fluid perivitellus and then begins to feed on the nonfluid perivitellus that constitutes most of the embryo reserves. An additional source of nutrients may be the numerous spermatozoa located inside the egg that are present in most genera of Ampullariidae (Ranjah 1942; Demian and Ibrahim 1971; Koch et al. 2009), although the main source of energy donors and nutrients is constituted by the complex perivitellus (Heras et al. 1998; Dreon et al. 2006).

We report here for the first time the presence of parasperm in the genus *Asolene*. Three types of sperm cells have been described in *Pomacea*: eupyrenic or eusperm (fertile, with a corkscrew head, straight middle piece, and long tail or flagellum), apyrenic (without chromatin, fusiform body, and multiflagellate) and oligopyrenic (with incomplete chromatin, pear-shaped body, and a single long flagellum) (Sachwatkin 1920; Andrews 1965; Gamarra-Luques et al. 2006; Winik et al. 2009). The latter has only been observed in *P. canaliculata*, but the morphology of the two former sperm cells corresponds closely to the eusperm and parasperm observed in *A. pulchella* in this study. The difference in size of the eusperm is greater between the two *Pomacea* species (e.g. 15  $\mu\text{m}$  vs. 39  $\mu\text{m}$ ) than between *P. canaliculata* and *A. pulchella* (39  $\mu\text{m}$  vs. 45  $\mu\text{m}$ ). The size of the apyrenic parasperm is similar for the two *Pomacea* species (22–25  $\mu\text{m}$ ) and is less than half the length of that of *A. pulchella* (52  $\mu\text{m}$ ). The apyrenic parasperm of the two *Pomacea* species has three to five flagella while that of *A. pulchella* shows seven to nine flagella. The motility of the *A. pulchella* parasperm is higher than that of the eusperm; Sachwatkin (1920) and Andrews (1964) mentioned differences between the two sperm types that are consistent with our observations. Winik et al. (2001) have speculated about a “carrier” function of the parasperm in *P. canaliculata*, in which these infertile cells could help in the transport of the fertile cells.

A common feature of the aquatic egg masses of apple snails is the notable swelling of the egg capsules during embryonic development (Bartelt 1970; Demian and Ibrahim 1971; Heiler et al. 2008). This is probably related to the marked increase in the concentration of  $\text{Ca}^{2+}$  and  $\text{Na}^{+}$  in the perivitelline fluid that Bartelt (1970) observed in *M. cornuarietis*, since the water permeability of the egg masses increases only slightly during development in that species. The newly laid eggs of *Asolene* and *Marisa* are very much smaller than those of *L. ovum* (2.25 and

2–3 mm vs. 5 mm, respectively), but the increase in volume is greater in the former (four and eight fold vs. 2.74-fold, respectively). The hatchlings of the Neotropical genera are also quite a lot smaller than those of *Lanistes* (1.4 and 1.3 mm vs. 3.0 mm). The embryos that are ready to hatch are very much smaller than the egg capsules in all cases and they crawl on the inner layer. The rupture of the egg capsule does not need any mechanical pressure from the embryo, as seems to occur in the ampullariids with calcareous egg shells (Pizani et al. 2005), although Koch et al. (2009), suggested that in *P. canaliculata* the embryo erodes the egg capsule prior to hatching. In *A. pulchella* hatching apparently occurs as a result of the radular action of embryo combined with the high pressure of the perivitelline fluid.

The minimum duration of development in *A. pulchella* (7 days) was comparable with those of the other two genera of apple snails (7–7.5 days) at similar temperatures (25–27 °C). However, the mean duration in *A. pulchella* was 10.8 days, and some egg masses began to hatch 16 days after oviposition. On the other hand, there was a very high degree of within-egg mass asynchrony in the hatching of *A. pulchella*. This may be due to the spatial arrangement of the eggs within each egg mass; probably, the inner eggs receive less oxygen and this delays development, a common effect in gelatinous egg masses of aquatic gastropods (e.g. Marois and Croll 1991; Cohen and Strathmann 1996). Perhaps, the asynchrony was partly increased by the removal of hatchlings from the egg masses as they hatched; probably their feeding on the egg mass may have increased the oxygenation of the inner parts of the egg mass or provoked premature hatching of the more retarded embryos. Bartelt (1970) stated that water aeration shortens the duration of development from one to 2.5 days in the similarly shaped egg masses of *M. cornuarietis* but commented that development is uniform within an egg mass at a given temperature.

Although the *A. pulchella* hatchlings are apparently fully developed, they are not fully competent during the first weeks of independent life. They only began to feed on senescent plant material in their third week; this contrasts with the behavior of *P. canaliculata* hatchlings that began to graze on fresh lettuce two to three days after hatching (pers. observations) and they are able to feed on it even if they are prematurely freed from the egg shell (Koch et al. 2009). No evidence of a lung was observed at the time of hatching in *A. pulchella*, whereas it appears on the fifth day of development in *M. cornuarietis* (Demian and Yousif 1973). The competence for air breathing in *A. pulchella* (attained at 8–10 weeks of age) seems to be very much delayed as compared with that of *P. canaliculata* hatchlings that began to breathe air within 3 h of hatching (Seuffert and Martín 2009). This evidence indicates that *Asolene* shows a lesser degree of specialization in air breathing than other lineages of

apple snails, as is also indicated by the weak development of the siphon (Michelson 1961).

Among the ampullariids, calcareous egg shells are only present in *Pomacea*, *Pomella*, and *Pila*, which deposit their egg masses above the waterline (Hayes et al. 2009). The habit of ovipositing above the waterline apparently evolved independently in New World (*Pomacea* and *Pomella*) and Old World (*Pila*) ampullariids. However, egg shells of these ampullariids share a unique trait since they are entirely composed of vaterite, an unstable and infrequent polymorph of calcium carbonate (Tompa 1980). The presence of abundant and scattered calcite microcrystals in the egg masses of *A. pulchella* represents a condition not previously observed in the Ampullariidae and opens new questions about the pathways of evolution of aerial egg masses in the Ampullariidae.

The results obtained in the present study indicate that even within the group of apple snails that lay gelatinous egg masses, the details of the egg mass structure and the development process (especially the presence of microcrystals and the late appearance of the lung), constitute a rich source of valuable traits for comparative and taxonomic studies in the future.

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