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# First description of egg masses, oviposition and copulation of a neglected apple snail endemic to the Iguazú and Alto Paraná Rivers

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## ABSTRACT

The Ampullariidae have attracted persistent scientific interest, although this has been mostly concentrated on a few invasive species. *Pomacea americanista* (Von Ihering, 1919) **new combination** is a neglected species as there has been no published information about its anatomy and natural history since its description in 1919. Our aim was to describe its egg masses, oviposition and copulation to compare them with those of other Ampullariidae. We made observations on egg masses in the Iguazú River and tributaries and on snails reared in the laboratory. *Pomacea americanista* exhibited the same reproductive mode as other *Pomacea* species: aerial egg masses composed of hundreds (mean 186.12) of eggs with calcareous shells (mean 3.1 mm in diameter) that are deposited on emergent substrates, mostly rocks. The egg masses were a characteristic pale pink that distinguishes them from those of other species of *Pomacea*. Embryonic development took nearly 17 days at 26 °C. Hatchlings had a smooth shell with 1½ whorls (mean 2.6 mm in diameter) and began to breathe the air and feed within a few hours. Copulation was subaquatic and long lasting (mean 16.13 h) and both sexes were highly promiscuous. The evidence gathered supports the hypothesis that *P. americanista* belongs to the genus *Pomacea*.

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Rio de la Plata Basin

## Introduction

The Ampullariidae, commonly known as apple snails, are freshwater snails of tropical and subtropical wetlands around the globe (Hayes et al. 2015). Even though they have attracted persistent scientific interest, enhanced in recent decades by the invasive character of some species (Horgan et al. 2014), this has concentrated on only a few of them and little is known about most of the species and genera (Hayes et al. 2015). Von Ihering (1919) described *Ampullaria americanista* from the Iguazú Falls (Argentina-Brasil) and Encarnación City (Paraguay). It constitutes a paradigmatic example of a neglected apple snail. Despite its large size and the fact that its type locality is in a highly visited and sampled national park (Parque Nacional Iguazú), no information about its anatomy and natural history has been published since its description. In her revision of the Ampullariidae of Argentina, Hylton Scott (1958) placed this species in the genus *Asolene* and the subgenus *Pomella* together with *Ampullaria megastoma* Sowerby, 1825 because they share a big shell with a reduced spire and an operculum smaller than the expanded aperture. Berthold (1991) reinstated *Pomella* as a full genus, reuniting these two species from the Rio de la Plata basin with *Asolene fairchildi* Clench, 1933 from Surinam (Cowie and Thiengo 2003).

Until recently, it was unknown if the egg masses of these species were aerial and calcareous (as in *Pomacea*

and *Pila*) or gelatinous and subaquatic (as in *Asolene*, *Marisa*, *Felipponea* and *Lanistes*; Hayes et al. 2009). Based on snails and egg masses collected together in the Uruguay River by another naturalist, Von Ihering (1919) suggested that the egg masses of *Pomella megastoma* were of the latter type. However, Hayes et al. (2009) reported that this species deposits masses of eggs with calcareous eggshells above the waterline. On the basis of its genetic and reproductive anatomy similarities and the same type of egg mass, Hayes et al. (2012) placed *Pomella megastoma* in the clade *Pomacea* and synonymised *Pomella* with *Pomacea*, an arrangement maintained in a recent review of the Ampullariidae (Hayes et al. 2015). However, the eggs and egg masses of *Pomella americanista* have remained unknown to date. On the basis of the information obtained in the present study we conclude that *Pomella americanista* belongs to the genus *Pomacea* and propose *Pomacea americanista* (Von Ihering, 1919) as a new combination.

*Pomacea americanista* is endemic to the Alto Paraná and Iguazú Rivers and its known geographical distribution is restricted to an extent of 450 km along these rivers (Hylton Scott 1958). Anecdotal observations, the type locality at the Iguazú Falls and the neritoid shell shape all suggest that *P. americanista* dwells in fast-flowing water on hard

substrates. The southernmost part of this range of *P. americanista* has been impounded by the Yaciretá hydroelectric complex, which has been responsible for the extinction of several species of rapids-dwelling endemic snails (Vogler et al. 2014). The Itaipú hydroelectric dam impounded 200 km of the Alto Paraná River upstream of the confluence with the Iguazú River and submerged the Guayrá Falls, possibly inhabited by *P. americanista*. Other large hydroelectric dams are in operation upstream of the zone inhabited by *P. americanista* and have caused changes in the hydrological cycles of these rivers. The restricted geographic distribution and the habitat requirements suggest that, at odds with other *Pomacea* spp. that are successful invaders (Horgan et al. 2014; Hayes et al. 2015), this species may be vulnerable (Martín et al. 2013).

Our aim was to describe the eggs, egg masses, hatchlings and copulation behaviour of *P. americanista*, to compare them with those of other apple snails from the Neotropical Region and to provide a basis for a better comprehension of the systematic position of this species and its conservation status.

## Materials and methods

### Study area and collection sites

The study area belongs to the southern extreme of the Paranaense Rainforest Ecoregion (Di Bitetti et al. 2003). The climate is humid subtropical with 1731 mm of precipitation and no dry season (Climate-Data.org 2016); the mean annual temperature is 21.2 °C with monthly means between 16 °C (July) and 26 °C (January).

In the summer of 2014 we sampled egg masses of *P. americanista* in two sites located in the Iguazú River basin (Misiones Province, Argentina): Dos Hermanas Falls (DHF, Lower Trail, Parque Nacional Iguazú, 25° 41.02' S, 54°26.73' W) and the San Francisco Stream (SFS, Municipality of Comandante Andresito, 25°36.7' S, 54°2.07' W). San Francisco Stream discharges into the Iguazú River 83 km upstream of the Iguazú Falls.

At each site we took scaled digital photographs of fresh egg masses (i.e., no eggs had hatched) and recorded the substrata on which they were laid. Egg masses were collected at the SFS and taken to the laboratory. We also searched intensively for adult apple snails in the same places where we collected the egg masses. We brought a sample of live snails to the laboratory for observation.

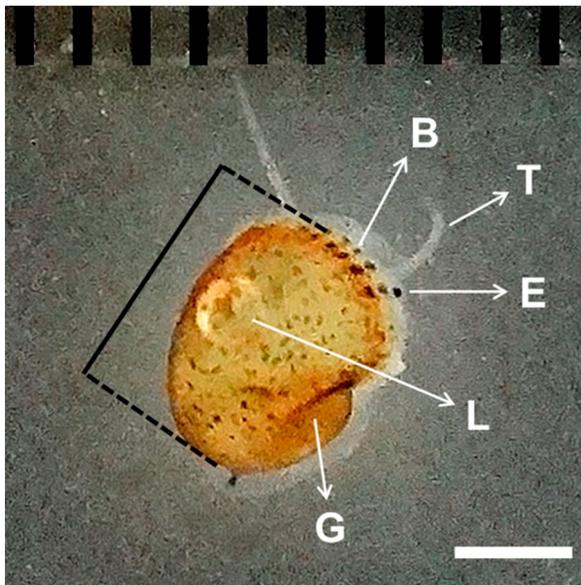
We also took two *P. megastoma* females from the Uruguay River at the port of Colón City (32°12.91' S, 58°8.23' W, Entre Ríos Province, Argentina) in January 2014. These females laid two egg masses in the laboratory, which were incubated at 25 °C until hatching. The size of the eggs and of the hatchlings were recorded as for *P. americanista* (see below).

### Rearing of *P. americanista* in the laboratory

The two egg masses from the SFS were incubated at 26 ± 1 °C and under a 14 h photoperiod (light from 6.00 to 20.00 Argentina Time [ART]) until hatching, and the hatchlings were allowed to fall into a plastic pot filled with tap water to a level of 3 cm. The hatchlings from the two egg masses were transferred to plastic trays 25 × 17 cm filled with water up to 4 cm and reared for 2 weeks with fish food flakes (VitaFish Premium). After this period 25 hatchlings from each field-collected egg mass were randomly selected and transferred to individual plastic aquaria with 200 cm<sup>3</sup> of CaCO<sub>3</sub>-saturated tap water. Thereafter, the hatchlings were fed daily with fresh lettuce *ad libitum*. Water was changed once a week and the aquaria were cleaned of lettuce debris and faeces. At the age of 2.5 months the juvenile snails were transferred to individual glass aquaria with 2300 cm<sup>3</sup> of CaCO<sub>3</sub>-saturated tap water.

The 25 snails from each field egg mass were numbered with paint on the shell and sexed (on the basis of the shape of the operculum and, when possible, the observation of the penis sheath, as in *P. canaliculata*; Burela and Martín 2007, 2009). Shell width was measured with callipers in the same way as for hatchlings (see below). After the snails reached 4 months of age they were given the opportunity to copulate: once a week all 50 snails were put together in a plastic circular arena for 7 hours (diameter 90 cm, water depth 14 cm) and then returned to their individual aquaria.

After the snails began to copulate, the glass aquaria were checked twice a day (at 9.00 and at 18.00 ART) for egg masses on the walls. When possible, observations of the copulation and oviposition processes were made and video-recorded. Two egg masses from each female were selected for further study. When the mucus that holds the eggs together was still fresh, the egg masses were gently detached and reattached on the underside of the plastic lid of a pot filled with 3 cm of water and then incubated at 26 ± 1 °C until hatching. Preliminary observations in these incubation pots showed that if egg masses were not wetted regularly no eggs hatched, even though the embryos were fully developed. Hence, every 2 days the egg masses were lightly sprayed with tap water to enhance hatchability. Every day each egg mass was checked for hatching and dropping of hatchlings into the water; the number of hatchlings, embryonated eggs (non-hatched but with a shelled embryo) and non-developed eggs (non-hatched without a recognisable embryo) of each egg mass was recorded after 1 week without new hatchlings. Unhatched egg masses were followed for 45 days and then the eggs were dissected to search for shelled embryos. We took scaled digital photographs of each egg mass and of 10 1-day-old hatchlings from one egg mass of each female.



**Figure 1.** One-day-old hatchling of *Pomacea americanista* showing how the shell width was measured (bracket). B—spiral band; E—eye; G—mid-gut gland; L—air bubble in the lung; T—cephalic tentacle. Scale bar = 2 mm.

Eggs and 9-day-old hatchlings obtained in the laboratory were fixed in 96% ethanol, cleaned by sonication and conditioned in preparation for the electron microscope (SEM LEO, 40 EVO-XVP).

#### Measurements and statistical analyses

The diameters of 10 eggs and the length and width of each egg mass, either from the field or from laboratory snails, were measured on scaled digital photographs. The shell width of the hatchlings from egg masses incubated in the laboratory was measured from the border of the aperture to the farthest point of the body whorl (Figure 1). The duration of development was calculated from the date of oviposition to the day when at least 10 hatchlings were recorded. Hatchability was calculated as the number of hatchlings divided by the total number of eggs (hatched, embryonated and non-developed eggs).

The diameters of the eggs from the laboratory were analysed through nested ANOVAs, with the females as the main random factor and their two egg masses as the nested factor. The diameter of the eggs from the

field, shell width of hatchlings, egg mass hatchability, duration of development and number of eggs per egg mass were analysed through one-way ANOVAs, with the egg masses as a random factor. The egg masses from the field and the laboratory were compared in terms of their length and width, and the diameter of their eggs with *t* tests. Homogeneity of variances was checked with Levene's tests. Pearson's correlation coefficient (*r*) was estimated for selected pairs of variables.

#### Results

In the two places searched (SFS and DHF) a single species of apple snail was found that was compatible with the previous descriptions of *P. americanista* and only one type of egg mass was found (Figure 2). The snails in the laboratory laid egg masses that were similar to those found in the field. The hatchlings that were obtained from egg masses collected at SFS became adults that were similar to those found in the field at both places.

Most of the egg masses observed and photographed in the field ( $n = 46$ ) were found on emergent basaltic boulders or on the pillars of bridges (85%) and a few on tree trunks (11%). Two egg masses were found on woody stems less than 1 cm in diameter; in these two cases the egg masses partially enclosed the stem. In 82.6% of the cases the substrate surface on which egg masses were found was bare, with the substrate of the remaining cases being covered by mosses or algae. Some of the egg masses that were photographed apparently became submerged totally since we did not observe them on subsequent days; in one case an egg mass was submerged by an increase in water level from one day to the next (Figure 2A–B). All the egg masses except one were laid on nearly vertical surfaces emerging directly from the water. Twenty-three of 32 egg masses on bridge pillars in stretches with strong currents were located on the downstream side.

The egg masses were usually longer than wider both in the field ( $5.73 \pm 1.51$  cm vs  $2.36 \pm 0.36$  cm; mean  $\pm$  SD) and in the laboratory ( $5.02 \pm 1.19$  cm vs  $1.50 \pm 0.35$  cm). The egg masses from the laboratory were



**Figure 2.** Egg masses of *Pomacea americanista* at the Iguazú Falls. **A**, Egg mass on a basaltic rock at 8.00 a.m. 16 December 2014; **B**, same egg mass at 9.00 a.m. 17 December 2014; **C**, egg mass on a basaltic rock. Scale bar = 10 mm.

significantly narrower than those of the field ( $t_{46} = 8.28$ ,  $P < 0.001$ ). The maximum number of layers of eggs was three in the laboratory and six in the field.

All the copulations observed in the laboratory were subaquatic. In a typical copulation the male approached the female and mounted its shell, crawled on it and positioned itself on the right side of the female's last whorl. The male then introduced its penis sheath inside the right side of the mantle cavity of the female after which both remained almost motionless; sometimes the female crawled on the substrate or detached its foot and retracted it partially. These copulations lasted several hours:  $16.13 \pm 5.48$  hours ( $n = 18$ ), with a recorded maximum of 26 hours. The copulating snails sporadically breached the water surface with their respiratory siphons and performed ventilation movements, repeatedly retracting the cephalopodium. Eighteen copulations of seven virgin females that lasted less than 6.67 h were not followed by oviposition. On average, the females copulated in  $43.05 \pm 23.6\%$  of their opportunities after they reached maturity (first copulation followed by an egg mass); the males tried to copulate in  $56.0 \pm 28.5\%$  of the opportunities after their first attempt to copulate. The maximum number of actual copulations or copulation attempts observed for females and males were 19 and 84, respectively.

Several females were observed laying eggs on the glass walls of the aquaria in the laboratory. Before beginning a typical oviposition event the female crawled above the waterline until its shell was totally out of the water. After a period of several minutes without any evidence of activity, the eggs began to appear one by one through the gonopore on the right side of the female's cephalopodium (Figure 3). The eggs appeared at intervals of 29 to 40 seconds and moved steadily through a groove on the foot until near its end, where they clustered in several layers. Up to seven eggs were seen simultaneously on the groove, sometimes closely in contact. During oviposition the female slid slowly backwards and the egg cluster gradually came in contact with the substratum. Almost all the egg masses (260 out of 263) were found during the morning inspection of the aquaria.

The egg masses were composed of hundreds of eggs with calcareous shells. In the laboratory, the number of eggs per egg mass was highly variable ( $186.12 \pm 111.03$ ,  $n = 42$ ) (Figure 4A), as seemed to be the case in the field. Egg number was highly correlated to the width of the egg mass ( $r = 0.661$ ,  $n = 24$ ,  $P < 0.001$ ) but not to the length ( $r = 0.278$ ,  $n = 24$ ,  $P = 0.188$ ). Individual eggs were almost spherical, although in most cases the area of contact with other eggs was slightly flat. The eggshell surface looked smooth when observed macroscopically but SEM micrographs showed that it was highly porous. In such micrographs

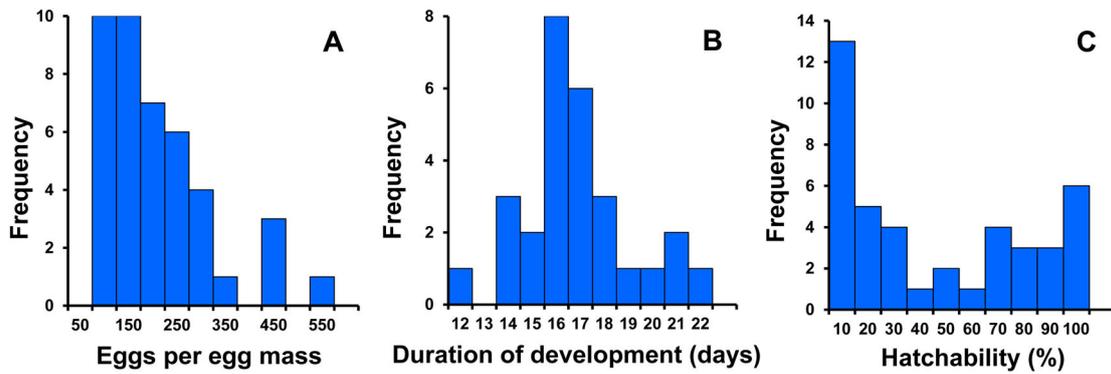


**Figure 3.** Female of *Pomacea americanista* laying eggs in the laboratory. Scale bar = 10 mm.

most eggs were usually smoother and more pointed at one end than at the other (Figure 5).

The grand mean of the egg diameter was  $3.105 \pm 0.404$  mm, although eggs from the field and the laboratory differed significantly ( $3.461 \pm 0.336$  and  $2.912 \pm 0.292$ , respectively;  $t_{678} = 22.203$ ,  $P < 0.001$ ). Laboratory egg diameter exhibited significant variation between egg masses of the same female ( $F_{22,396} = 21.36$ ,  $P < 0.001$ ) and among females ( $F_{21,22} = 2.854$ ,  $P < 0.01$ ). There was no significant relationship between female size at maturity and laboratory egg diameter of the first ( $r = 0.132$ ,  $n = 22$ ,  $P = 0.558$ ) and second egg mass ( $r = 0.082$ ,  $n = 22$ ,  $P = 0.715$ ), although the correlation between the two egg masses was significant ( $r = 0.504$ ,  $n = 22$ ,  $P = 0.017$ ). The diameter of eggs differed significantly among egg masses from the field ( $F_{23,216} = 55.885$ ,  $P < 0.001$ ).

The colour of recently laid egg masses, in which the eggshells had not yet dried, varied from light pink to pink with an orange tinge (Figure 3 and Figure 6A, respectively). When the eggshells became dry the external colour was paler and whitish but the perivitelline fluid remained light pink. The exposed area of each egg frequently showed a series of concentric areas of different tones around the most exposed point of the egg. These concentric areas were observed in both field and laboratory egg masses (Figure 2C and Figure 6B), but they were usually more complex and visible in the field. During the last days of their development, the general colour of the eggs became greyish or brownish (Figure 6B). This was apparently due to the



**Figure 4.** Frequency histograms of variables from egg masses of *Pomacea americanista* in the laboratory. **A**, Number of eggs per egg mass; **B**, duration of embryonic development (days); **C**, hatchability of egg masses (%).

pigmentation of the embryos that became visible from the outside, but also because of a decrease in the remaining perivitelline fluid (see below). Dissections of eggs that did not complete their development showed that the perivitelline fluid still had the initial pale pink colour.

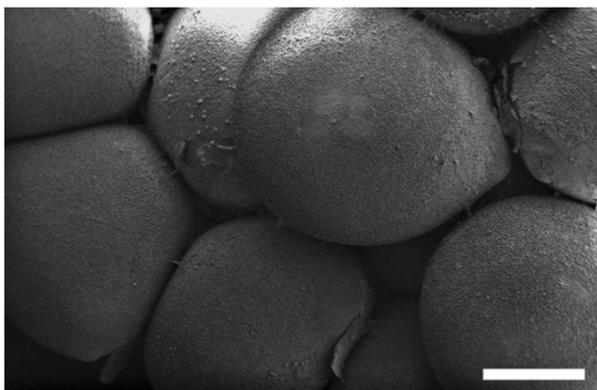
Development at  $26 \pm 1$  °C took  $16.82 \pm 2.28$  days until the hatching of the first 10 eggs, but some egg masses took up to 22 days (Figure 4B). Most of the eggs of an egg mass hatched within 3 days, without a definite pattern of hatching. The duration of development was not significantly related to the number of eggs in the egg mass ( $r = 0.065$ ,  $P = 0.742$ ,  $n = 28$ ) nor to its mean egg diameter ( $r = -0.067$ ,  $P = 0.779$ ,  $n = 20$ ). At the moment of hatching most of the perivitelline fluid has been consumed and the eggshell looked white.

On average the hatchability of the egg masses was  $39 \pm 36\%$  in the laboratory; 33.3% failed completely and a few exhibited hatchability higher than 95% (Figure 4C). The number of eggs with shelled embryos that did not hatch was very low (no more than three per egg mass). The percentage of hatching was positively related to the number of eggs in the egg mass ( $r = 0.511$ ,  $P < 0.001$ ,  $n = 42$ ) (Figure 7). The mean hatchability of the first egg mass from couples from

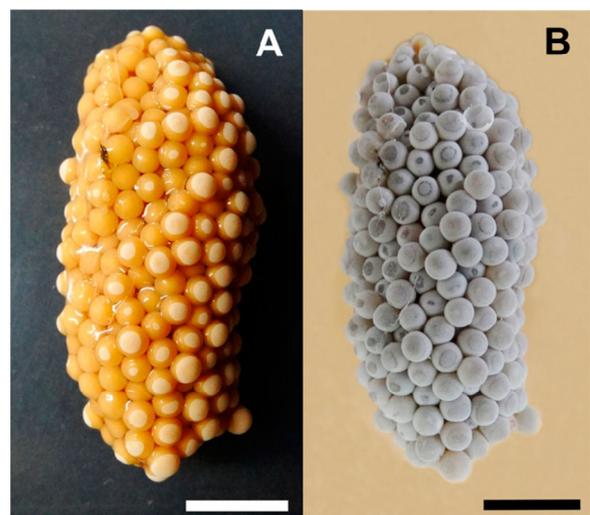
the same egg mass (0.38) and from parents from different egg masses (0.46) were not significantly different ( $t_{23} = -0.568$ ,  $P = 0.266$ ).

The hatchlings exhibited a well-developed shell, foot, operculum, cephalic and labial tentacles and eyes (Figure 1). The pigmentation of the cephalopodium was light grey with a melanic posteropedal streak. The mantle showed a more definite pattern of round to elongated dark spots on an almost colourless background. The pre-hatching shell had no evidence of pigmentation but immediately after hatching six to 10 dark brown spiral bands appeared in the growing edge of the shell of most hatchlings. A few hours after dropping to the water the hatchlings had an air bubble in the lung (Figure 1) and began to feed on lettuce. During the first days after hatching the mid-gut gland showed a definite red-brownish colour resembling that of the perivitelline fluid in the last part of development.

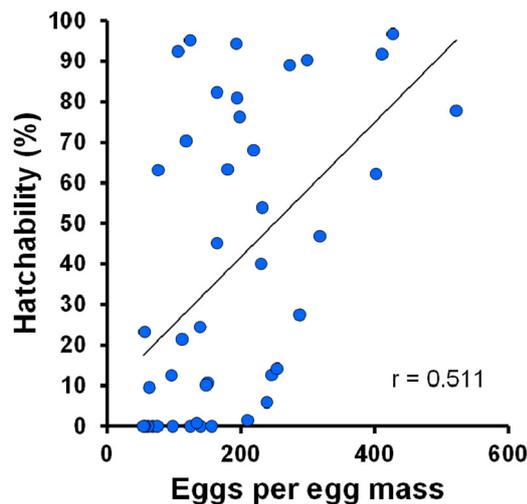
At hatching the shell exhibited  $1\frac{1}{2}$  whorls and the umbilicus was open (Figure 8A–B). The unhatched eggs, which were dissected, contained embryos with



**Figure 5.** SEM micrograph of an egg mass of *Pomacea americanista*. Scale bar = 1 mm.



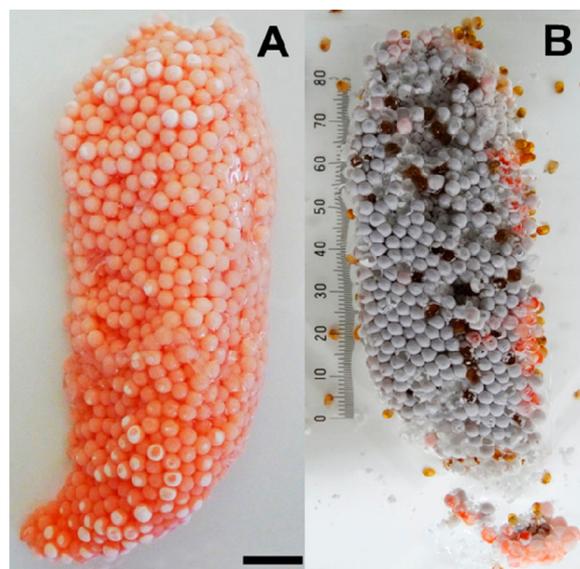
**Figure 6.** Egg mass of *Pomacea americanista* in the laboratory. **A**, Recently laid (less than 12 hours); **B**, same egg mass at hatching. Scale bars = 10 mm.



**Figure 7.** Scatterplot of hatchability (%) vs number of eggs per egg mass of *Pomacea americanista* in the laboratory.

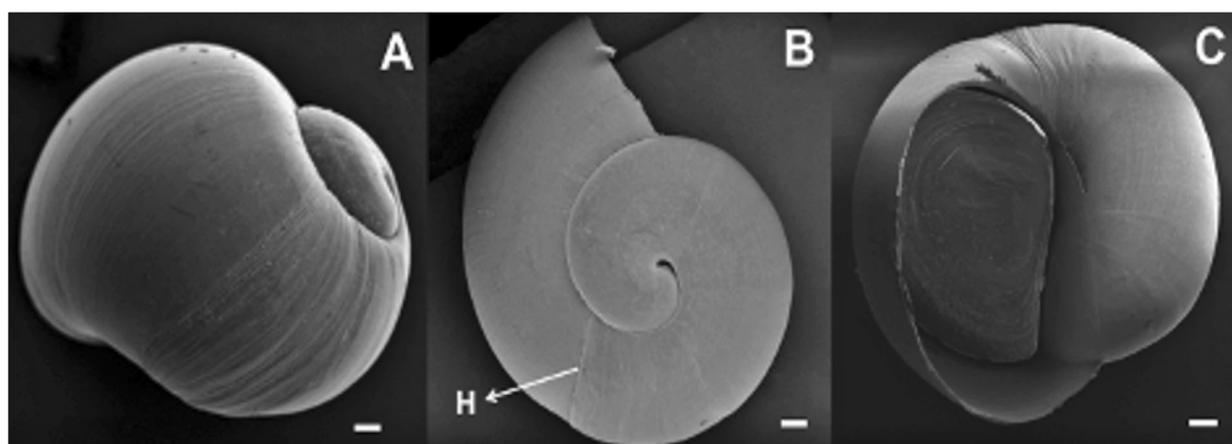
shells of variable size and number of whorls, indicating teleoconch growth before hatching. A hatching mark dividing the pre- and post-hatching teleoconch was evident as a series of microscopic lines (Figure 8A, C). The operculum was concave and its shape and its size were similar to those of the aperture (Figure 8B). The protoconch and the pre-hatching teleoconch were smooth, showed no evidence of periostracal hairs and were undistinguishable. No hairs were found on 9-day-old hatchlings either, but 3 weeks after hatching spiral rows of hairs were visible with the naked eye. The grand mean maximum shell width at hatching was  $2.613 \pm 0.229$  mm; there was significant variation among hatchlings of different laboratory females ( $F_{14,135} = 22.157$ ,  $P < 0.001$ ).

The observations of *P. megastoma* egg masses, although limited, showed some clear differences to those of *P. americanista*. The colour of the whole egg mass at the moment of hatching was more whitish (Figure 9A–B) than that of *P. americanista*; however,



**Figure 9.** Egg mass of *Pomacea megastoma* in the laboratory. **A**, Recently laid (less than 12 hours); **B**, same egg mass at hatching (viewed through the aquarium wall). Scale bar = 10 mm.

the colour of the perivitelline fluid observed in broken eggs was pink, but with a more definite orange tinge than in *P. americanista*. The mean diameter of the eggs of *P. megastoma* was  $3.78 \pm 0.15$  mm ( $n = 20$ ) and the shell width of the hatchlings was  $3.07 \pm 0.19$  mm ( $n = 20$ ). The wetting of the egg masses was not necessary for hatching and, in fact, it was sometimes harmful since it allowed the development of fungal hyphae on the egg mass surface. The hatchlings of *P. megastoma* were very similar in shape to those of *P. americanista*, but the pigmentation of the cephalopodium was more whitish and showed a more conspicuous melanic posteropedal streak. A few hours after hatching most hatchlings had an air bubble in the lung. The colour of the mid-gut gland after hatching was red and lacked the brownish tinge of *P. americanista* hatchlings.



**Figure 8.** SEM micrographs of 9-day-old hatchlings of *Pomacea americanista*. **A**, Dorsal view; **B**, apical view; **C**, apertural view. H—hatching line. Scale bars = 200  $\mu$ m.

## Discussion

Our observations on the eggs and egg masses of *P. americanista* show that its reproductive mode is the same as that described for other *Pomacea* species: aerial egg masses composed of dozens to thousands of eggs with calcareous eggshells that are deposited on exposed emergent substrates. The only exception is *Pomacea urceus* (Müller, 1774), which broods the eggs and hatchlings inside the shell while aestivating buried in mud (Burky 1974). Even though the eggs of *P. americanista* flatten slightly at their contact points with other eggs, they clearly belong to the 'loose and spherical' not the 'tight and polygonal' type of Hayes et al. (2009). The eggs of *P. megastoma* also belong to the 'loose and spherical' type considered as the more derived within the *Pomacea* lineages (Hayes et al. 2009).

Females of *P. americanista* mostly lay their egg masses on vertical hard substrates at sites that receive direct sunlight and rain during the nearly 17 days of embryonic development. Some degree of wetting by rain, dew or spray seems necessary for the eggs to hatch normally. Wetting egg masses of other *Pomacea* spp. has harmful effects on hatchability, probably because of alteration of the eggshell (Turner 1998; Pizani et al. 2005) or fungal growth as in *P. megastoma*. In contrast to these species, *P. americanista* inhabits a very rainy region (more than 150 mm per month during summer), which may explain its tolerance to, or even need of, wetting.

The hatchability of the egg masses showed a strongly bimodal distribution with many egg masses that failed completely and a similar number that showed hatchability that was higher than 70%. Fujio et al. (1991) showed that inbreeding depression could reduce egg mass hatchability in *P. canaliculata* but in the present case this effect can be disregarded since the hatchability of egg masses produced by couples from the same and from different field egg masses was not different. The low hatchability of egg masses was not due to the inability of the embryos to break the eggshell since the eggs were all artificially broken to count the embryos ready to hatch. The relationship between the number of eggs and the hatchability of an egg mass seems to be driven by the existence of an edge effect by which the most exposed eggs are more prone to fail in early stages of development.

Most *P. americanista* egg masses in the field were deposited on clean flat surfaces such as rocks and bridge pillars. In contrast, females of *Pomacea maculata* prefer to oviposit on plant material and on rounded surfaces rather than on flat ones (Kyle et al. 2011). We never observed egg masses belonging to *P. americanista* on herbaceous plants although these were common in the places where we searched.

Probably most herbaceous plants are not strong enough to support the weight of a mature *P. americanista* female (34 to 68 g; Gurovich, pers. obs.), as they are for the smaller and lighter *P. paludosa* (Turner 1996).

Based on our laboratory data on the number of eggs per egg mass and the rate of egg laying, a female would spend 75 minutes on average to deposit an egg mass. Taking into account the bigger size of the egg masses from the field, that time would be 3.6 times higher in the field (i.e., 4.5 hours). This is close to the time estimated for *P. canaliculata* (up to 5 hours; Estebenet and Martín 2002). Most egg masses in the laboratory seemed to have been deposited between sunset and dawn. This nocturnal oviposition behaviour is similar to that observed in other *Pomacea* spp. and may be related to the avoidance of terrestrial or flying predators and of heat stress and desiccation during the extended oviposition events (Estebenet and Martín 2002; Heiler et al. 2008).

The number of eggs per egg mass in *P. americanista* is similar to the number in *P. canaliculata* (Table 1), being closer to the lower extreme of the range reported for non-brooding species of *Pomacea* (from 10 to more than 2000). The mean diameter of eggs of *P. americanista* is 3.1 mm, which is close to that of *P. canaliculata* and *P. scalaris* and bigger than those of *P. maculata*. On the other hand, the mean diameter of *P. megastoma* eggs was 3.8 mm, close to the 4.4 mm of *P. paludosa*, which are the biggest reported for non-brooding *Pomacea*. The shell diameters of hatchlings of *P. americanista* and *P. megastoma* are similar to those of *P. canaliculata* and *P. bridgesii*, and intermediate in size between those of *P. maculata* and *P. paludosa*. The number of eggs per egg mass in non-brooding *Pomacea* is inversely related to egg size, and this in turn is positively related to hatchling size (Hayes et al. 2015). The two species treated in the present study fall within this general trend.

**Table 1.** Number of eggs per egg mass, egg diameter and hatchling size in non-brooding species of the genus *Pomacea*.

<i>Pomacea</i> species	Eggs per egg mass	Egg diameter (mm)	Hatchling size (mm)
<i>P. americanista</i>	186.12 <sup>a</sup>	3.1 <sup>a</sup>	2.6 <sup>a</sup>
<i>P. megastoma</i>	–	3.8 <sup>a</sup>	3.1 <sup>a</sup>
<i>P. canaliculata</i>	159 to 265 <sup>b</sup>	2.24 to 3.47 <sup>c</sup>	2.53 <sup>b</sup> , 2.6 <sup>d</sup>
<i>P. maculata</i>	2064 <sup>e</sup>	1.94 <sup>e</sup>	1.2 (height) <sup>d</sup>
<i>P. paludosa</i>	10 to 80 <sup>f</sup>	4.4 <sup>g</sup>	3.8 <sup>g</sup>
<i>P. bridgesii</i>	50 to 200 <sup>h</sup>	–	2.4 <sup>h</sup>
<i>P. scalaris</i>	9 to 302 <sup>i</sup>	2.69 <sup>i</sup>	1.74 <sup>i</sup>

<sup>a</sup>Present study.

<sup>b</sup>Martín and Estebenet (2002).

<sup>c</sup>Estebenet and Cazzaniga (1993).

<sup>d</sup>Hayes et al. (2012).

<sup>e</sup>Barnes et al. (2008).

<sup>f</sup>Turner (1996).

<sup>g</sup>Hanning (1979).

<sup>h</sup>Coelho et al. (2012).

<sup>i</sup>Wu et al. (2011).

The smaller size of the eggs and egg masses of *P. americanista* in the laboratory was probably related to the fact that we estimated these variables using the first egg masses of each female. Probably the egg diameter and the number of eggs per egg mass increase during the reproductive life of the females since they continue growing after maturity (Gurovich, unpubl. data). The diameter of eggs is also very variable among egg masses, females and populations in *P. canaliculata* (Estebenet and Cazzaniga 1993; Martín and Estebenet 2002).

The eggs and egg masses of *P. americanista* exhibit a characteristic pale pink colour that allows them to be distinguished from the other species of *Pomacea* dwelling in the same area (*P. maculata* and *P. canaliculata*), in which the colour is more reddish and bright. The colour is probably due to the carotenoid astaxanthin, which has been found in other *Pomacea* spp. and that can exhibit variation in colour depending on the conjugate carbohydrates (Heras et al. 2007). The bright pink to reddish colour of the egg masses of some *Pomacea* has been characterised as aposematic and is related to neurotoxic and antinutritive properties of the perivitelline proteins (Dreon et al. 2013). Even though the egg masses of *P. americanista* do not appear as clearly aposematic to the human eye, we did not see any evidence of predation on them.

The eggs of *P. americanista* showed concentric bands of different tones around the most exposed point. The pattern was not present in the recently laid eggs; in the field, where humidity and temperature are more variable, it was usually more complex and visible, indicating that the bands probably arise during the desiccation of the eggs and depend on the speed at which it occurs. These bands have not been described in other species of *Pomacea* but some published photographs show traces of the same effect (e.g., Rawlings et al. 2007; Hayes et al. 2009).

In contrast to those apple snails with subaquatic egg masses (Tiecher et al. 2014), the hatchlings of *P. americanista* attain autonomy rapidly once they fall into water, as they begin to breathe air and to feed within a few hours, as also occurs in other apple snails with aerial egg masses (Seuffert and Martín 2009). Probably, as occurs in *P. canaliculata* (Koch et al. 2009), during the first days after hatching they also make use of the remains of the perivitelline fluid stored intracellularly in the mid-gut gland.

Development duration is under strong influence of temperature for apple snails with both aerial and aquatic egg masses (Seuffert et al. 2012; Tiecher et al. 2015). Development of *P. americanista* takes 16.82 days at 26 °C, 4 days longer than *P. canaliculata* at the same temperature (13 days; Seuffert et al. 2012). For aerial eggs with calcareous shells a longer duration was associated with a bigger egg size (Wu et al. 2011), probably because more time is needed to attain a

bigger hatching size. However, the development duration in *P. megastoma* is a little shorter (c. 16 days; Gurovich, pers. obs.) than in *P. americanista*, although the eggs of the former are bigger.

The shell of *P. americanista* at hatching has  $1\frac{1}{8}$  whorls while those of *P. canaliculata* and *P. maculata* show  $1\frac{3}{4}$  and  $1\frac{1}{4}$  whorls, respectively, as measured on SEM micrographs of Hayes et al. (2012). In all three cases the protoconch and pre-hatching teleoconch are smooth and devoid of periostracal hairs, which appear a few days after hatching in *P. canaliculata* (Estebenet et al. 2006) and after 2 weeks in *P. americanista*.

Copulation of *P. americanista* follows the same pattern as in other *Pomacea* species: it is subaquatic and long lasting, and both males and females are highly promiscuous, copulating repeatedly under laboratory conditions (Burela and Martín 2007, 2009, 2011). The mean duration of copulation (16.13 hours) is near to that recorded for *P. canaliculata* (12.8 hours) and it is very variable as in that species.

Our observations on two of the species from the former genus *Pomella* showed that their eggs and egg masses are not qualitatively different from those of other species of *Pomacea*. The duration of copulation in *P. americanista* is also similar to that in *Pomacea* (reviewed in Burela and Martín 2011). This evidence supports the hypothesis that *P. americanista* belongs to the genus *Pomacea* (Hayes et al. 2012) and not to one of the other Neotropical genera that lay gelatinous egg masses underwater (such as *Felipponea*, *Marisa* and *Asolene*; Hayes et al. 2009) and in which copulations are short (less than 6 hours; Burela and Martín 2011; Tiecher et al. 2014). The name *Pomacea americanista* (Von Ihering, 1919) **new combination** was used previously in two instances (Martín et al. 2013, 2015) but these publications are not recognised as valid publications since they are not registered in the Official Register of Zoological Nomenclature (International Commission on Zoological Nomenclature 2012). Our laboratory observations (Gurovich, unpubl. data) also showed that the siphon in *P. megastoma* and *P. americanista* is far from being reduced or almost absent as has been reported (Hylton Scott 1943, 1958). These two species feature a shell with a reduced spire and an operculum smaller than the expanded aperture, and both seem to prefer microhabitats with hard substrates and strong currents. As yet there is not enough evidence to determine if these shared traits are due to convergence or to a common ancestry.

Even though the hatchability of the egg masses of some *Pomacea* spp. may not be strongly affected by short submergences, the egg masses usually detach or disintegrate if submergence occurs when they are fresh (Turner 1998; Pizani et al. 2005; Horn et al. 2008). Moreover, cannibalistic behaviour occurs when

egg masses become submerged and frequently results in the loss of the whole egg mass (Horn et al. 2008; Saveanu and Martín 2014). Some egg masses are probably lost every year because of natural floods, but anthropogenic interference with hydrological cycles may be increasing the loss of egg masses. During periods of high energy consumption there is a weekly increase in water levels because of the higher discharge from hydroelectric dams located upstream, and therefore egg masses laid during periods of low energy consumption (e.g., non-working days) are at the risk of being submerged (Figure 2B–C). This may represent a further threat to a species that can be considered as vulnerable because of its restricted geographical range and its habitat specialisation (Martín et al. 2013).

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