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

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Abstract

The freshwater snails *Pomacea* lay conspicuous aerial eggs clutches that are ignored by most predators. Egg biochemical defenses in the apple snail *P. canaliculata* (Lamarck, 1822) are provided by multifunctional proteins. We analyzed the eggs of a sympatric species, *P. maculata* Perry,1810, studying the gross composition, toxicity, hemagglutinating activity, as well as its antinutritive and antidigestive properties. Eggs are mostly composed of polysaccharides, mainly galactogen, and proteins, followed by lipids and non-soluble calcium. Two perivitellins account for ~85 % dw of the egg protein. The major lipids are phospholipids and sterols. A suite of potential defenses were 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 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.

Keywords: animal defense; egg composition; indigestible polysaccharide; protease inhibitors; antinutritive; antidigestive; apple snails; *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 later 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 which is believed to deter predators (i.e. aposematic) (Fuhrman et al. 1969; Heras et al. 2008; Winters et al. 2014). Chemical defenses are usually non-proteinaceous compounds, however, recent studies described neurotoxic proteins and peptides inside eggs in two species, the apple snail *Pomacea canaliculata* (Lamarck,1822) (with aposematic eggs) and the black widow spider, *Lactrodectus 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, while some are also present 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 as a consequence of aquaculture activities they spread and soon became an invasive species in Southeastern Asia, North America and Spain (Cowie 2002; López et al. 2010). Like most *Pomacea* species, they lay calcareous and conspicuously colored egg masses on hard surfaces above the waterline (Heras et al. 2007), a strategy that opposes to the deposition of eggs in gelatinous masses under the water, as most other ampullarids (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 eggs clutches have no reported predators in their native range and only one predator (the 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 amount 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 which 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 give 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 their main components involved in the embryo defense.

Materials and Methods

Clutch and egg characteristics

In the reproductive season, female *Pomacea* snails venture out of water and lay calcareous eggs forming masses cemented on emergent hard surfaces (Heras et al. 2007), which hatch between 1 and 3 weeks later (Seuffert et al. 2012). In *P. macualta*, egg clutches are very large and comprise from a few hundred to more than 4,500 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). Average hatching efficiency ranges from 33.1-70.8 % (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 with 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 laid and kept at -20 °C until processed.

Sample preparation

Whole egg homogenate was prepared on ice-cold 20 mM Tris-HCl, pH 7.4, keeping a 3:1 v/w buffer:sample ratio as previously described (Pasquevich et al. 2014). Homogenate was

sequentially centrifuged to obtain the egg soluble fraction; from now on perivitelline fluid, PVF (Pasquevich et al. 2014). Homogenate was used to quantify macromolecules and for lipid and polysaccharide extraction, while PFV was used in the determination of soluble ions, glucose, protein analyses and functional assays. Three independent pools of 3 clutches each (9 clutches in total) were used in every experiment.

Dry weight, ashes and minerals

To determine dry weight and ash content, pre-weighted 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 weighted. For mineral analysis, the homogenate and PVF were prepared as described in section 2.2 but using milli-Q water instead of buffer. Electrolyte concentrations were determined in a Konelab 60I Prime (Wiener Lab), soluble Na^{+} (100-200 mEq/L), Cl^{-} (50-150 mEq/L) and K^{+} (2-10 mEq/L) with a selective ion analyzer; soluble Mg^{+2} with a colorimetric method (Wiener Lab). Ca^{+2} was determined with a Ca-Color kit (Wiener lab., Argentina) using either the whole homogenate (i.e. with the egg shell) to determine total Ca^{+2} or the PVF for soluble Ca^{+2}. Percentage of the ions w/w (dry weight) 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 van Handel (1965) method. Monosaccharide composition of polysaccharide was determined by gas chromatography (GC-FID) after digestion in methanolic-HCl, and derivatization with hexamethyldisilazane/ trimethylchlorosilane/pyridine (Sigma-Aldrich) as previously described

(Ituarte et al. 2010). Standard monosaccharides (Sigma-Aldrich, St. Louis, MO, USA) were silylated and analyzed under the same conditions.

Protein analysis

Total protein concentration was determined from homogenate following the method of Markwell (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 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 second dimension, the IPG strips were sealed on the top of 1.5 mm thick 12% polyacrylamide gels, with MW 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 weight (MW)-isoelectric point (pI) coordinates were estimated using Image Master 2-D Platinum software (GE Helthcare Life Science). Tentative identity of spots was made by comparison with the MW-pI coordinates previously obtained for the sister species *P. canaliculata* (Sun et al. 2012) and for purified *P. maculata* proteins (Pasquevich et al. 2014).

Native-PAGE was performed in 4-20% gradient polyacrylamide gels in a miniVE Electrophoresis System (GE Healthcare, Life Science). *P. canaliculata* PVF was also analyzed by Native-PAGE for comparison. High molecular weight standards (Amersham Biosciences) were run

in the same gels. Gels were stained with Coomassie Blue G-250 staining (Echan and Speicher 2002) and protein bands were quantified by calibrated scanning densitometry using 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., USA).

Non-polar lipid classes were separated in one-dimension double-development high-performance thin-layer chromatography (HPTLC) using hexane/diethyl ether/glacial acetic acid (80:20:1.5 v/v/v) to separate non-polar 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% orthophosphoric 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 by (Beninger and Lucas 1984), which were calculated for aquatic invertebrates: Carbohydrates: 4.1 kcal/ g or 17.2 kJ/g; proteins: 4.3 kcal/g or 17.9 kJ/g, and lipids: 7.9 kcal/g or 33.0 kJ/g.

Visible spectrum of perivitelline fluid

Absorption spectrum of PVF was recorded every 1nm between 350 nm to 650 nm in an Agilent 8453 UV/Vis diode array spectrophotometer (Agilent Technologies). Three independent samples were measured. Sample buffer was used as 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 weight: 16±1.1 g) were injected intraperitoneally (i.p.) with a single dose of 200 µL of phosphate buffer 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 96h after injection, statistical analysis was performed by PROBIT using EPA-Probit analysis program v1.5 statistical software of the US *Environmental Protection Agency* (US EPA), based on Finney’s method (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 substrate. Chymotrypsin assay was

performed following Wirnt and Bergmeyer (1974) method using 3.5 μg of the enzyme and N-benzoyl-L-tyrosine ethyl ester (BTEE) as substrate. Elastase type IV was analyzed using 6.25 μg of enzyme and the substrate Succinyl-Ala-Ala-Ala-p-nitroanilide ($SucAla_3$-pNA) (Bieth et al. 1974). Subtilisin A was tested with BAEE, using 125 μg of the enzyme at 50°C, a modification of the Schwert and Takenaka method (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-Wilks normality test. An unpaired *t* test was performed to compare enzymatic activity with and without co-incubation of PVF. A *P* value 0.05 was taken as the level of significance.

Hemagglutinating activity

Rabbit erythrocytes were obtained from animal facilities at Universidad Nacional de La Plata. Blood samples were obtained by cardiac puncture and collected in sterile Alsever's solution (100 mM glucose, 20 mM NaCl, and 30 mM sodium citrate, pH 7.2) (Sigma-Aldrich, St. Louis, MO, USA). Prior to use, erythrocytes were washed by centrifugation at 1500 g for 10 min in 20 mM phosphate buffer, 150 mM NaCl, pH 7.4. Hemagglutinating activity was determined using a two-fold 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 mM sodium phosphate monobasic buffer with 6.7 mM sodium chloride, pH 6.9, or with a solution of 0.02 mg/mL of pancreatin (Sigma-Aldrich) in 50 mM 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 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 µg of reducing sugars.

Results

Dry weight, ashes, minerals and egg energy

Dry weight represents 18.07±1.15 % of the total egg, while ashes represent 10.69 ±1.20 % w/w wet weight, i.e. 57.3 ±0.4 % w/w of egg dry weight (dw).

From the biochemical composition it was possible to calculate the equivalent calories of just-layed eggs which was 4.04 Kcal/g dw, 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 % dw and soluble ions 1.34 ±0.06 % dw. In particular, total Ca^{+2} represents 1.18 ±0.12 % while non-soluble Ca^{+2}, calculated as the difference between total and soluble Ca^{+2}, represents 1.05 ±0.15 % dw.

Carbohydrate composition

Polysaccharides represent the main carbohydrate of the eggs, accounting for 76.41 ± 1.83 % dw, while free glucose represent only 0.056 ± 0.005 % dw. 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 (Figure 1).

Protein composition

Total proteins represent 18.7 ±2.4 % dw. PVF proteins comprise 3 fractions: PV1, PV2 and PV3 (Figure 2). A comparison of protein pattern between this species and *P. canaliculata* is shown in Figure 2. The Native-PAGE shows that PV3 fraction differs markedly between both species, for example, 113 kDa and 58 kDa bands were only detected in *P. maculata*, while 87 kDa and 80 kDa bands were only identified in *P. canaliculata*. The proportions of the proteins 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 (Table S1)[1]. A comparison between the proteomic patterns of *P. maculata* (Figure 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 spot #4 and #5) identified in the *P. canaliculata* map (Sun et al. 2012) were not detected in *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 % dw, mostly phospholipids and free sterols (Figure 4). Polar lipids were represented by phosphatidylethanolamine (PE) and phosphatidylcholine (PC) and an unidentified polar compound

[1]Supplementary material: Table.

with an Rf between phosphatidylserine (PS) and PC (Figure 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, while astaxanthin monoester and diester represented 16.81 ±1.79 % and 33.46 ±4.64 %, respectively (Figure 4C).

Perivitelline fluid spectral features

The PVF visible absorption spectrum is shown in Figure 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 which 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. Besides, they presented extreme abduction of the rear limbs and were not able to support their own body weight (paresis). When mice tried to raise their tail muscles showed spastic movements (tremors). After 30 h post-inoculation mice showed flaccid paralysis of the rare limbs which were unable to support the body weight, 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 for 50 % of death (LD_{50} 96h) determined was 1.7 mg/kg (Figure S1)[2].

[2] Supplementary material: Figure S1

Protease inhibition

Protease inhibition tests (Figure 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 the PVF.

Hemagglutinating activity

Taking into account 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 (Figure S2)[3].

Polysaccharide resistance to in vitro digestion

P. maculata egg polysaccharide resistance to digestion was assayed using α-amylase and pancreatin. The digestive enzymes readily degrade 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 (Figure S3)[4]. This result indicates that neither α-amylase nor pancreatin can degrade the most abundant egg sugar.

Discussion

Egg biochemical composition

Apple snail eggs have a direct development and therefore the embryos rely on the contents of the perivitelline fluid to sustain growth until hatching (yolk contribution is negligible).

[3] Supplementary material: Figure S2.
[4] Supplementary material: Figure S3.

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 *Pomacea maculata* eggs and found that carbohydrates were the major component (76.4 % dry weight) followed by proteins (18.7 %), but, unlike eggs of other aquatic invertebrates, only a small amount of lipids was detected (1.2 %). This agrees with the composition of other gastropods (Livingstone and Zwaan 1983; Heras et al. 1998) and was not surprising as the energy metabolism of many gastropods are carbohydrate-based and contrasts with that found in other mollusks 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 mollusks (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 Zwaan 1983). Galactogen content (~70% dw) was considerably higher than that reported in other gastropod eggs that are usually in the 30-40% dw range (Raven 1972; Heras et al. 1998). To be able to use this sugar source, embryos need a set of specific β-glycosidades as 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 which must be linked in a different way than the usual α(1→4) glyosidic bond of other reserve polysaccharides, as it was not degraded by α-amylase. Galactans as heteropolymers were also reported in other gastropods, such as *Biomphalaria glabrata* (Say,1818) (Livingstone and Zwaan 1983). In contrast, eggs of other species that store carbohydrates such as fish (Terner 1979) and *Drosophila* (Gutzeit et al. 1994)

they do so in the form of glycogen. Indeed, glycogens are the universal storage polysaccharides amongst 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 components are 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 weights and the same position spots in 2-DE maps though it is more concentrated in *P. maculata*. On the other hand, PV3 consists of a heterogeneous fraction in both species (Garín et al. 1996; Pasquevich et al. 2014) but with 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 employed 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* egg 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 freshwater. Calcium is the major ion in just-laid eggs, and it occurs mostly in a non-soluble state. This large amount of calcium agrees with the fact that *Pomacea* eggs are truly cleidoic (Pizani et al. 2005), and surrounded by a calcareous shell, which seems 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 for the developing snail shell during organogenesis (Tompa 1980). Inorganic components of *Pomacea* eggs have not been reported before precluding further comparisons.

4.2. 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 in 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 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 *Lactrodectus tredecimguttatus* (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, *P. scalaris* (d'Orbigny, 1832) PVF lacks this 400-kDa protein (Ituarte et al. 2008) and no neurotoxicity was observed in its PVF (SI *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), a remarkable behaviour that suggests the presence of deterrents within the eggs. *P. 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 slightly stronger (Heras et al. 2008). The observed differences in toxicity among *Pomacea* species can be understood in light of 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, while *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* (Oliver, 1804) and *Pomacea urceus* (Müller, 1774) (Uhlenbruck et al. 1973; Baldo and Uhlenbruck 1974). While agglutinating activity has been associated with plant embryo defenses against predation, for instance 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 issue 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). *P. 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 these information 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, that leads to malabsorption as 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).

Conclusion

As a whole this study provides insights into the unusual reproductive strategy of *Pomacea* snails that highlights the presence of multiple and overlapping biochemical defensive components that enhance the embryo survival in harsh conditions. This seems a key acquisition in the success that *Pomacea* snails have achieved for their invasion and spread in 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 *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 defenses.

Acknowledgements

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Figure captions

Figure 1. GC chromatogram of PVF polysaccharides from *P. maculata*. Polysaccharide composition by acidic hydrolysis and derivatization of free monosaccharides. **Panel A:** Standard: galactose (peaks ¨a¨) and glucose (peaks ¨b¨); **Panel B:** polysaccharide.

Figure 2. Native-PAGE of *P. maculata* and *P. canaliculata* PVF. *Std:* molecular weight standard*; Pm*: perivitellin fluid of *P. maculata* (lanes 1 and 2); *Pc*: perivitellin fluid of *P. canaliculata*. (lanes 3 and 4) Lanes 1, 3: 100µg; lanes 2, 4: 10 µg. Unshared PV3 protein bands between the species are indicated by arrowheads.

Figure 3. 2-DE of the perivitellin fluid of *P. maculata.* Numbered spots correspond to tentatively identified proteins *(see Supplementary Table).*

Figure 4. Thin layer chromatography of PVF lipid classes of *P. maculata* eggs. PVF: Perivitelline fluid lipids **A)** Polar lipids. PE: phosphatidylethanolamine; PC: phosphatidylcholine; PS: phosphatidylserine; SM: sphingomyelin; UPL: unidentified polar lipid. **B)** HPTLC of non-polar 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 mono- ester; Asx De: astaxanthin diester (identified using the data of Pasquevich et al. (2014)).

Figure 5. **Perivitelline fluid absortion spectrum.** Data are the mean of three independent measures (full line) ± SD (dotted lines).

Figure 6. **Protease inhibition capacity of *P. maculata* PVF.** Protease specific activity without PVF (-PVF) and after the incubation with *P. maculata* PVF (+ PVF). Values represent the mean ± SD (*n*=3). ***$P<0.0001$; *$P<0.05$.

Table 1. Major soluble ions of P. maculata eggs.

Ion	Relative %	% dw
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 % of soluble ions and as % w/w egg dw.

Results are the mean of three replicates ± 1SD.

Table 2. Relative percentage of perivitellin fractions PV1, PV2 and PV3 in *P. maculata* and *P. 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: Results are the mean of three replicates ±1SD. (*$P < 0.05$; †$P < 0.01$).

Table 3. Lipid class composition of *Pomacea maculata* eggs.

Lipid	Relative %
HC + ES	2.48 ±0.48
TG	Traces
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: Results are the mean of triplicate analysis ± 1SD. HC: hydrocarbons; ES: esterified sterols; TG: triacylglycerols; FFA: free fatty acids; ST: free sterols; PE: phosphatidylethanolamine; PC: phosphatidylcoline. HC and SE were quantified together.

Figure 1

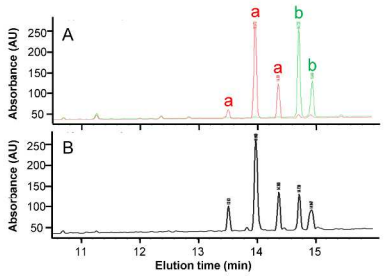

Figure 1.GC chromatogram of PVF polysaccharides from P. maculata. Polysaccharide composition by acidic hydrolysis and derivatization of free monosaccharides. Panel A: Standard: galactose (peaks ¨a¨) and glucose (peaks ¨b¨); Panel B: polysaccharide.
Figure 1
297x420mm (300 x 300 DPI)

Figure 2

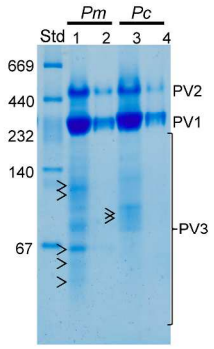

Figure 2. Native-PAGE of P. maculata and P. canaliculata PVF. Std: molecular weight standard; Pm: perivitellin fluid of P. maculata (lanes 1 and 2); Pc: perivitellin fluid of P. canaliculata. (lanes 3 and 4) Lanes 1, 3: 100µg; lanes 2, 4: 10 µg. Unshared PV3 protein bands between the species are indicated by arrowheads.

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297x420mm (300 x 300 DPI)

Figure 3

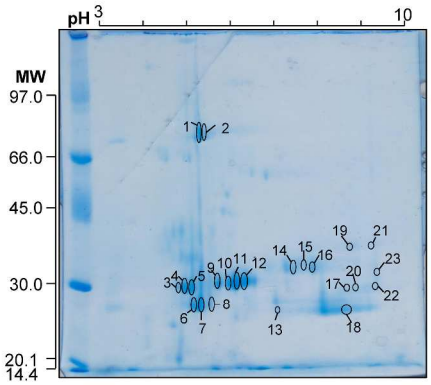

Figure 3. 2-DE of the perivitellin fluid of P. maculata. Numbered spots correspond to tentatively identified proteins (see Supplementary Table).

Figure 3
297x420mm (300 x 300 DPI)

Figure 4

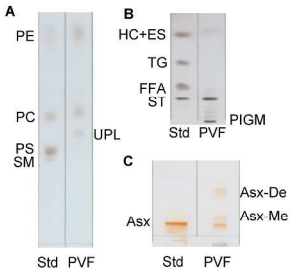

Figure 4. Thin layer chromatography of PVF lipid classes of P. maculata eggs. PVF: Perivitelline fluid lipids A) Polar lipids. PE: phosphatidylethanolamine; PC: phosphatidylcholine; PS: phosphatidylserine; SM: sphingomyelin; UPL: unidentified polar lipid. B) HPTLC of non-polar lipids. ES: esterified sterols, HC: hydrocarbons, FFA: free fatty acid; TG: triacylglycerols; ST: free sterols, PIGM: pigment. C) HPTLC of pigments. Asx: free astaxanthin; Asx Me: astaxanthin mono- ester; Asx De: astaxanthin diester (identified using the data of Pasquevich et al (2014)).

Figure 4
297x420mm (300 x 300 DPI)

Figure 5

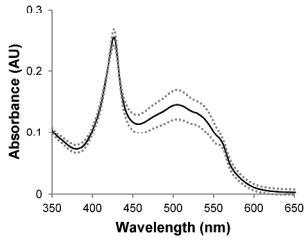

Figure 5. Perivitelline fluid absortion spectrum. Data are the mean of three independent measures (full line) ± SD (dotted lines).
Figure 5
297x420mm (300 x 300 DPI)

Figure 6

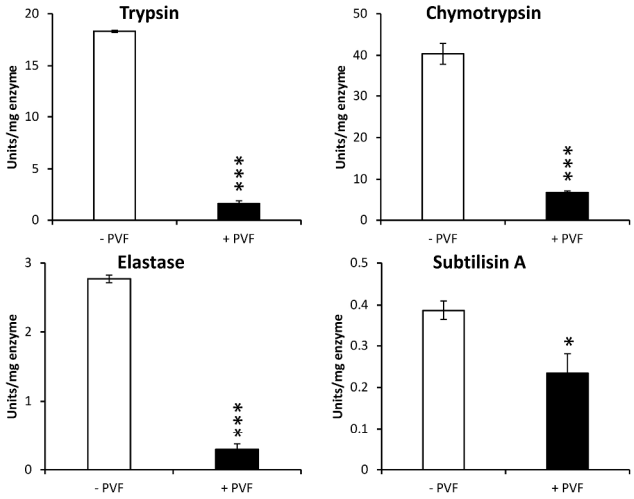

Figure 6. Protease inhibition capacity of P. maculata PVF. Protease specific activity without PVF (-PVF) and after the incubation with P. maculata PVF (+ PVF). Values represent the mean ± SD (n=3). ***p<0.0001; *p<0.05.

Figure 6
297x420mm (300 x 300 DPI)