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ARTICLE

The eggs of the apple snail *Pomacea maculata* are defended by indigestible polysaccharides and toxic proteins

M.L. Giglio, S. Ituarte, M.Y. Pasquevich, and H. Heras

Abstract: The freshwater snails *Pomacea* Perry, 1810 lay conspicuous aerial egg clutches that are ignored by most predators. Egg biochemical defenses in the apple snail *Pomacea canaliculata* (Lamarck, 1822) are provided by multifunctional proteins. We analyzed the eggs of a sympatric species, *Pomacea maculata* Perry, 1810, studying the gross composition, toxicity, hemagglutinating activity, and its antinutritive and antidigestive properties. Eggs are mostly composed of polysaccharides (mainly galactogen) and proteins, followed by lipids and nonsoluble calcium. Two perivitellins account for ~85% dry mass of the egg protein. The major lipids are phospholipids and sterols. A suite of potential defenses was determined, including strong lethal neurotoxicity on mice and moderate antidigestive and lectin activities. Remarkably, their polysaccharides were refractive to in vitro digestion by digestive glycosidases. This study characterized ~99% of egg composition and identified multiple potential defenses, provided not only by proteins but also by polysaccharides. This is the first evidence to our knowledge that reserve sugars may be involved in defenses, giving further insight into the unusual reproductive strategy of these well-defended snail eggs.

Key words: animal defense, egg composition, indigestible polysaccharide, protease inhibitors, antinutritive, antidigestive, apple snails, *Pomacea maculata*.

Résumé : Les escargots d'eau douce *Pomacea* Perry, 1810 pondent des œufs à l'air bien en évidence auxquels la plupart des prédateurs ne s'attaquent pas. Les mécanismes de défense biochimiques des œufs chez l'ampullaire brune *Pomacea canaliculata* (Lamarck, 1822) sont assurés par des protéines multifonctionnelles. Nous avons analysé les œufs d'une espèce sympatrique, *Pomacea maculata* Perry, 1810, pour en étudier la composition globale, la toxicité, l'activité hémagglutinante et les propriétés antinutritionnelles et antidigestives. Les œufs sont majoritairement composés de polysaccharides (principalement galactogènes) et de protéines, suivis par des lipides et du calcium non soluble. Deux périvitellines constituent ~85 % en masse sec des protéines des œufs. Les principaux lipides sont des phospholipides et des stérols. Une série de mécanismes de défense potentiels ont été cernés, dont une forte neurotoxicité létale pour les souris et des activités antidigestives et de la lectine modérées. Fait à noter, leurs polysaccharides étaient réfractaires à la digestion in vitro par des glycosidases digestives. L'étude a caractérisé ~99 % de la composition des œufs et cerné plusieurs mécanismes de défense assurés non seulement par des protéines, mais également par des polysaccharides. Il s'agit des premiers indices à notre connaissance de l'intervention possible de sucres en réserve dans les mécanismes de défense, ce qui jette un nouvel éclairage sur la stratégie de reproduction inhabituelle de ces œufs d'escargot bien défendus. [Traduit par la Rédaction]

Mots-clés : mécanisme de défense des animaux, composition des œufs, polysaccharides non digestibles, inhibiteurs de protéase, antinutritionnel, antidigestif, ampullaires brunes, *Pomacea maculata*.

Introduction

Oviparous species usually follow one of two reproductive strategies to mitigate the risk of egg predation: producing such abundant offspring that enough will survive or produce a small offspring with mechanisms to ensure embryo survival (Purcell et al. 1999; Dumont et al. 2002; Winters et al. 2014). The latter involves, among others, parental care, hiding or guarding eggs, or maternal investment to produce eggs with noxious chemicals (secondary metabolites) sometimes laced with conspicuous coloration that is believed to deter predators (i.e., aposematic) (Fuhrman et al. 1969; Heras et al. 2008; Winters et al. 2014). Chemical defenses are usually nonproteinaceous compounds; however, recent studies described neurotoxic proteins and peptides inside eggs of two species, the apple snail *Pomacea canaliculata* (Lamarck, 1822) (with aposematic eggs) and the black widow spider (*Latrodectus tredecimguttatus* (Rossi, 1790)) (Heras et al. 2008; Dreon et al. 2013; Li et al. 2013). Embryo protection by defensive proteins, however, is a strategy much more developed among plants, which provide seeds with an array of proteinase inhibitors, antinutritive factors (i.e., resistant to digestion), and lectins as defense against predation (Chrispeels and Raikhel 1991; Christeller 2005; Chye et al. 2006). On the contrary, in animals, such maternal investment on this varied array of proteinaceous defenses was only reported in *P. canaliculata* eggs, whereas some are also pres-

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ent in the egg foam of the frog *Engystomops pustulosus* (Cope, 1864) (Fleming et al. 2009; Dreon et al. 2010, 2013).

The apple snail *Pomacea maculata* Perry, 1810 are freshwater, amphibious snails native to South America (Rawlings et al. 2007), but because of aquaculture activities, they have spread and soon became an invasive species in Southeastern Asia, North America, and Spain (Cowie 2002; López et al. 2010). Like most species of the genus *Pomacea* Perry, 1810, they lay calcareous and conspicuously colored egg masses on hard surfaces above the waterline (Heras et al. 2007), a strategy that is opposite to the deposition of eggs in gelatinous masses under the water, like most other ampullariids (Hayes et al. 2009). The shift from aquatic to aerial oviposition, which has seldom occurred in animals, has been considered a key feature for the diversification and spread of *Pomacea* species (Hayes et al. 2009). This unusual reproductive strategy exposes the eggs to sunlight, desiccation, high temperatures, and terrestrial predators (Heras et al. 2007, 2008). Notably, these large egg clutches have no reported predators in their native range and only one predator (the fire ant, *Solenopsis geminata* (Fabricius, 1804)) in their invasive range, which is probably related to their bright coloration (aposematic) advertising the presence of noxious components (Snyder and Snyder 1971; Yusa et al. 2000; Stevens 2015). Furthermore, common predators of adult apple snails, such as rats, avoid eating *Pomacea* eggs and the albumen gland, a female gland of the reproductive tract that synthesizes and stores the egg perivitellinic proteins and large amounts of calcium for the calcareous egg shell (Yusa et al. 2000; Dreon et al. 2002; Catalán et al. 2006).

Pomacea egg defenses are provided by the perivitelline fluid (PVF) surrounding the embryos, mostly composed of polysaccharides and glyco-lipo-carotenoprotein complexes called perivitellins (Heras et al. 2007, 2008). In particular, it was found that some perivitellins were not only a source of nutrients for the embryo, but were also involved in the defense system against environmental stressors and predators. Moreover, recently the first apple snail egg proteome was characterized in *P. canaliculata*, revealing several other new perivitellins that could also be involved in embryo defenses (Sun et al. 2012). To our knowledge, no study has examined the general biochemical composition and defense system of *Pomacea* eggs other than those of the sympatric species *P. canaliculata*.

The information of the egg composition and bioactive compounds of *Pomacea* snails is of utmost importance to understand the defense mechanisms in the reproductive strategy of these species and to shed some light on their role in the diversification and spread of apple snails. Thus, the aim of the present work is to study the general composition of *P. maculata* eggs and analyze functional aspects of the main components involved in the embryo defense.

Materials and methods

Clutch and egg characteristics

During the reproductive season, female *Pomacea* snails venture out of the water and lay calcareous egg masses cemented on emergent hard surfaces (Heras et al. 2007), which hatch between 1 and 3 weeks later (Seuffert et al. 2012). In *P. maculata*, egg clutches are very large and comprise from a few hundred to more than 4500 eggs (egg diameter = 1.9 ± 0.03 mm) (Barnes et al. 2008). These clutches are conspicuously pink–red to orange–pink colored when recently laid and become whitish during development (Hayes et al. 2012). Mean hatching efficiency ranges from 33.1% to 70.8% (Barnes et al. 2008; Burks et al. 2010). All these *P. maculata* egg characteristics (large clutches, with large number of eggs and high fecundity rates) are related to their invasiveness (Barnes et al. 2008).

Sample collection

Adult females of *P. maculata* were collected in the Paraná River in San Pedro (33°30′35.97″S, 59°41′52.86″W), Buenos Aires province, Argentina, and kept in the laboratory. Voucher specimens were deposited in the Museo de La Plata Collection (MLP 13749). Eggs were collected within 24 h of being laid and were kept at –20 °C until processed.

Sample preparation

Whole egg homogenate was prepared on ice-cold 20 mmol/L Tris-HCl, pH 7.4, keeping a 3:1 (*v*:*m*) buffer:sample ratio as previously described (Pasquevich et al. 2014). The homogenate was sequentially centrifuged to obtain the egg soluble fraction; henceforth referred to as PVF (Pasquevich et al. 2014). The homogenate was used to quantify macromolecules and for lipid and polysaccharide extraction, whereas the PFV was used in the determination of soluble ions, glucose, protein analyses, and functional assays. Three independent pools of three clutches each (nine clutches in total) were used in every experiment.

Dry mass, ashes, and minerals

To determine dry mass and ash content, pre-weighed egg masses were sequentially heated at 100 °C for 24 h and at 550 °C for 5 h, and the products of each step were weighed. For mineral analysis, the homogenate and PVF were prepared as described above but milli-Q water was used instead of a buffer. Electrolyte concentrations were determined in a Konelab 60I Prime (Wiener lab, Santa Fe, Argentina); soluble Na^+ (100–200 meq/L), Cl^- (50–150 meq/L), and K^+ (2–10 meq/L) were determined with a selective ion analyzer; soluble Mg^{2+} was determined by a colorimetric method (Wiener lab). Ca^{2+} was determined with a Ca-Color kit (Wiener lab) using either the whole homogenate (i.e., with the egg shell) to determine total Ca^{2+} or the PVF to determine soluble Ca^{2+}. Percentage of the ions (*m*/*m*; dry mass) and relative percentage of soluble ions were calculated.

Carbohydrate analysis

PVF soluble glucose was determined by a colorimetric method using glucose oxidase (Wiener lab). Total polysaccharide concentration was calculated gravimetrically from eggs following the method of van Handel (1965). Monosaccharide composition of polysaccharide was determined by gas chromatography (GC–FID) after digestion in methanolic HCl and derivatization with hexamethyldisilazane–trimethylchlorosilane–pyridine (Sigma–Aldrich, St. Louis, Missouri, USA) as previously described (Ituarte et al. 2010). Standard monosaccharides (Sigma–Aldrich) were silylated and analyzed under the same conditions.

Protein analysis

Total protein concentration was determined from the homogenate following the method of Markwell et al. (1978). A standard curve was prepared using bovine serum albumin (Sigma–Aldrich). Absorbance data were collected using an Agilent 8453 UV/Vis diode array spectrophotometer (Agilent Technologies).

PVF proteins were analyzed qualitatively by two-dimensional electrophoresis (2-DE) analysis and quantitatively by a native polyacrylamide gel electrophoresis (PAGE) analysis. Two-dimensional electrophoresis was carried out with an immobilized pH gradient (IPG) – isoelectric focusing (IEF) in the first dimension and sodium dodecyl sulfate (SDS) – PAGE in the second dimension (Görg et al. 1988). The IEF was performed using an Ettan IPGphor III (GE Healthcare) and 7 cm linear pH 3–10 Immobiline dry strips (GE Healthcare) as previously described (Pasquevich et al. 2014). For SDS–PAGE in the second dimension, the IPG strips were sealed on the top of 1.5 mm thick 12% polyacrylamide gels, with molecular mass standards (GE Healthcare) run in parallel. Vertical electrophoresis was carried out at 120 mV. Gels were stained with a colloidal suspension of Coomassie Brillant Blue G (Sigma–Aldrich). Protein molecular mass – isoelectric point (pI) coordinates were estimated using Image Master 2-D Platinum software (GE Healthcare, Life Science). Tentative identity of spots was made by comparison with the molecular mass – pI coordinates previously obtained for the sister species *P. canaliculata* (Sun et al. 2012) and for a purified *P. maculata* protein (Pasquevich et al. 2014).

Native PAGE was performed in 4%–20% gradient polyacrylamide gels in a miniVE Electrophoresis System (GE Healthcare, Life Science). *Pomacea canaliculata* PVF was also analyzed by native PAGE for comparison. High molecular mass standards (Amersham Biosciences) were run in the same gels. Gels were stained with Coomassie Blue G-250 (Echan and Speicher 2002) and protein bands were quantified by calibrated scanning densitometry using the ImageJ software (Schneider et al. 2012).

Lipid analysis

Lipids were extracted following the method of Bligh and Dyer (1959) and total lipid content was determined gravimetrically. In short, egg homogenate was extracted for 1 min with a mixture of methanol–chloroform–water (1:1:0.9, *v*:*v*:*v*). The lipid fraction was transferred to pre-weighed glass vials and evaporated under nitrogen atmosphere at 50 °C and weighed to the nearest 0.1 μg on a microbalance (Mettler M5; Mettler Instrument Corp., Columbus, Ohio, USA).

Nonpolar lipid classes were separated in one-dimensional double-development high-performance thin-layer chromatography (HPTLC) using hexane – diethyl ether – glacial acetic acid (80:20:1.5, *v*:*v*:*v*) to separate nonpolar lipids and hexane–acetone (80:20, *v*:*v*) run up to 3 cm from the bottom edge to resolve pigments. Polar lipid classes were separated by thin-layer chromatography (TLC) on pre-coated plates (Merck KGaA, Darmstadt, Germany) using chloroform – methanol – diethyl ether – water (65:25:4:4, *v*:*v*:*v*:*v*). Lipids were revealed with 10% cupric sulfate in 8% *o*-phosphoric acid (Touchstone et al. 1983) and quantified by calibrated scanning densitometry using the ImageJ software (Schneider et al. 2012).

Energy conversion factors

We employed the energy conversion factors described in Beninger and Lucas (1984), which were calculated for aquatic invertebrates as follows—carbohydrates: 4.1 kcal/g or 17.2 kJ/g; proteins: 4.3 kcal/g or 17.9 kJ/g; lipids: 7.9 kcal/g or 33.0 kJ/g.

Visible spectrum of PVF

Absorption spectrum of PVF was recorded every 1 nm between 350 and 650 nm in an Agilent 8453 UV/Vis diode array spectrophotometer (Agilent Technologies). Three independent samples were measured. Sample buffer was used as a blank. Data were normalized at 280 nm.

Toxicity test

All studies performed with animals were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council 2011) and were approved by the Comité Institucional de Cuidado y Uso de Animales de Experimentación of the School of Medicine, UNLP (Assurance No. P08-01-2013). Animals were obtained from the Experimental Animals Laboratory of the School of Veterinary Science, UNLP. Groups of five female BALB/cAnN mice (body mass = 16 ± 1.1 g) were injected intraperitoneally (i.p.) with a single dose of 200 μL of phosphate-buffered saline (PBS) or the same volume of a serial dilution of five concentrations of PVF. Median lethal dose (LD_{50}) was determined by a lethality test 96 h after injection. Statistical analysis was performed by PROBIT using the EPA–Probit analysis program version 1.5 of the US Environmental Protection Agency (US EPA), based on the method of Finney (1971).

Protease inhibition

Protease inhibition capacity of the PVF from *P. maculata* eggs was assayed using several proteases from vertebrates and bacteria. Proteases were incubated with 67–76 μg of PVF proteins for 5 min and then assayed for enzymatic activity with specific substrates; incubations without PVF proteins were included as controls. All enzymes and substrates were provided by Sigma. Trypsin activity was assayed following the method of Schwert and Takenaka (1955) using 4 μg of the enzyme and *N*-benzoyl-L-arginine ethyl ester (BAEE) as the substrate. Chymotrypsin assay was performed following the method of Wirnt and Bergmeyer (1974) using 3.5 μg of the enzyme and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate. Elastase type IV was analyzed using 6.25 μg of enzyme and the substrate succinyl-Ala-Ala-Ala-*p*-nitroanilide (Suc-Ala_3-pNA) (Bieth et al. 1974). Subtilisin A was tested with BAEE using 125 μg of the enzyme at 50 °C, which is a modification of the method of Schwert and Takenaka (1955). Results were expressed as enzyme specific activity. Three replicates of three independent pooled PVF samples were measured. Normal distribution of the data was checked using the modified Shapiro–Wilk normality test. An unpaired Student's *t* test was performed to compare enzymatic activity with and without co-incubation of PVF. A *P* value of 0.05 was taken as the level of significance.

Table 1. Major soluble ions in eggs of the apple snail *Pomacea maculata*.

Ion	Relative %	% dm
Na^+	44.99±1.87	0.61±0.03
K^+	21.05±2.61	0.26±0.05
Cl^-	23.81±1.23	0.32±0.01
Ca^{2+}	8.17±0.27	0.11±0.01
Mg^{2+}	1.98±0.04	0.03±0.00

Note: Data expressed as relative percentage (%) of soluble ions and as percentage of egg dry mass (% dm; *m*/*m*). Values are mean (±1 SD) of three replicates.

Hemagglutinating activity

Rabbit erythrocytes were obtained from animal facilities at UNLP. Blood samples were obtained by cardiac puncture and collected in sterile Alsever's solution (100 mmol/L glucose, 20 mmol/L NaCl, and 30 mmol/L sodium citrate, pH 7.2) (Sigma–Aldrich). Prior to use, erythrocytes were washed by centrifugation at 1500*g* for 10 min in 20 mmol/L phosphate buffer, 150 mmol/L NaCl, pH 7.4. Hemagglutinating activity was determined using a twofold serial dilution of *P. maculata* PVF proteins (3.4 mg/mL) following the method previously described by Dreon et al. (2013). Three independent pooled PVF were assayed.

Resistance of polysaccharides to digestive enzymes

Isolated polysaccharides were treated either with a solution containing 0.12 U/mL of α-amylase (Sigma–Aldrich) in 20 mmol/L sodium phosphate monobasic buffer with 6.7 mmol/L sodium chloride, pH 6.9, or with a solution of 0.02 mg/mL of pancreatin (Sigma–Aldrich) in 50 mmol/L potassium phosphate dibasic, pH 7.5. The samples were incubated at 25 °C for 3 min using starch (Sigma–Aldrich) under the same conditions as the positive control. Degradation of polysaccharides were measured by the 3,5-dinitrosalicylic acid method (Miller 1959). After incubation at 100 °C for 15 min, the reducing sugars produced were detected measuring the absorbance at 540 nm using an Agilent 8453 UV/Vis diode array spectrophotometer (Agilent Technologies). A standard curve was made using maltose (Sigma–Aldrich). Results are expressed as micrograms (μg) of reducing sugars.

Results

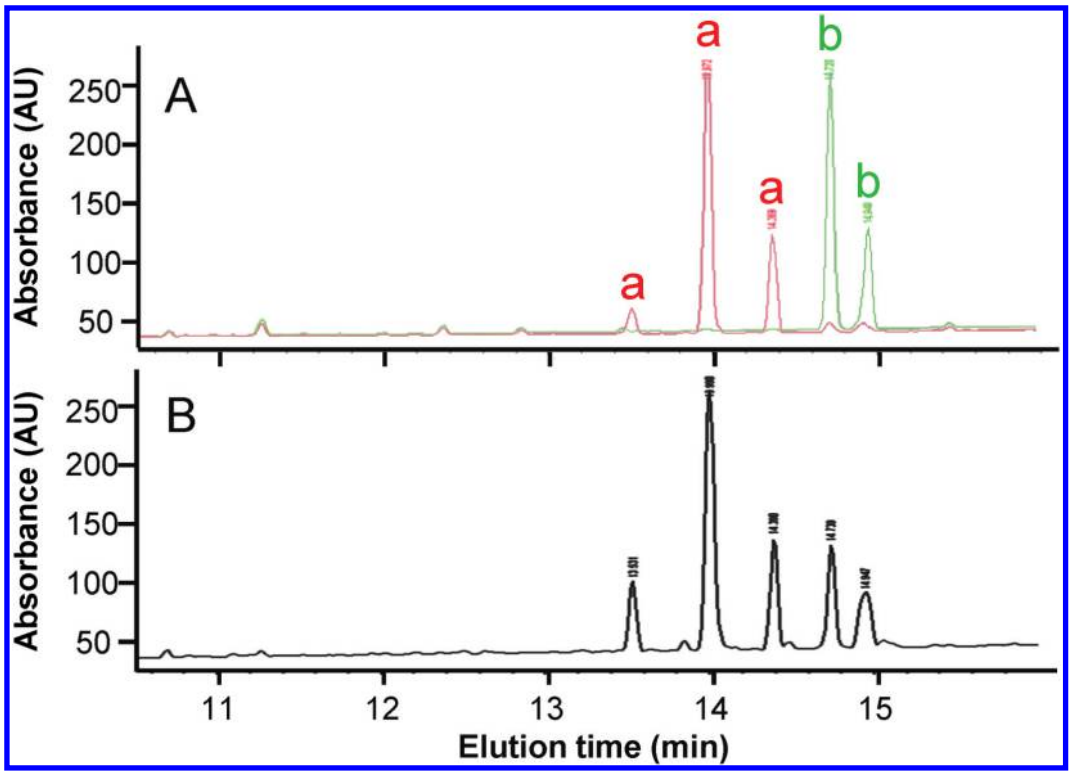
Dry mass, ashes, minerals and egg energy

Dry mass represents 18.07% ± 1.15% of the total egg, whereas ashes represent 10.69% ± 1.20% (*m*/*m*) wet mass, i.e., 57.3% ± 0.4% (*m*/*m*) egg dry mass (dm).

From the biochemical composition, it was possible to calculate the equivalent calories of just-laid eggs, which was 4.04 kcal/g dm, corresponding mostly to carbohydrates (3.14 kcal/g) followed by proteins (0.80 kcal/g) and lipids (0.10 kcal/g).

Major soluble ions present in the eggs are summarized in Table 1. As a whole, total ions measured (Ca^{2+}, Mg^{2+}, K^+, Na^+, and Cl^-) represent 2.6% ± 0.2% dm and soluble ions represent 1.34% ± 0.06% dm. In

Fig. 1. Typical chromatogram of PVF polysaccharides from the apple snail *Pomacea maculata*. Polysaccharide composition was determined by acidic hydrolysis and derivatization of free monosaccharides followed by gas chromatography. (A) Standard: galactose (peaks "a") and glucose (peaks "b"); (B) polysaccharide. Figure appears in color on the Web.



particular, total Ca^{2+} represents 1.18% ± 0.12%, whereas nonsoluble Ca^{2+}, calculated as the difference between total and soluble Ca^{2+}, represents 1.05% ± 0.15% dm.

Carbohydrate composition

Polysaccharides represent the main carbohydrate of the eggs, accounting for 76.41% ± 1.83% dm, whereas free glucose represents only 0.056 ± 0.005% dm. The GC analysis of the polysaccharides showed two groups of peaks matching with the standards for D-galactose and D-glucose, representing 68.31% ± 4.45% and 31.69% ± 4.45%, respectively (Figs. 1A, 1B).

Protein composition

Total proteins represent 18.7% ± 2.4% dm. PVF proteins comprise three fractions: PV1, PV2, and PV3 (Fig. 2). A comparison of protein pattern between this species and *P. canaliculata* is shown in Fig. 2. The native PAGE shows that the PV3 fraction differs markedly between both species; for example, 113 and 58 kDa bands were only detected in *P. maculata*, whereas 87 and 80 kDa bands were only identified in *P. canaliculata*. The proportions of protein fractions are compared in Table 2. The concentration of PV1 and PV2 are significantly different between both species.

Based on a previous report for *P. maculata* perivitellin-1 (PmPV1) (Pasquevich et al. 2014) and on the proteomic analysis of *P. canaliculata* PVF (Sun et al. 2012), it was possible to tentatively identify many spots from the proteomic map of *P. maculata* PVF (Supplementary Table S1).[1] A comparison between the proteomic patterns of *P. maculata* (Fig. 3) and *P. canaliculata* (Sun et al. 2012) PVF indicates that although in general they are similar, there are remarkable differences; for instance, the apoptosis-inducing factor (2-DE spot #3) and kunitz-like protease inhibitor (2-DE spots #4 and #5) identified in the *P. canaliculata* map (Sun et al. 2012) were not detected in the *P. maculata* 2-DE profile. Likewise, some spots in *P. maculata* PVF are not detected in *P. canaliculata* PVF (Sun et al. 2012), such as the 30 kDa (pI 6.5), 34 kDa (pI 7.1), and 27 kDa (pI 8.2) spots. Further proteomic analysis is needed to characterize the full PVF proteome.

Lipid composition

Lipids are a minor component of the eggs, representing 1.25% ± 0.11% dm; they are mostly phospholipids and free sterols (Figs. 4A–4C). Polar lipids were represented by phosphatidylethanolamine (PE) and phosphatidylcholine (PC) and an unidentified polar compound with an R_f value between phosphatidylserine (PS) and PC (Fig. 4A). Table 3 summarizes the lipid composition of egg. Carotenoid pigments were previously identified as free astaxanthin and two sterified forms (Pasquevich et al. 2014). We found that free astaxanthin represented 49.73% ± 3.56% of total pigments, whereas astaxanthin monoester and astaxanthin diester represented 16.81% ± 1.79% and 33.46% ± 4.64%, respectively (Fig. 4C).

PVF spectral features

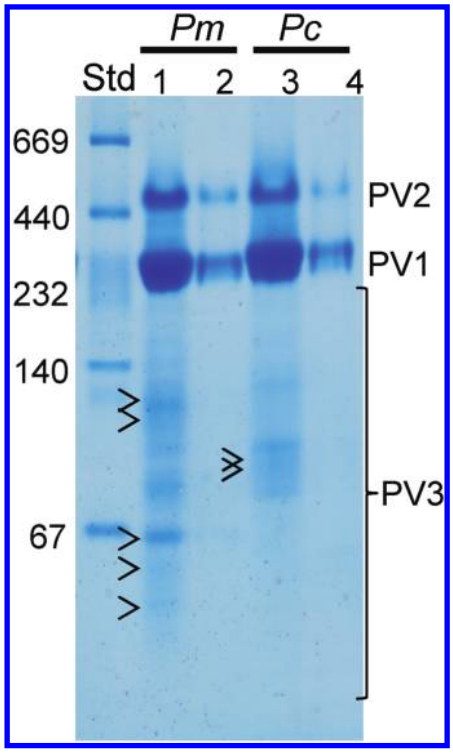
The PVF visible absorption spectrum is shown in Fig. 5. The PVF absorbs in a wide range of the visible spectrum (350–650 nm), showing a peak at 427 nm and another wide peak at 505 nm that exhibits fine structure. These features of the PVF spectrum are similar to those previously reported for its major perivitellin, PmPV1 (Pasquevich et al. 2014).

Toxicity

Mice injected i.p. with *P. maculata* PVF showed remarkable behavioral changes after 16–20 h. These included weakness and lethargy, half-closed eyes, tachypnea, and hirsute hair. They also presented extreme abduction of the rear limbs and were not able to support

[1]Supplementary figures and table are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjz-2016-0049.

Fig. 2. Native PAGE of PVF from apple snails *Pomacea maculata* and *Pomacea canaliculata*. Std: molecular mass standard (kDa); *Pm*: PVF from *P. maculata* (lanes 1 and 2); *Pc*: PVF from *P. canaliculata* (lanes 3 and 4). Lanes 1 and 3: 100 μg; lanes 2 and 4: 10 μg. Unshared PV3 protein bands between the species are indicated by arrowheads. Figure appears in color on the Web.



their own body mass (paresis). When mice tried to raise their tails, their tail muscles showed spastic movements (tremors). Thirty hours after inoculation, mice showed flaccid paralysis of the rare limbs, which were unable to support the body mass, whereas the forelimbs remained functional. Interestingly, survivors were fully recovered after 96–120 h, even after severe symptomatology. Death in the majority of mice came after 40 h of injection.

The PVF lethal dose causing 50% mortality (LD_{50} 96 h) was determined to be 1.7 mg/kg (Supplementary Fig. S1).[1]

Protease inhibition

Protease inhibition tests (Fig. 6) showed that PVF causes a significant decrease in the activity of all enzymes assayed ($P < 0.0001$ for trypsin, chymotrypsin, and elastase; $P < 0.05$ for subtilisin), clearly demonstrating the protease inhibitory capacity of PVF.

Hemagglutinating activity

Accounting for the presence of hemagglutinating activity in other *Pomacea* species, we tested for *P. maculata* PVF hemagglutinating capacity using rabbit erythrocytes. Positive reaction was observed above 1.7 mg/mL of PVF proteins, though a mild hemagglutinating activity was already observed at 0.85 mg/mL, indicating the presence of active lectins (Supplementary Fig. S2).[1]

Polysaccharide resistance to in vitro digestion

Pomacea maculata egg polysaccharide resistance to digestion was assayed using α-amylase and pancreatin. The digestive enzymes readily degrade the control starch (α-amylase: $P < 0.001$; pancreatin: $P < 0.0001$). However, they were not able to release reducing sugars from the samples, as observed by colorimetry (Supplementary Fig. S3).[1] This result indicates that neither α-amylase nor pancreatin can degrade the most abundant egg sugar.

Table 2. Relative percentage of perivitellin fractions PV1, PV2, and PV3 in the apple snails *Pomacea maculata* and *Pomacea canaliculata*.

Fraction	*P. maculata*	*P. canaliculata*
PV1	63.8±3.6*	69.8±4.0*
PV2	22.7±1.7**	18.9±2.0**
PV3	13.6±2.2	11.3±2.2

Note: Values are mean (±1 SD) of three replicates.
*, $P < 0.05$; **, $P < 0.01$.

Discussion

Egg biochemical composition

Apple snail eggs have a direct development and therefore the embryos rely on the contents of PVF to sustain growth until hatching (yolk contribution is negligible). PVF primary functions are thought to be protection of the developing embryo from predators and physical stresses, as well as providing nutrition (Dreon et al. 2006; Heras et al. 2007; Hayes et al. 2015).

In this study, we characterized the biochemical composition of nearly 99% of the dry matter of *P. maculata* eggs and found that carbohydrates were the major component (76.4% dm) followed by proteins (18.7% dm); however, unlike eggs of other aquatic invertebrates, only a small amount of lipids was detected (1.2% dm). This agrees with the composition of other gastropods (Livingstone and de Zwaan 1983; Heras et al. 1998) and was not surprising because the energy metabolism of many gastropods is carbohydrate-based and contrasts with that found in other molluscs such as in bivalve eggs that usually contain proteins and lipids as the major components (Holland 1978). This difference may be related with the life histories of these molluscs (Heras et al. 1998).

The most abundant carbohydrate in *P. maculata* eggs is the polysaccharide galactogen, which is also the major component of those of *P. canaliculata* (Heras et al. 1998) and many other gastropod eggs. This polysaccharide is assumed to serve as an energy source in reproduction in pulmonate snails and some Caenogastropoda (Livingstone and de Zwaan 1983). Galactogen content (~70% dm) was considerably higher than that reported in other gastropod eggs, which are usually in the 30%–40% dm range (Raven 1972; Heras et al. 1998). To be able to use this sugar source, embryos need a set of specific β-glycosidades because galactogen is a β(1→3)- or β(1→6)-linked chain of D-galactose units (Goudsmit 1972). In this regard, it is interesting to note that besides D-galactose, *P. maculata* galactogen also contains a significant amount of D-glucose that must be linked in a different way than the usual α(1→4) glyosidic bond of other reserve polysaccharides because it was not degraded by α-amylase. Galactans as heteropolymers were also reported in other gastropods, such as the bloodfluke planorb (*Biomphalaria glabrata* (Say, 1818)) (Livingstone and de Zwaan 1983). In contrast, eggs of other species that store carbohydrates such as fish (Terner 1979) and the fruit fly *Drosophila* (Gutzeit et al. 1994) do so in the form of glycogen. Indeed, glycogens are the universal storage polysaccharides among metazoan eggs and, thus, it has long been a rather puzzling fact that the gastropod store egg sugars as galactogen (Urich 1994). We performed experiments to test a possible explanation for this (see below).

The second most abundant *P. maculata* egg component is proteins, which were separated into three fractions: PV1, PV2, and PV3 (named PmPV1, PmPV2, and PmPV3 in *P. maculata*; Pasquevich et al. 2014). PV1 fraction includes a single particle and has been previously described for both *P. maculata* (PmPV1) and *P. canaliculata* (PcOvo) (Dreon et al. 2004, 2008; Pasquevich et al. 2014). PV2 is also a single particle, which was only characterized for *P. canaliculata* (PcPV2) (Garín et al. 1996; Heras et al. 2008; Frassa et al. 2010; Dreon et al. 2013, 2014). Here we show that in both species, PV2 presents similar molecular masses and the same position spots in 2-DE maps, although it is more concentrated in *P. maculata*. On the

Fig. 3. Two-dimensional electrophoresis of apple snail *Pomacea maculata* PVF, where MM is molecular mass (kDa). Numbered spots correspond to tentatively identified proteins (see Supplementary Table S1).[1] Figure appears in color on the Web.

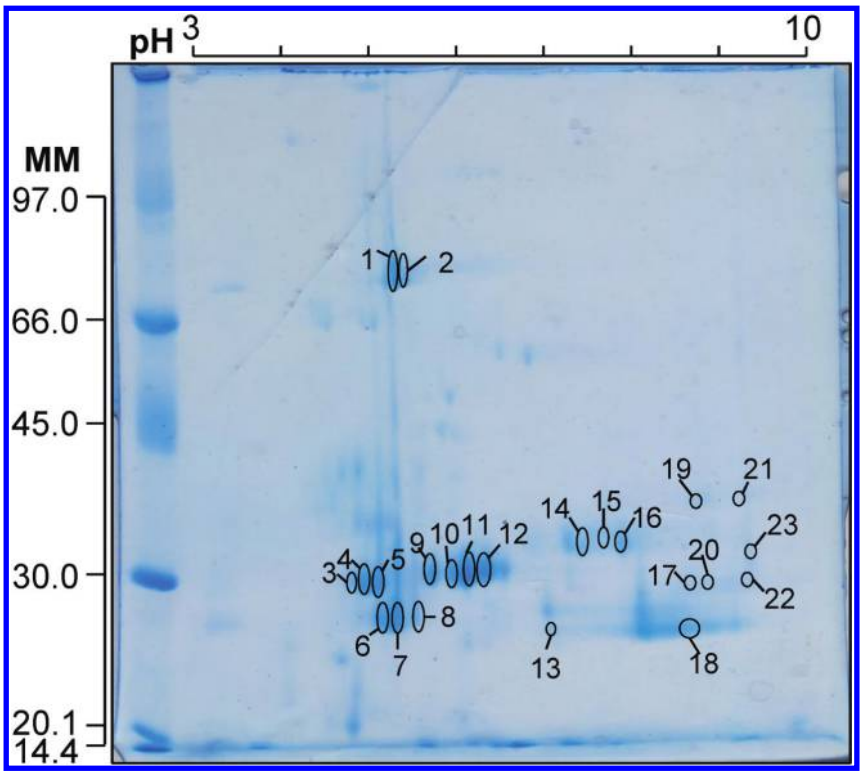


Fig. 4. Thin-layer chromatography of PVF lipid classes from apple snail *Pomacea maculata* eggs. (A) Polar lipids. PE: phosphatidylethanolamine; PC: phosphatidylcholine; PS: phosphatidylserine; SM: sphingomyelin; UPL: unidentified polar lipid. (B) One-dimensional double-development high-performance thin-layer chromatography (HPTLC) of nonpolar lipids. ES: esterified sterols; HC: hydrocarbons; FFA: free fatty acid; TG: triacylglycerols; ST: free sterols; PIGM: pigment. (C) HPTLC of pigments. Asx: free astaxanthin; Asx-Me: astaxanthin monoester; Asx-De: astaxanthin diester (identified using the data from Pasquevich et al. 2014). Figure appears in color on the Web.

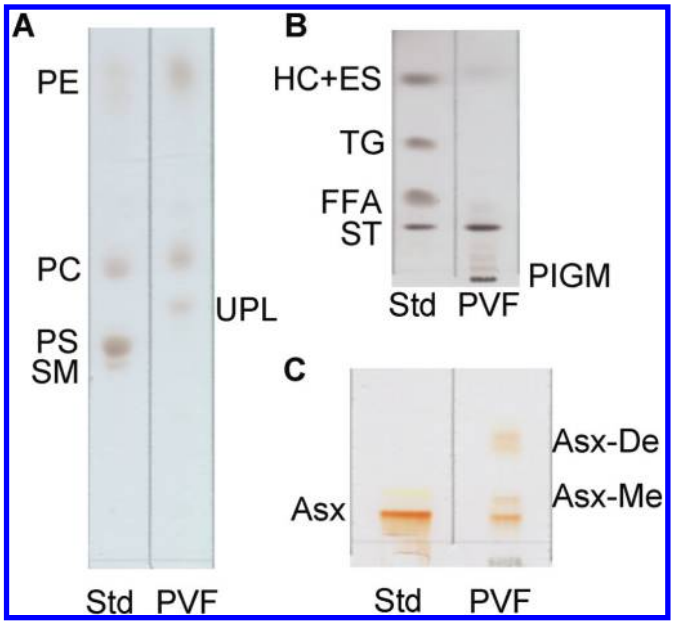


Table 3. Relative percentage of lipids in eggs of the apple snail *Pomacea maculata*.

Lipid	Relative %
HC + ES	2.48±0.48
TG	Trace amount
FFA	3.42±1.18
ST	21.28±4.80
Carotenoids	8.77±1.39
PE	32.97±4.55
PC	18.81±1.05
Unidentified polar lipid	12.26±0.57

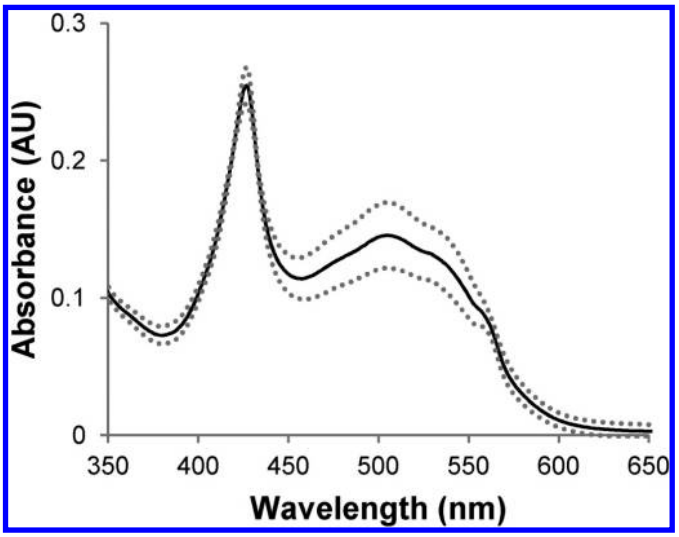
Note: Values are mean (±1 SD) of three replicates. HC, hydrocarbons; ES, esterified sterols; TG, triacylglycerols; FFA, free fatty acids; ST, free sterols; PE, phosphatidylethanolamine; PC, phosphatidylcoline. HC and ES were quantified together.

other hand, PV3 consists of a heterogeneous fraction in both species (Garín et al. 1996; Pasquevich et al. 2014), but with a different protein pattern. As a whole, these findings suggest that these two related species have important differences in their egg protein profiles. This new biochemical information could be used as a characteristic to distinguish between these closely related *Pomacea* species as was previously suggested (Pasquevich et al. 2014).

Lipids are a minor component within *P. maculata* eggs, mostly represented by structural lipids and pigments. This is similar to *P. canaliculata* eggs and further supports the notion that snails do not use lipids as a major energy reserve during reproduction (Heras et al. 1998).

The *P. maculata* egg inorganic ion composition resembles that of fresh water. Calcium is the major ion in just-laid eggs and it occurs mostly in a nonsoluble state. This large amount of calcium agrees with the fact that *Pomacea* eggs are truly cleidoic (Pizani et al. 2005)

Fig. 5. Absorption spectrum of apple snail *Pomacea maculata* PVF. Data are the mean of three independent measures (solid line) ± SD (dotted lines).



and are surrounded by a calcareous shell, which seems to be an adaptation to the aerial oviposition strategy of these species (Hayes et al. 2015). Besides, direct development implies that calcium needs to be stored to supply the developing snail shell during organogenesis (Tompa 1980). Inorganic components of *Pomacea* eggs have not been reported before, thus precluding further comparisons.

Role of polysaccharides and proteins as *P. maculata* egg defenses

Pomacea maculata snails combine several reproductive strategies, as they deposit many clutches with huge number of eggs with high hatchability during every reproductive season (Barnes et al. 2008). Here we show that these conspicuously colored eggs are also chemically defended. Previous reports have shown that *Pomacea* snails have developed an array of defensive strategies that is unique among animals, which include a cocktail of neurotoxic, antinutritive, and antidigestive proteins (Dreon et al. 2006; Heras et al. 2007; Hayes et al. 2015). This, together with their bright coloration, presumably a warning signal, is probably the reason why the eggs have virtually no predators (Heras et al. 2007).

In particular, egg proteins with neurotoxic activity have only been reported in two species, the black widow spider (Li et al. 2013) and the apple snail *P. canaliculata* (Heras et al. 2008). In the latter, the toxic effect was assigned to the second most abundant perivitellin, PcPV2 (Heras et al. 2008; Dreon et al. 2013). Remarkably, *Pomacea scalaris* (d'Orbigny, 1832) PVF lacks this 400 kDa protein (Ituarte et al. 2008) and no neurotoxicity was observed in its PVF (S. Ituarte, unpublished data). In the present study, we report that the eggs of *P. maculata* are toxic to mice. As mentioned, rodents are among the few predators of apple snails and avoid eating eggs, as well as the adult female albumen gland (Yusa et al. 2000), which is a remarkable behavior that suggests the presence of deterrents within the eggs. *Pomacea maculata* have a 400 kDa perivitellin similar to PcPV2, which was therefore named PmPV2 (Pasquevich et al. 2014). Moreover, *P. maculata* PVF administration to mice, as a model rodent, causes the same neurological and behavioral symptoms as those reported for *P. canaliculata*, but were slightly stronger (Heras et al. 2008). The observed differences in toxicity among *Pomacea* species can be understood in regards to the phylogeny of the group, as *P. maculata* and *P. canaliculata* belong to a separate clade than *P. scalaris*, which has developed different defenses in this rapidly diversifying group (Hayes et al. 2015). However, the eggs of the three species have varied agglutinating activity. In *P. scalaris*, a strong hemagglutinating activity was associated with the major perivitellin, PsSC, whereas *P. canaliculata* showed a mild hemagglutinating activity for both PcPV2 and the whole PVF (Ituarte et al. 2012; Dreon et al. 2013, 2014). Here we report the presence of hemagglutinating activity for the PVF of *P. maculata*. Hemagglutinating activity of eggs seems widespread in the family, and was also reported for other ampullariid snails, namely *Pila ovata* (Olivier, 1804) and *Pomacea urceus* (Müller, 1774) (Uhlenbruck et al. 1973; Baldo and Uhlenbruck 1974). Although agglutinating activity has been associated with plant embryo defenses against predation, e.g., acting as an antidigestive system (Hajos et al. 1995; Peumans and Van Damme 1995), or against pathogens (Ituarte et al. 2012), the role of egg hemagglutinating capacity in defense is still unknown in ampullariids. Further work is needed to shed light on this topic in *Pomacea* species.

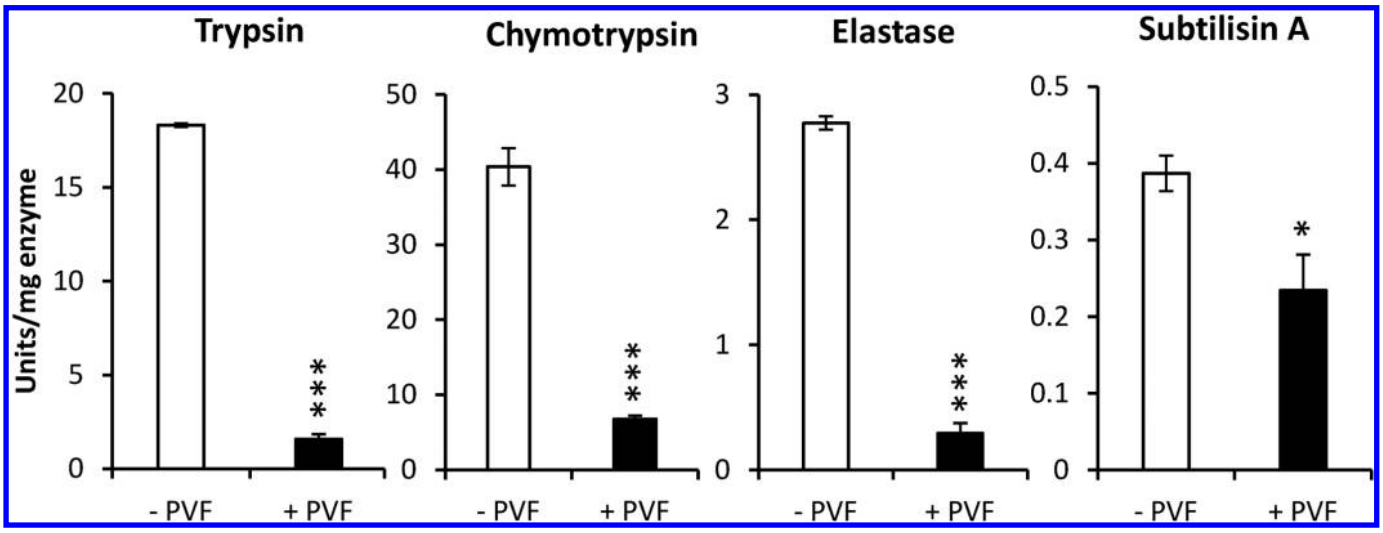
Regardless if the agglutinating activity plays an antidigestive role as in seeds, an antidigestive effect of the PVF was reported for *P. canaliculata* due to a strong antiprotease activity (Dreon et al. 2010). This protease inhibition activity was ascribed to the presence of Kunitz-like proteins in *P. canaliculata* PVF proteome (Sun et al. 2012). In the present work, antiprotease activity was observed for *P. maculata* PVF, which inhibits not only animal digestive enzymes but also bacterial proteases. Thus, in addition to a neurotoxin, the presence of an antiprotease activity could be also part of the egg defense system against predation and pathogens.

Aerial oviposition exposes the eggs to sunlight, air, and high temperatures. To cope with these environmental stressors, one possible adaptation would involve the provision of antioxidant and photoprotective molecules to the embryo. The association of the astaxanthin pigment with PcOvo and PmPV1 has been related with a strong antioxidant activity (Dreon et al. 2006; Pasquevich et al. 2014). *Pomacea maculata* eggs have this carotenoid in free and esterified forms, suggesting an antioxidant activity. Besides, the PVF absorbs light throughout most of the visible range. This behavior, also present in the purified PV1 fraction, was related with a photoprotective effect for the embryo against sunlight radiation at the beginning of development (Dreon et al. 2006; Pasquevich et al. 2014).

The present study provides evidence that, in addition to proteins, carbohydrates may also be indigestible in apple snail eggs, limiting predator ability to digest nutrients. In this respect, it is known that plant β-linked polysaccharides, such as cellulose and hemicelluloses, are food components refractive to animal digestion unless some complex biochemical adaptations are present to exploit them (Karasov et al. 2011). These adaptations include the production of cellulases (endogenous or through symbiotic microbiota), which is a common strategy in gastropods. However, it has been reported that even animals with β-glycosidases are not able to digest galactogen (Myers and Northcote 1958). In fact, only snail embryos and hatchlings are known to catabolize galactogen (Weinland 1953; Myers and Northcote 1958; Goudsmit 1976). Here we found that neither α-amylase nor pancreatin could degrade *P. maculata* galactogen in an in vitro assay. All this information has lead us to suggest that the storing of galactogen instead of glycogen within gastropod eggs could represent a biochemical adaptation that has the advantage of rendering polysaccharides indigestible for a predator; an antinutritive strategy that would complement the protein defenses of eggs, which leads to malabsorption because indigested food passes quickly through the digestive tract. Further analysis is necessary to confirm this hypothesis.

Likewise, the high amount of galactogen could have other roles to cope with the aerial ovipositon such as retaining water, which would protect the eggs against desiccation (Dreon et al. 2006), or providing for the high viscosity of the PVF, which has been suggested as a potential antimicrobial defense (Ituarte et al. 2010).

Fig. 6. Protease inhibition capacity of apple snail *Pomacea maculata* PVF. Protease specific activity without PVF (–PVF) and after incubation with *P. maculata* PVF (+PVF). Values represent means ± SD (n = 3). ***, $P < 0.0001$; *, $P < 0.05$.



Conclusion

As a whole, this study provides insights into the unusual reproductive strategy of *Pomacea* snails, which highlights the presence of multiple and overlapping biochemical defensive components that enhance the survival of the embryo in harsh conditions. This seems a key acquisition in the success that *Pomacea* snails have achieved for their invasion and spread into new areas. Toxic, antidigestive, antinutritive, and hemagglutinating properties would conform an effective egg defense against predators, supported by the fact that only one predator was reported to predate on *Pomacea* eggs in nature (Yusa 2001). We provide some evidence to support a hypothesis seeking to explain, for the first time to our knowledge, the widespread use of galactogen in gastropod eggs: it may be involved in egg defense.

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