**Parents living in water, embryos developing in air: respiratory adaptations to use both environments in the freshwater gastropod *Pomacea figulina* (Spix in Wagner, 1827) (Gastropoda, Ampullariidae)**

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ABSTRACT

The ability to use oxygen from both air and water has been considered key to the colonization of terrestrial environments by invertebrates. The freshwater gastropod *Pomacea figulina* (Spix in Wagner, 1827)generally lives submerged, although females lay their eggs mainly above water during night-time, on the stems of aquatic plants, and the embryos develop inside the air-exposed, calcareous egg capsules. However, little is known about the physiological adaptations that the ovipositing females and the developing embryos have developed to breath in air. In this study, we report sexual differences in the aerial and aquatic oxygen consumption of adults and embryonic respiration in aerially developing egg clusters. We found that males and females of this species can obtain oxygen from both water and air, although uptake rates for both sexes were much lower in air than in water. Females in air consumed oxygen 42% faster than males, which may be related with the habit of egg laying above water level. Developing embryos inside of calcareous eggs obtained oxygen faster when submersed underwater than when exposed to air, although embryos near hatching were better able to use oxygen from both air and water than embryos at earlier stages of development. Our data suggest that for *P. figulina*, laying eggs in the terrestrial environment may be more a strategy for protecting embryos from predators than a respiratory adaptation.

Key words: embryos, oxygen consumption, gastropods, apple snail*.*

INTRODUCTION

Water and air exhibit very different physical properties relevant to gas exchange (Simcic *et al*., 2010).Thus, the successful colonization of the terrestrial environment by several groups of aquatic invertebrates (e.g. molluscs and arthropods) (Anger, 1995; Little, 1983; Taylor & Carefoot, 1993) has required a number of physiological as well as morphological adaptations for breathing air (Bliss & Mantel, 1968; Taylor & Carefoot, 1993). For instance, some species can use oxygen equally well from both air and water using a lung (e.g. Ampullariids). Other species exhibit behavioral changes that serve to maintain an adequate water balance (Edney, 1968; Warburg, 1964); however, reproductive adaptations have also been reported. Generally, invertebrate adults and embryos develop in the same environment, though there are cases in which one stage is aquatic while the other is terrestrial (Ehlinger & Tankersley, 2004). In most of these semi-aquatic or semiterrestial species, embryonic development is still normally associated with water (Anger, 1995; Simoni *et al*., 2011). Less commonly, some aquatic species have become adapted to laying eggs under more terrestrial conditions (e.g. amphipods, Wildish, 1979).

The success of early development in terrestrial environments requires not only adjustments to the spawning process by adults, but also the ability of the embryonic stages to tolerate terrestrial conditions and then make a successful transition to an aquatic environment (Simoni *et al*., 2011). Although some marine invertebrates provide maternal care for their embryos by adjusting the surrounding environment to reduce various environmental stresses (Chaparro *et al*., 2009; Fernandez *et al*., 2002), such direct maternal care is absent in non-brooding species, and embryos may instead be protected by egg capsules and other enveloping structures that help to maintain an aqueous environment for embryonic development (Pechenik, 1986). Particularly, enclosed development aids embryonic respiration by facilitating the diffusion of oxygen into the fluid that bathes them. All these cases require the species to have morpho-functional capabilities that facilitate the availability and use of oxygen (Micallef & Bannister, 1967; Simcic *et al*., 2010). Although adults of some species can utilize oxygen from both air and water (Innes *et al*., 1984; Simonik & Henry, 2014; Tagliarolo *et al*., 2013), little is known about the capacity for oxygen uptake in the terrestrial environment by embryonic stages (Simoni *et al*., 2011).

Members of the family Ampullariidae, known as apple snails, are freshwater gastropods widely distributed throughout the humid tropics and subtropics (Hayes *et al*., 2015). Due to human activities, *Pomacea* spp. have become major pests of wetland rice in many countries (Cowie, 2002). This invasive condition has been related, among other traits, to their “amphibious” capacity (Andrews, 1965; García-Ulloa *et al*., 2008; McClary, 1964; Thiengo, 1987), as adult and juveniles apple snails have the capacity to use oxygen from both water and air due to the simultaneous presence of a ctenidium and a ‘lung’. The lung is formed from a dorsal fold of the mantle cavity and its floor bears a slit-like pulmonary aperture that is kept closed by the opposition of its two lips; the roof is thinner than the floor and is formed by a well vascularized epithelium (Andrews, 1965; Thiengo, 1987). Although *Pomacea* females are primarily aquatic, during the reproductive season they lay their calcareous eggs in a cluster (egg mass) that they attach to a variety of solid substrates that extend above the water line, including reeds and other aquatic vegetation in the lentic and lotic environments in which they live (Cowie, 2002; Hayes *et al*., 2015). To accomplish this, females leave the water (Barnes *et al*., 2008) and remain exposed to air, mainly during night-time (Albrecht *et al*., 1996; Halwart, 1994). Ovipositing females may therefore have better functional capabilities to use oxygen from the air than males, since males do not make this transition, they should not be expected to have such capabilities. However, previous reports found no differences in the oxygen uptake between *Pomacea* males and females, e.g. Santos & Mendes (1981) did not identify any differences in *P. lineata* and Seuffert & Martin (2009) found no differences in the use of the lung in *P. canaliculata*, based on the frequency on which the siphon was exposed to air. Although Seuffert & Martin (2009) have suggested the possible existence of gender differences in metabolic rates and in characteristics of the respiratory organs that could influence air breathing behavior for *P. canaliculata*, this possibility has not yet been explored.

Once egg masses are attached to an aerial substrate, *Pomacea* embryos develop between 7 – 28 days depending on aerial temperatures inside a calcium carbonate covering (the eggshell) that is filled with perivitelline fluid, which is ingested during development (Hayes *et al*., 2015). During early development, *Pomacea* embryos are enclosed in their egg cases exposed to aerial conditions; however, eggs could be underwater, in those areas where intense rain period increase the volume of lentic or lotic environments (Pizani *et al*., 2005). While the fluid that bathes them allow embryos to breath dissolved oxygen during development, little is known whether aerial or aquatic oxygen can diffuse through the calcareous cover and be used by developing embryos. Investigations carried out during the embryonic development in *Pomacea* sp. have shown that immersion for prolonged periods (e.g. up to 60 days) results in development delayed, high mortalities and decreased hatching rate (Pizani *et al*., 2005; Taylor *et al*., 1996). However, these studies did not show whether embryos could use the diffused air or aquatic oxygen. To date, no studies have been published on the aerobic capabilities of encapsulated embryos or recently hatched juveniles in *Pomacea*.

In the present study, we set out to determine whether females of the ampullariid snail *Pomacea figulina* (Spix in Wagner, 1987) are better able to use aerial oxygen than males, since females must emerge from water for egg-laying. *Pomacea figulina* is widely distributed in tropical areas. The costs for aerial exposure were determined in terms of water loss and oxygen debt. We also sought to examine the embryonic capabilities of using aerial oxygen for this species. Our results give new insights of metabolic gender differences, and how embryos succeed using diffused oxygen irrespective of their origin.

MATERIALS AND METHODS

*Obtaining biological material*

Adults and egg masses containing embryos of *P. figulina* in various stages of development were collected from the Itupararanga dam (Sorocaba, São Paulo, Brazil; 23˚ 37’ 24’’ S, 47˚ 19’ 55’’ W) associated with the roots of floating plants *Eichhornia crassipes* (Mart) Solms and *Pistia stratiotes* L. Adults were kindly identified for us as *P. figulina* by Dr. S. Thiengo (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil), based on the morphology of the male reproductive system and the egg masses that were deposited (Hayes *et al*., 2015). Adults with shell lengths >25 mm were selected to ensure that they were reproductively active, particularly for females (based on studies conducted in *P. canaliculata*: Gamarra-Luques *et al.,* 2013 and Tamburi & Martín, 2009). In the laboratory, collected adult specimens were marked individually and kept for 1 week at 22°C in a large and well aerated 15 L aquarium filled with fresh water (Klarina®), before beginning measurements of oxygen consumption. The experimental temperature was chosen within the average temperature range observed in the Itupararanga dam (14 – 26 °C; Beghelli *et al*., 2014). During this period, animals were fed *ad libitum* with lettuce leaves (*Lactuca sativa*). Collected egg masses representing all stages of embryonic development were marked (i.e. numbered on plant substrate to which they were) and kept exposed to the air, under the same temperature conditions as the adults. Early developmental stages were identified as: morula-trochophore, early embryos (without a measurable shell) and when shell was present, we identified them according to shell size. At the end of the experiments, embryonic developmental stages were confirmed by direct microscopic examination.

*Oxygen consumption rate (OCR) measurements in adults*

In order to understand the physiological differences between females and males in terms of their aerobic response, aquatic and air oxygen consumption rates (OCRs) were recorded in adults of *P. figulina* (n = 10 females and n = 12 males) using hermetically sealed 135 ml glass flasks (Duran Shott) as experimental chambers in the dark at 22 ± 0.5°C. The dissolved oxygen concentrations (aerial/aquatic) were determined non-invasively using a Fibox 3 oxygen sensor system (Precision Sensing gmbH9, Germany), calibrated with Na2SO3, according to manufacturer’s protocol). For aquatic OCR measurements, experimental chambers were filled with fresh water oxygen saturated by air bubbling, and each adult was carefully introduced into an experimental chamber. Animals were pre-acclimated under the same conditions during one week. The water for OCR measurements was saturated with oxygen by bubbling with air, but aeration was terminated 15 min prior to start of the experiment to help eliminate bubbles before sealing the chambers. During aquatic OCR measurements, dissolved oxygen concentration was measured at the start of each experiment and were measured every 30 min up to 120 min. OCR measurements were stopped when oxygen levels in the respiration chambers reached values of approximately 30% below the initial concentration. Afterwards, OCR in air was measured in the same specimens used for aquatic OCR. Adults were directly deposited into chambers without water and hermetically closed. Oxygen measurements were carried out as indicated previously for adults in water. Submersed snails were active during the record of OCR, they exposed the siphon and moving around the chamber but they were less active when exposed to air. In parallel, with each set of OCR measurements made in water and air, we also monitored changes in oxygen levels in two flasks without animals as controls. The volumes for each adult snail were subtracted from the respiration chamber volume to establish the actual volume of water or air used during OCR measurements. These data were later used to correct measurements obtained from chambers containing animals.

After OCR measurements in water and air, adults were sexed using a stereomicroscope; individuals with a well-developed male reproductive system, with a sheath and pouch of the penis, and associated glands on the right margin of the mantle cavity were considered males, while those with a poorly developed penis sheath and the presence of a pink albumen gland were identified as females (Thiengo *et al*., 2011). Dry tissue weight for each specimen was determined by removing the soft tissue from each shell and placing it in a pre-weighed, individually numbered foil pan. After heating the tissue at 60 ºC for approx. 48 h to ensure removal of tissue water, the pans were cooled and weighed again on a balance to the nearest 0.001 g. OCR was expressed as mg O2 h-1 g-1 of dry tissue weight.

*Adult water loss and oxygen uptake in water*

In order to understand the effects of aerial exposition, adults of *P. figulina* (n = 10) collected from the Itupararanga dam (São Paulo, Brazil) were immediately taken back to the laboratory, where shell size and total wet weight of each specimen was determined. Later, these individuals were deposited in a plastic box (40x20x10 cm) whose top was covered by a net that prevented the access of potential predators. Several holes were drilled into the base of the box, to drain water in the case of rain. The box was then placed outdoors in the shade since the animals were collected associated with the roots of floating aquatic plants and therefore, did not receive direct light. Animals were maintained under these conditions for 24-48 h. Five individuals were removed from the box at random every 24 hours and each animal was weighed, in order to estimate the amount of water lost during exposure to air. The animals were then sealed within respiration chambers (Shott Duran, 135 ml) and the OCR in water was measured, in order to identify potential changes in OCR resulting from prior exposure to air. OCRs were determined using the methodology described above for adults. The difference in wet weight before and after exposure was used to estimate weight loss, allowing us to determine whether water loss could explain any recorded changes in weight-specific OCR. From each animal, the dry tissue weight was then obtained as described previously.

*OCR measurements in egg masses*

Individual egg masses (n = 21) at different stages of embryonic development (Koch *et al*., 2009) were carefully removed from the plant substrate to which they were attached and then introduced into hermetically sealed respiration chambers (135 ml) to measure aerial OCR. The next day, aquatic OCR was determined using the same egg masses. Measurements were made as described in the previous section for air and aquatic OCR in adults. After the OCR measurements, the total wet weight for each egg mass was determine to the nearest 0.001g, and then between 10 and 20 calcareous eggs were carefully separated from the rest of the egg mass, weighed to obtain the average weight per egg. As each egg contain one embryo, the egg weight was used as a proxy to estimate the number of eggs per egg mass. The total number of embryos contained in every egg mass used for OCR quantifications could be estimated.

Using a stereomicroscope at 40X magnification, 15 calcareous eggs from each egg mass were carefully opened, allowing us to identify the development stages of the enclosed embryos. Embryos were categorized as being either in the T (morula - trochophora) or EE (early embryos without a measurable shell) developmental stages (Koch *et al*., 2009). In cases where embryos had visible shells, we measured their longest dimension (Fig. 1) at 50X magnification by using an AxiomCam MRC camera attached to a stereomicroscope. Embryo shell lengths were then determined using AxionVision 4.5 image processor software. Egg mass volumes were estimated by measuring water displacement when added into a known volume of fresh water that was pipetted into a test tube. This information was used to calculate the actual volume of air in the respiration chamber, which was used to estimate OCR per embryo. The OCR would have expressed as per standardized embryo weight, but the real embryo weight was not possible to obtain.

*Data analysis*

Paired *t*-tests were performed to compare aquatic and aerial OCR in females and males, respectively. Student’s unpaired *t*-test were performed to compare aerial OCR between females and male, during immersion and dehydration, and between aerial and aquatic OCR in egg masses. Bartlett’s K-squared test was used beforehand for checking equal variances. The relationship between aerial and aquatic OCR and embryos size were modeled by fitting power analysis. Significant level was set at *P* = 0.05.

RESULTS

*OCR in adult snails of* Pomacea figulina

Adults of *Pomacea figulina* were able to breathe both when under water and when exposed to air (Fig. 2a, b). However, OCR in water was 95% higher than in air for both sex (Paired *t*-test; Female: *t* = 5.33, df = 9, *P* < 0.001; Male: *t* = 4.89, df = 11, *P* < 0.001). Aerial oxygen consumption was significantly different between sex, females consumed more oxygen than males (*t*-test: t = 2.74, df =20, *P* = 0.01).

*OCR in adults after air exposure*

OCR in water did not differ significantly between snails that had been exposed to air for 24-48 hours and then returned to water for measurements and snails kept continuously immersed (Fig. 3; *t*-test; *t* =0.71, df = 27, *P* = 0.48). The average respiration rate was 2.12 ± 0.49 mg O2 h-1 g- 1 for snails previously exposed to 24-48 h of air exposure. In addition, individuals that were desiccated for 24-48 h showed a 16% decrease from 4.63 ± 1.34 to 3.90 ± 1.19 g (mean ± SD) in weight due to water loss.

*Embryonic stage and OCR air rate*

Oxygen consumption by embryos showed the same trend as that was observed in adults: enclosed embryos at all stages of development were able to use oxygen from both air and water (Fig. 4), but they consumed oxygen, on average, 87% faster in water than in air (*t*-test; *t* = -5.75, df = 42, *P* < 0.001). As embryos advanced in development, mean OCR in water increased 50-fold, from 1 x 10-5 mg O2 h-1 embryo-1 for early embryos to 50 x 10-5 mg O2 h -1 embryo-1 for embryos approximately 2 mm in shell length (Fig. 5a, *r*2 = 0.41, *P* < 0.01). Embryos also get more oxygen from the air over time by about 13-fold (Fig. 5b, *r*2 = 0.42, *P* < 0.01), with minimum values of 6 x 10-6 mg O2 h-1 embryo-1 for trochophores to a maximum of 8 x 10-5 mg O2 h-1 embryo-1 for individuals close to 2 mm shell length.

DISCUSSION

The use of both aquatic and terrestrial environments by any species requires the ability to obtain adequate oxygen from both air and water. This ability could be present through all stages of development or could be concentrated at just one or a few stages in the life cycle. In many amphibious species, juveniles and adults tend to be less dependent on the aquatic environment than the earlier stages of development (Anger, 1995). In contrast, the juvenile and adult stages of *Pomacea figulina* and other apple snails are usually found in lentic and lotic water bodies (Cowie, 2002; Hayes *et al*., 2015; Seuffert & Martín, 2012) and they are almost constantly submerged, whereas embryonic development takes place in air. The present research indicates that adults of *P. figulina* —and especially females—can use oxygen from both air and water, although oxygen consumption was more intense when submerged rather than when exposed to air. The desiccation experiment showed no effect on rates of oxygen consumption, even when snails were exposed to air for up to 48 hours and their body weight declined during that time through water loss. A similar situation was observed during early development, while embryos are bathed in the perivitelline fluid, they succeed using aerial or aquatic diffused oxygen through the calcareous cover.

*Sex influence on aerial/aquatic metabolic rates*

In general, our results indicate that *P. figulina* adults make greater use of oxygen from water than from air, despite the substantially higher concentration of oxygen in air than in water. This difference could be related to the less active behavior of *P. figulina* observed in air. While snails were submersed, they were most active during the record of OCR, exposing the siphon and actively moving. Similar results have been reported in the ampullariid gastropod *Marisa cornuarietis* (Freiburg & Hazelwood, 1977) with higher OCR in water than in air; however, in other members of the family, OCRs were similar under air and water, or even higher when they were exposed in air rather than in water (*P. paludosa*, Freiburg & Hazelwood, 1977; *P. maculata,* Mueck *et al*., 2019; *P. lineata*, Santos & Mendes, 1981). The discrepancy may suggest a species-specific response, in which *Pomacea figulina* has less physiological capacity to utilize oxygen from the air than other species in the family Ampulariidae. Individuals of this species have rarely been observed out of the water, and in an aquarium the animals do not leave the water, except when females lay eggs masses (E.P.A. personal observations). In the field, predation by water birds might be one of the reasons why these snails are not seen outside of the water. In the region of the Itupararanga Dam, 50 species of water birds, i.e. potential predators (Smith, 2020), have been registered. Therefore, this high predation pressure could result in *P. figulina* specimens being more physiologically adapted to live constantly under water.

In this study, male and female *P. figulina* showed no significant difference in their ability to utilize oxygen from water, as previously reported for several other *Pomacea* species (e.g. *P. paludosa*, Freiburg & Hazelwood, 1977; *P. lineata*, Santos & Mendes, 1981; *P. canaliculata.* Seuffert & Martín, 2009), even though males remain fully submerged all the time. However, in this study, females of *P. figulina* were significantly more efficient than males at using oxygen when exposed to air. This finding seems reasonable in that males are nearly always submerged, while females occasionally make excursions above the water line.

During the laying period, mothers seem to make direct use of oxygen from air, without generating any measurable oxygen debt (Fig. 3). This was an unexpected finding, since females consumed oxygen at much lower rates in air than in water, an oxygen debt would have been expected. Indeed, Mueck *et al*., (2019) demonstrated in *P. maculata* that an aestivation period (i.e. aerial exposure) of 55 days did not result in any oxygen debt, as similar respiration rates --before and after re-submersion water-- were observed. In *P. figulina* the behavior of floating below the water line has been constantly observed in animals kept in an aquarium, and the animals return to the bottom abruptly (E.P.A, personal observation). In the congeneric species *P. paludosa*, juveniles and adults are indeed able to obtain oxygen from the air even while submerged, using the siphon, especially when individuals are exposed to water with low oxygen saturation (McClary, 1964); this capacity would account for the OCR values that we recorded for females of *P. figulina* during the aerial measurements, something that is supported by the results of our air-exposure water-loss experiment: once the partially desiccated animals were returned to the aquarium, the rates of oxygen consumption in water did not differ significantly from those recorded from animals that had been kept submerged for the entire time. Therefore, future research could address this unexpected finding and identify the mechanism that allows *P. figulina* to be exposed to air without any oxygen debt.

*Aerial/aquatic exposure of egg masses*

Since the enclosed stage of early development takes place above the water level, oxygen used by embryos must come from the air. *Pomacea figulina* embryos are surrounded by perivitelline fluid, which may play an important role in maintaining humidity and in facilitating the diffusion of oxygen from the air for its subsequent use by embryos, in addition to serving as a major source of nutrition (Heras *et al*., 1998). However, while our results clearly indicated that embryos of this species are able to use oxygen from the air throughout its development, that ability is less than their ability to use oxygen from water. The increased capabilities for air oxygen consumption by advanced embryonic stages during the aerial phase of development seems reinforced by studies with the related species *P. canaliculata* (Seuffert & Martín, 2009); these authors found that the lung was already present and apparently well-developed before hatching in that species. The same authors found no air bubbles in pre-hatching snails that had been artificially removed from their calcareous egg shells, which seems to indicate that the lung is not yet functional in the last stage of development within the egg mass, and became functional in juveniles newly released from eggs and recently having entered the aquatic environment.

Although egg masses are likely to be submerged in water only occasionally (e.g., during intense rains) the greater ability to use the oxygen in water was found for all stages of *P. figulina* development, with an evident increase in such capacities as development proceeded within the egg covering. However, our OCR results related to developmental progress should be viewed with caution. OCR measurements were taken with one day apart between air and aquatic conditions, which could cause stress on embryos; furthermore, OCRs were expressed per embryo and not by weight. Increases in the embryonic OCR as development progresses have been largely reported in many aquatic invertebrates (e.g. *Ostrea chilensis*, Chaparro *et al*., 2006; *Crepipatella dilatata*, Segura *et al*., 2010). Indeed, advanced embryos of *P. paludosa* did not suffer any developmental difficulties when they were permanently immersed (Turner, 1998); however, in *P. canaliculata*, development was delayed by 50% under water causing up to a 32% reduction in hatching success (Pizani *et al*., 2005). In our results, rates of oxygen consumption were higher for embryos in the more advanced stages of pre-hatching development, both in the use of oxygen from air and from water. This seems reasonable, as metabolic demands increase with development (e.g. *Armases miersii*, Simoni *et al*., 2011; *Crepipatella dilatata*, Segura *et al*., 2010). However, the well-developed ability of pre-hatching *P. figulina* snails to use oxygen from water while they are still developing in the terrestrial environment could also be adaptive, in considering that once the calcareous egg shell breaks, juveniles go immediately from an availability of oxygen in air to an oxygen demand from the aquatic environment. In addition, it should also be considered that even the eggs are in terrestrial environmental, the embryo’s oxygen exchange occurs, during all its development, with a liquid medium, the perivitelline fluid. In contrast, in juveniles of the ampullariid gastropod *Asolene platae* (referred by Tiecher *et al*., 2014 as *A. pulchella)*, which embryonic development occurs entirely under water (Tiecher *et al*., 2016), the lung develops only after 8-10 weeks of independent life. However, the development occurs entirely underwater (Tiecher *et al*.*,* 2014). The limited ability of some *Pomacea* species to extract oxygen from the air could indicate a condition associated to an adaptative down-regulation of metabolic rate related to a dependence on water, which is vital for the early stages of development.

The egg mass oviposition above the water by female *P. figulina* could be more related to the vulnerability of developing embryos to aquatic predators, including themselves, than to any physiological advantage of developing in air. Apple snails are voracious eaters (Boland *et al*., 2008; Qiu & Kwong, 2009; Wong *et al*., 2010), predating on animal tissue including eggs and juveniles of other snails as well as on macrophytes or detritus (Carter *et al*., 2018; Karraker & Dudgeon, 2014; Kwong *et al*., 2009; Saveanu *et al*., 2017; Wood *et al*., 2006). Indeed, some apple snails, such as *P. maculata* (Horn *et al*., 2008), or *A. platae* (Tiecher *et al*., 2014), even consume egg masses of their own species. As a consequence of such cannibalism, experiments with egg masses of *P. maculata* have shown that egg masses kept underwater were consumed almost entirely by conspecifics, and to an extent that hatching success was reduced to approx. 1% (Horn *et al*., 2008). The predation of egg masses by apple snails is clearly not accidental (Tiecher *et al*,. 2014), supporting the hypothesis that predation of egg masses by ampullariid gastropods and other predators represents a significant evolutionary pressure favoring oviposition in air (Turner, 1998). In contrast, in air, *Pomacea* eggs virtually have no predators due to the presence of a proteinase inhibitor, which limits the predator’s digestive capacity to use the egg nutrients, altering the gastrointestinal function and morphology (Dreon *et al*., 2010; Giglio *et al.,* 2016, Giglio *et al*., 2018). These results may help explain why *P. figulina* has evolved to favor the deposition of their egg masses in air rather than in water.

In summary, we have shown that the apple snail *Pomacea figulina* can use oxygen from both water and air, both as adults and in the pre-hatching stages of development. However, adults of both sexes were better at obtaining oxygen from water than from air, keeping with the fact that juveniles and adults of this species spend most of their life submerged in water. Only females need to leave the water occasionally for oviposition, which represents only a small fraction of their lifespan. On the other hand, oviposition out of water and development of the early phases in the air seems to reflect a strategy for avoiding cannibalism and predation from other species on egg masses, rather than a need to obtain oxygen from air for successful embryonic development; indeed, embryos also obtained oxygen more readily from water than from air

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Figure captions

**Figure 1.** Embryos artificially extracted from eggs, showing how shell length was measured. A) early shelled embryo, B) pre-hatching juvenile.

**Figure 1. Black and white.** Embryos artificially extracted from eggs, showing how shell length was measured. a) early shelled embryo, b) pre-hatching juvenile.

**Figure 2.**Oxygen consumption rate (OCR) (mean ± SD) in aquatic and aerial conditions for A) Females (Paired *t*-test; Female: *t* = 5.33, df = 9, *P* < 0.001, n = 10); B)Males(Paired *t*-test; *t* = 4.89, df = 11, *P* < 0.001, n = 12).

**Figure 3.**OCR (mean ± SD) measured for adult individuals under immersion and following desiccation (*t*-test; *t* = 0.71, df = 27, *P* = 0.48). Individuals in desiccation conditions were previously exposed for 24-48 hours in air, out of contact with water. OCR was then determined immediately after the animals were returned to water. Snails in immersion conditions were continuously kept in water.

**Figure 4.** OCR (mean ± SD) in water and air for the same egg masses, regardless of developmental stage of the enclosed embryos (*t*-test; *t* = -5.75, df = 41, *P* < 0.001, n = 21).

**Figure 5.** Relationship between OCR and embryonic developmental stage and size; A) OCR in water and B) in air. Regression equation was estimated using embryos with a measurable shell. “T” represents trochophore stage and “EE” represents early embryo.