A peer-reviewed version of this preprint was published in PeerJ on 29 October 2018.

View the peer-reviewed version (peerj.com/articles/5314), which is the preferred citable publication unless you specifically need to cite this preprint.

Giglio M, Garro C, Caviedes-Vidal E, Heras H. 2018. Egg perivitelline fluid of the invasive snail *Pomacea canaliculata* affects mice gastrointestinal function and morphology. PeerJ 6:e5314 https://doi.org/10.7717/peerj.5314
Egg perivitelline fluid of the invasive snail *Pomacea canaliculata* affects mice gastrointestinal function and morphology

Matías Giglio 1, 2, Cintia Garro 3, 4, Enrique Caviedes-Vidal Corresp. 3, 4, Horacio Heras Corresp. 1, 2

1 Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina
2 Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), Consejo Nacional de Investigaciones Científicas y Técnicas y Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina
3 Instituto Multidisciplinario de Investigaciones Biológicas de San Luis (IMIBIO-SL), Consejo Nacional de Investigaciones Científicas y Técnicas y Universidad Nacional de San Luis, San Luis, San Luis, Argentina
4 Departamento de Bioquímica y Ciencias Biológicas, Universidad Nacional de San Luis, San Luis, San Luis, Argentina

Corresponding Authors: Enrique Caviedes-Vidal, Horacio Heras
Email address: ecv@uns1.edu.ar, h-heras@med.unlp.edu.ar

Background. *Pomacea* Apple snails are freshwater, amphibious snails native from South America. Some species such as *P. canaliculata* have become a driver of ecosystemic changes in wetlands and an important rice and taro pest after its introduction to Asia and other parts of the world. Females deposit colored eggs clutches above the waterline, a reproductive strategy that exposes the eggs to harsh conditions and terrestrial predation. However, eggs have no reported predators in their native range, probably by the acquisition of unparalleled biochemical defenses based on a set of proteins (perivitellins) that nourish embryos and protect them from predators and abiotic factors. Notably, ingestion of egg perivitelline fluid (PVF) decreases rat growth rate and alters their gastrointestinal morphology. The aim of the study is to determine the effect of apple snail egg PVF on mice gut digestive activity, morphology and nutrient absorption. Methods. Carbohydrate digestion by intestinal disaccharidases (sucrase-isomaltase and maltase-glucoamilase) was evaluated *ex vivo* in mice gavaged with 1 or 4 doses of PVF. Gut morphological changes and absorptive surface were also determined. In addition, alteration on nutrient absorption rates, transport pathways and intestinal permeability was evaluated by luminal perfusions of small intestine with radiolabeled L-proline (absorbed by paracellular and transcellular pathways) and L-arabinose (absorbed exclusively by paracellular pathway). Results. PVF affected mice which displayed large morphological changes in the small intestine epithelium inducing the appearance of shorter and wider villi as well as fused villi. This resulted in a diminished absorptive surface, notably in the proximal portion. Likewise, the activity of disaccharidases diminished in the proximal portion of the intestine. Total absorption of L-proline increased in treated mice in a dose-dependent manner. There were no differences neither in the ratio paracellular-to-
transcellular absorption of L-proline nor in gut permeability as revealed by the clearance of L-arabinose. Discussion. Oral administration of apple snail PVF to mice adversely alters gut morphophysiology by reducing the intestinal absorptive surface, affecting enzymes of sugar metabolism and increasing the absorption rate of nutrients without affecting the relative contribution of the absorption pathways or gut permeability. These results further support the notion that Pomacea snail eggs possess a passive anti-predator defense targeting the digestive system.
Egg perivitelline fluid of the invasive snail *Pomacea canaliculata* affects mice gastrointestinal function and morphology

Matías L. Giglio†1,2, Cintia Garro‡3,4, Enrique Caviedes-Vidal*3,4, Horacio Heras*1,2.

1 Instituto de Investigaciones Bioquímicas de La Plata, INIBIOLP (CONICET-UNLP). La Plata, Argentina.
2 Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, La Plata, Argentina.
3 Instituto Multidisciplinario de Investigaciones Biológicas de San Luis, Consejo de Investigaciones Científicas y Técnica, San Luis, Argentina.
4 Departamento de Bioquímica y Ciencias Biológicas, Universidad Nacional de San Luis, San Luis, Argentina.

‡ These authors contributed equally to this work.

* Shared Corresponding authors:

Horacio Heras Ph: +54 (221)4824894 ext.119. E-mail: h-heras@med.unlp.edu.ar
Enrique Caviedes-Vidal Ph: +54 (266) 4520300 ext. 6611. E-mail: ecv@unsl.edu.ar
ABSTRACT

Background. Pomacea Apple snails are freshwater, amphibious snails native from South America. Some species such as *P. canaliculata* have become a driver of ecosystemic changes in wetlands and an important rice and taro pest after its introduction to Asia and other parts of the world. Females deposit colored eggs clutches above the waterline, a reproductive strategy that exposes the eggs to harsh conditions and terrestrial predation. However, eggs have no reported predators in their native range, probably by the acquisition of unparalleled biochemical defenses based on a set of proteins (perivitellins) that nourish embryos and protect them from predators and abiotic factors. Notably, ingestion of egg perivitelline fluid (PVF) decreases rat growth rate and alters their gastrointestinal morphology. The aim of the study is to determine the effect of apple snail egg PVF on mice gut digestive activity, morphology and nutrient absorption.

Methods. Carbohydrate digestion by intestinal disaccharidases (sucrase-isomaltase and maltase-glucoamilase) was evaluated *ex vivo* in mice gavaged with 1 or 4 doses of PVF. Gut morphological changes and absorptive surface were also determined. In addition, alteration on nutrient absorption rates, transport pathways and intestinal permeability was evaluated by luminal perfusions of small intestine with radiolabeled L-proline (absorbed by paracellular and transcellular pathways) and L-arabinose (absorbed exclusively by paracellular pathway).

Results. PVF affected mice which displayed large morphological changes in the small intestine epithelium inducing the appearance of shorter and wider villi as well as fused villi. This resulted in a diminished absorptive surface, notably in the proximal portion. Likewise, the activity of disaccharidases diminished in the proximal portion of the intestine. Total absorption of L-proline increased in treated mice in a dose-dependent manner. There were no differences
neither in the ratio paracellular-to-transcellular absorption of L-proline nor in gut permeability as revealed by the clearance of L-arabinose.

Discussion. Oral administration of apple snail PVF to mice adversely alters gut morphophysiology by reducing the intestinal absorptive surface, affecting enzymes of sugar metabolism and increasing the absorption rate of nutrients without affecting the relative contribution of the absorption pathways or gut permeability. These results further support the notion that *Pomacea* snail eggs possess a passive anti-predator defense targeting the digestive system.
INTRODUCTION

*Pomacea* (Caenogastropoda: Ampullariidae), commonly known as apple snails, is a genus of freshwater snails native from South America (Hayes et al. 2009b; Hayes et al. 2012). In contrast to most aquatic gastropods, *Pomacea* snails are amphibious, laying calcareous and conspicuously colored egg masses above the waterline (Heras et al. 2007; Heras et al. 2008), a reproductive strategy considered as a key acquisition associated with their successful diversification and dispersion (Hayes et al. 2009a). Conversely, this reproductive strategy exposes the eggs to harsh conditions like sunlight, desiccation and terrestrial predators (Heras et al. 2007). To cope with that embryos are surrounded by a perivitelline fluid (PVF) that nourish and protects them, mainly composed of polysaccharides and proteins (perivitellins) (Garín et al. 1996; Giglio et al. 2016; Hayes et al. 2015; Heras et al. 2007; Heras et al. 1998). The protective functions described for perivitellins include antioxidant, photoprotective, antiprotease, antinutritive and toxic (Dreon et al. 2004; Dreon et al. 2010; Heras et al. 2008). The presence of noxious proteins is advertised by the egg conspicuous coloration (aposematic coloration) considered a warning signal to potential predators. This complex defensive system is very effective and in fact these apple snail eggs have no predators reported in their native range and only one, the fire ant *Solenopsis geminata*, in southeastern Asia where *P. canaliculata* snails has been introduced and became an invasive species (Yusa et al. 2000).

As the epithelial cells along the digestive tract are fully exposed to food contents, they are a possible target site for defensive proteins. In this regard, plants have evolved a wide array of toxic dietary lectins that interact with the membrane glycoproteins of the luminal side of the gut of predators having an important role in plant seed defenses against predation (herbivory) (Peumans & Van Damme 1995). In animals, however, similar embryo defenses have only been described in *Pomacea* snails (Dreon et al. 2014; Pasquevich et al. 2017). In fact, oral
administration of *P. canaliculata* PVF to rats causes a decrease in its growth rate and induces large morphological changes in the small intestine mucosa which reduce its absorptive surface. Furthermore, *P. canaliculata* PVF showed strong cytotoxic activity on Caco-2 cells in culture, indicating the presence of toxins somehow damaging enterocytes (Dreon et al. 2014). The antiprotease activity of *Pomacea* PVF has also been suggested as an antidigestive defense system (Giglio et al. 2016). Other digestive components that could be targeted by egg defenses are the membrane-bound digestive enzymes of enterocytes such as disaccharidases. However, there is no information on the effect of snail PVF on membrane-bound sugar digestion enzymes.

After sugar and protein digestion, the intestinal absorption of the resulting water-soluble nutrients (e.g. monosaccharides and amino acids) is performed by one of two pathways: transcellular, the transporter-mediated absorption of nutrients through enterocytes and paracellular where nutrients move passively through a small space between enterocytes. Paracellular pathway involves movement of solutes either by diffusion or by solvent drag through the tight-junctions of cells (Pappenheimer 1987). These pathways do not contribute equally to the water-soluble nutrients absorption among vertebrates (Caviedes-Vidal et al. 2008; Fasulo et al. 2013; Lavin et al. 2007). For instance in rodents, paracellular pathway accounts only for 20-30% of glucose and amino acids absorption (Brun et al. 2014; Lavin et al. 2007; Price et al. 2014). There is no information on the effect of PVF on rodent nutrient absorption. We hypothesize that PVF large alteration of small intestine morphology (Dreon et al. 2014) would be accompanied by changes of digestive physiology.

The aim of this study was to better understand the defensive mechanisms of apple snail eggs evaluating the effect of *P. canaliculata* egg extracts (PVF) on gut morphophysiology using mice as a murine model of a potential predator. In particular, we determined the effect of PVF on
mice ability to digest and assimilate carbohydrates, intestinal permeability of water-soluble
nutrients, and on the intestinal morphology and absorptive surface.

Mice exposed to *P. canaliculata* PVF showed strong morphological changes in their small
intestine, with a marked reduction of the absorption surface. At the same time, digestive function
was also altered with an increased absorption rate without changes in permeability and inhibition
of disaccharidases.

**MATERIALS AND METHODS**

**Ethics statement**

All experiments with mice have been made according to institutional animal use regulations and
approved animal use protocols by the Animal Care and Use Committee of the Universidad
Nacional de San Luis (UNSL), protocol number B205/15, and were carried out in accordance
with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

**Egg collection and perivitelline fluid preparation**

Fresh egg masses of *P. canaliculata* were collected from females raised in our laboratory
at Universidad Nacional de La Plata (UNLP) from a colony established with eggs from an
artificial pond at La Plata, Argentina (34°54’38” S; 57°56’17” W). Clutches were rinsed with
ice cold 20 mM Tris-HCl, pH 7.8, and homogenized in a Potter type homogenizer with a buffer:
sample ratio of 3:1 v/w. The crude homogenate were then sequentially centrifuged at 10,000 xg
for 30 min in an Avanti JE centrifuge (J25-50 rotor) and at 100,000 xg for 50 min in a Beckman
L8- centrifuge (Ti 70.1 rotor). The pellet was discarded and the supernatant was considered the
perivelline fluid (PVF). The total protein concentration of the PVF was determined by the method of Lowry et al. (1951).

**Mice**

All experiments were performed using adult male and female BALB/C mice (*Mus musculus*) (28.3 ± 0.5 g; mean ± SEM). They were held in cages at relatively constant temperature (22 ± 2 °C), relative humidity of 35 ± 3%, and with a lighting schedule of 13:11 h light: dark. Animals had *ad libitum* access to water and food.

**Gavage**

Absorption experiments and enzyme assays were performed with three groups of treated mice, 10 animals each (5 males and 5 females). One group was gavaged with 300 µL of PVF fraction (560 µg total protein) 12 h before perfusion (one-dose group). Another group of mice were gavaged with doses of 300 µL of PVF fraction (560 µg total proteins) every 24 h during 4 days before perfusion (four-dose group). The control group (10 mice) received the equivalent volume of water. Oral gavage was performed using a winged needle infusion set and were completed within 30 s.

**Luminal perfusions**

To examine tissue-level absorption, we used *in situ* intestinal luminal perfusions. The animals were anesthetized with isoflurane (1-5%) and oxygen delivered by a vaporizer (Surgivet/Anesco Isotec 4 N° de serie W621107). Mice body temperature during the procedure was maintained using a heating pad at 37 °C (Braintree Scientific Inc., Braintree, Massachusetts). The abdominal cavity was opened with a peritoneal incision and proximal and distal ends of the intestine were identified. The intestine was cannulated at ~ 1 cm from the stomach using a rat gavage needle as the enter cannula and an exit cannula was placed ~ 10 cm away from the first
one, both extremes were secured with sutures. The intestine was flushed with a pre-warmed saline solution (9%) to remove its contents. The saline solution was then evacuated with air. Finally the animal was perfused with a perfusion buffer containing 85 mM NaCl, 5 mM NaHCO$_3$, 2.5 mM KCl, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 10 mM D-glucose, 10 mM L-proline, 10 mM L-arabinose, using a perfusion pump (Watson-Marlow Alitea 400) during 2 hours at a rate of 1 mL min$^{-1}$. During the procedure the perfusate returned to a reservoir and was continuously recirculated. The solutions were labeled with a tracer amount of [1-$^{14}$C]-L-arabinose and [2,3-$^{3}$H]-L-proline (Perkin Elmer). The perfusate was weighted carefully before and after the perfusion. Subsamples (50 μL) of the perfusate collected before and after the perfusion were counted in 4 mL Ultima Gold TM scintillation cocktail (Perkin Elmer) in 8 mL glass scintillation vials with a scintillation counter (Wallac 1409 DSA, Perkin Elmer). At the end of the surgery the animal was euthanized with isoflurane at 5%. The intestine was dissected out and the length and circumference of the perfused segment was measured. After perfusion assay, the whole small intestine (i.e. perfused and non-perfused parts) was removed and divided in three portions of the same length named proximal, medial and distal. Samples of each portion were collected for the following assays.

Absorption of each probe was calculated from the decrease in total radioactivity during the experiment, normalized dividing by the duration (min) of the perfusion and by nominal surface area (cm$^2$) of the perfused section of intestine. For L-arabinose, we also calculated the clearance to account for the slight changes in probe concentration over the course of the experiment. To calculate clearance (µl min$^{-1}$ cm$^{-1}$), we divided absorption rate by [(C$_{initial}$ - C$_{final}$) / ln(C$_{initial}$ / C$_{final}$)], where C stands for probe concentration (Sadowski & Meddings 1993).
Clearance values for L-proline were not calculated because it is absorbed by both carrier-mediated and non-mediated mechanisms.

Since L-proline is absorbed through both paracellular and transcellular pathways, its absorption represents the total absorption, while L-arabinose absorption is only passive and represents the paracellular absorption (Chediack 2001). Using this information we estimate the proportion of paracellular and transepithelial nutrient absorption.

**Enzyme assays**

The activity of two intestinal disaccharidases were measured: sucrase-isomaltase (E.C. 3.2.1.10) and maltase-glucoamilase (E.C. 3.2.1.3), which catalyze the hydrolysis of sucrose and maltose, respectively. Enzyme activity was determined in control and treated animals in the three sections of the small intestine after the intestinal luminal perfusion. We used the colorimetric method developed by Dahlqvist (1968) and modified by Martínez del Río (1990). Briefly, tissues were thawed at 4 °C and homogenized for 30 s in 300 mM mannitol in 1 mM N-2-hydroxyethylpiperazine-Ν’-2-ethanosulfonic acid (Hepes)-KOH, pH 7.0, keeping a 1:100 (v:m) buffer:sample ratio, using a manual homogenizer (Fisher Scientific™ Laboratory Homogenizer, Model 125). The resulting homogenate was diluted 10 and 100 times for sucrose and maltase assays, respectively. Aliquots of 40 µL of diluted intestinal homogenates were incubated with 40 µL of 56 mM sucrose or 56 mM maltase in 0.1 M maleate/NaOH buffer, pH 6.5, at 37 °C for 20 min. The activity of the enzymes was measured following the amount of hydrolyzed glucose using the “Glucosa Liquid plus” kit (GT Laboratorios SRL) following manufacturer instructions. Reactions were allowed to stand for 20 min at room temperature and the absorbance measured at 505 nm. Enzyme activity (µmoles min⁻¹) was determined using a glucose standard curve.
Histological and morphological measurements

Histology was performed following that described in Dreon et al. (2014). In short, intestinal tissue samples from proximal, medial and distal portions were fixed in 10% neutral formaldehyde for 24 h, then dehydrated with ethanol and stored until processed. Samples were then completely dehydrated in ethanol 100% and embedded in paraffin wax. Sections (5-7 μm) were stained for general morphology analysis with with hematoxylin and eosin.

Ten to fifteen villi and crypts from small intestine mucosae were selected at random and their length and width measured. This data was employed to calculate mucosal absorptive surface area following the method of (Kisielinski et al. 2002). The surface area is calculated using mean values of the structures that define the mucosal unit, namely villus length and width, and crypt width. The mucosal-to-serosal amplification ratio $M$ was calculated as follows:

$$M = \frac{(\text{villous with } \ast \text{ villuos length}) + \left(\frac{\text{villous width}}{2} + \frac{\text{crypt width}}{2}\right)^2 - \left(\frac{\text{villous width}}{2}\right)^2}{\left(\frac{\text{villous width}}{2} + \frac{\text{crypt width}}{2}\right)^2}$$

Statistics

Statistical analyses were conducted with R statistical software and results are expressed as mean ± 1 SEM. The effect of PVF on parameters (i.e., enzyme activity, absorption, clearance and paracellular/transcellular ratio) was determined by one way analysis of variance (one way ANOVA) with post-hoc Tukey’s test. The F-values of these and other analyses of variance are presented in the text with the relevant degrees of freedom as subscripts. The significance level selected to accept difference for all statistical analysis performed was $\alpha < 0.05$.

RESULTS
Effect of PVF on mice gastrointestinal morphology

*P. canaliculata* PVF induced large morphological changes in mice small intestine. Villi from treated animals were shorter and wider than those of control mice, some with a “tongue-like” shape (Fig. 1). In addition, an augmented number of goblet cells was observed in the mucosae of treated animals (Fig. 1).

A comparison between the Kisielinsky parameter (*M*) of the proximal portion of intestines of the treated animals showed a decrease in the absorption surface (*F*_{2,227} = 22.14; *P* < 0.001). The decrease was evident in animals exposed to a single dose of PVF (*P* < 0.05) and more markedly after four PVF dosis (*P* < 0.05; Fig. 2). On the other hand, the absorptive surface of the medial portion of intestine was reduced only in animals receiving four doses of PVF (*F*_{2,216} = 33.95; *P* < 0.0001) (Fig. 2). Regardless of the doses administered, the distal portion showed no differences between control and treated animals (data not shown).

Effect of PVF on carbohydrate digestion

The activity of sucrase-isomaltase decreased at the proximal section of the intestine only in one-dose group (*F*_{2,25} = 4.55, *P* < 0.05, Fig. 3 A). In contrast maltase-glucoamilase activity exhibited a dose-dependent decrease at the proximal section of the intestine. (*F*_{2,26} = 3.76, *P* < 0.05, Fig. 3 B). The activity of the two disaccharidases in medial and distal sections of small intestine showed no differences between control and treated groups (*P* > 0.05) (Fig. 3 A-B).

Effect of PVF on intestine permeability

Clearance of L-arabinose shows no differences between the three groups (*F*_{2,23} = 0.96; *P* = 0.3973, Fig. 4 A). The absorption of L-proline, calculated per nominal intestinal area, showed
differences between control group and one-dose group in comparison to four-dose group ($F_{2,25}=19.95, P < 0.0001, \text{Fig.}4\ B$). The percentage of L-proline absorption by paracellular and transcellular did not differ among groups ($F_{2,46}=3.07; P = 0.0563, \text{Fig.}4\ C$).

**DISCUSSION**

**Effect of the PVF on mice intestine physiology and morphology**

When mice food was supplemented with *P. canaliculata* egg PVF, its gastrointestinal tract rapidly undergoes morphological changes, strongly affecting the proximal portion of the small intestine and to a lesser extent the medial portion. These changes resulted in a reduction of the absorption area in both proximal and medial regions of treated animals. Similar morphological effects in the proximal portion of the small intestine of rats ingesting apple snail PVF accompanied by an increased amount of goblet cells and enterocyte proliferation (Dreon et al. 2014). Moreover, similar effects on intestinal mucosa were observed by the oral ingestion of rat with plant toxins such as phytohemagglutinin toxic lectin resulting in disturbed gut morphology with villi shortening and rapid decreases in disaccharidase activities and macromolecular absorption capacity (Bardocz et al. 1995; Linderoth et al. 2006). In fasting animals and in animals fed with a low-quality diet, comparable morphological changes are associated with energy-saving physiological adjustments to reduce the high metabolic cost of the gastrointestinal tissues (Chediack et al. 2012; Cramp 2005; Thaysen & Thaysen 1949). In this regard, several antidigestive (digestion inhibition) and antinutritive (non-digestible) compounds were described in *Pomacea* PVF (Dreon et al. 2010; Giglio et al. 2016; Pasquevich et al. 2017) which combined could render a low-quality diet to a predator.

PVF ingestion not only adversely alters gut morphology buy, in parallel to this, the intestinal function became affected, particularly the activity of some digestive enzymes (antidigestive property). Previous reports showed the PVF inhibit proteases, notably *P. canaliculata* and *P. maculata* PVFs inhibit soluble proteases secreted to the intestinal lumen such as trypsin, chymotrypsin and elastase (Dreon et al. 2010; Giglio et al. 2016). Here we found that PVF has the capacity to also inhibit membrane-bound enzymes, particularly the brush-border
disaccharidases which further extend the enzyme inhibition capacity of apple snail eggs to enzymes of the carbohydrate metabolism. A decrease in intestinal maltase and sucrase activity was also observed in rats administered with phytohemagglutinin (Linderoth et al. 2006). In our experiments, after 12 h exposure to PVF, both maltase-glucoamylase and sucrase-isomaltase diminished its activity in the proximal region of intestine in comparison with control animals. However, while maltase-glucoamylase further diminished its activity after a longer exposure to PVF, these animals restored sucrase-isomaltase activity to control levels. This differential response to stressors was also observed in rodents exposed to temperature, diet and alkaloids (del Valle et al. 2006; del Valle et al. 2004; Pan et al. 1993). Along with morphological changes, a stronger response was observed in the proximal region while the medial and distal portions displayed lesser or no response, respectively. This gut behavior has been described for other oral toxins and is most probably due to the fact that this region is the first one in contact with the ingested toxin (Gavhane & Yadav 2012).

Plant dietary toxic lectins and fasting animals usually display a reduction of gut mucosal area which in term, causes a reduction of nutrient absorption (Ishiguro et al. 1984; Secor 2005a; Secor 2005b). However, apple snail PVF effect on mice small intestine is somewhat different: although the mucosal area is also reduced, aminoacid absorption increased after a long exposure to PVF by mechanisms still unclear. The fast changes in mice gut morphology might be an attempt to adapt to the PVF exposition, which is possible due to their large plasticity (Timmons et al. 2012). In addition, the increase in mucous secretion as suggested by the increased goblet cells in treated mice, is another adaptation isolating and protecting the intestinal surface from the toxic proteins. Further analysis is required to better understand this issue.
286 **Ecological implications**

Eggs are one of the most vulnerable life cycle stages and subjected to intense predation since they are a rich source of easy-to-obtain nutrients for predators (Dussourd et al. 1988).

Unlike this generalized observation *Pomacea canaliculata* snails have evolved an embryo defense system that is very effective against predators since only one egg predator has been reported so far (Dreon et al. 2013; Hayes et al. 2015; Yusa et al. 2000). As mentioned before, this is due to the presence of several overlapping defenses, many targeting the digestive system of putative predators, including antinutritive compounds, enzyme inhibitors, toxic dietary lectins, and toxins affecting intestinal cells (Dreon et al. 2014; Dreon et al. 2013; Dreon et al. 2010; Giglio et al. 2016; Ituarte et al. 2012). Similar combined defenses have been found in plant seeds but as far as we know, in animals this has only been reported in *Pomacea* eggs and partially in the túngara frog foam (Dreon et al. 2013; Dreon et al. 2010; Fleming et al. 2009). Unlike plant seeds, *Pomacea* eggs not only have noxious compounds but their noxiousness is advertised by pigmented proteins providing a conspicuous coloration, a true warning signal preventing predators from consuming them (Heras et al. 2007; Pasquevich et al. 2014).

**CONCLUSIONS**

The ingestion of PVF limits the ability of mice to digest and absorb nutrients altering gut morphophysiology. PVF decrease gut absorptive surface, inhibit disaccharidase activity and affect the absorption rate of nutrients in the small intestine of mice, without affecting the relative contribution of paracellular and transcellular pathways or gut permeability. These results further support the presence of passive anti-predator defenses in the eggs of *Pomacea* snails targeting more aspects of the digestive metabolism that previously thought. This defensive strategy is unparalleled in animals.
ACKNOWLEDGEMENTS

MLG, CG, EC, and HH are members of CONICET, Argentina. We thank PE Fernández and N Scelcio for their help with the histological techniques and analyses. We thank Dra. M Casais for logistic assistance in the Laboratorio de Biología de la Reproducción y Radioisótopos.

REFERENCES

Bardocz S, Grant G, Ewen SW, Duguid TJ, Brown DS, Englyst K, and Pusztai A. 1995. Reversible effect of phytohaemagglutinin on the growth and metabolism of rat gastrointestinal tract. *Gut* 37:353-360. DOI: 10.1136/gut.37.3.353.

Brun A, Price ER, Gontero-Fourcade MN, Fernandez-Marinone G, Cruz-Neto AP, Karasov WH, and Caviedes-Vidal E. 2014. High paracellular nutrient absorption in intact bats is associated with high paracellular permeability in perfused intestinal segments. *Journal of Experimental Biology* 217:3311-3317. DOI: 10.1242/jeb.104927.

Caviedes-Vidal E, Karasov W, Chediack J, Fasulo V, Cruz-Neto A, and Otani L. 2008. Paracellular absorption: a bat breaks the mammal paradigm. *PLoS One* 3. DOI: 10.1371/journal.pone.0001425.

Chediack JG, Funes SC, Cid FD, Filippa V, and Caviedes-Vidal E. 2012. Effect of fasting on the structure and function of the gastrointestinal tract of house sparrows (Passer domesticus). *Comparative Biochemistry and Physiology A* 163:103-110. DOI: 10.1016/j.cbpa.2012.05.189.
Chediack JGC-V, E.; Karasov, W.H. and Pestchanker, M. 2001. Passive Absorption of Hydrophilic Carbohydrate Probes by the House Sparrow Passer Domesticus The *Journal of Experimental Biology* 204:723-731.

Cramp RL, Franklin,C.E. and Meyer, E.A. 2005. The impact of prolonged fasting during aestivation on the structure of the small intestine in the green-striped burrowing frog, *Cyclorana alboguttata*. *Acta Zoologica (Stockholm)* 86:13-24.

Dahlqvist A. 1968. Assay of intestinal disaccharidases. *Analytical Biochemistry* 22:99-107. DOI: 10.1016/0003-2697(68)90263-7.

del Valle JC, Busch C, and López Mañanes AA. 2006. Phenotypic plasticity in response to low quality diet in the South American omnivorous rodent Akodon azarae (Rodentia: Sigmodontinae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 145:397-405. DOI: 10.1016/j.cbpa.2006.07.013.

del Valle JC, Mañanes AAL, and Busch C. 2004. Phenotypic flexibility of digestive morphology and physiology of the South American omnivorous rodent Akodon azarae (Rodentia: Sigmodontinae). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 139:503-512. DOI: 10.1016/j.cbpb.2004.10.013.

Dreon MS, Fernández PE, Gimeno EJ, and Heras H. 2014. Insights into embryo defenses of the invasive apple snail *Pomacea canaliculata*: Egg mass ingestion affects rat intestine morphology and growth. *PLoS Negl Trop Dis* 8:e2961. DOI: 10.1371/journal.pntd.0002961.

Dreon MS, Frassa MV, Ceolin M, Ituarte S, Qiu JW, Sun J, Fernández PE, and Heras H. 2013. Novel animal defenses against predation: A snail egg neurotoxin combining lectin and
pore-forming chains that resembles plant defense and bacteria attack toxins. *PLoS ONE* 8:e63782. DOI: 10.1371/journal.pone.0063782.

Dreon MS, Heras H, and Pollero RJ. 2004. Characterization of the major egg glycolipoproteins from the perivitellin fluid of the Apple Snail *Pomacea canaliculata*. *Mol Reprod Dev* 68:359-364.

Dreon MS, Ituarte S, and Heras H. 2010. The role of the proteinase inhibitor ovorubin in Apple Snail eggs resembles plant embryo defense against predation. *PLoS ONE* 5:e15059. DOI: 10.1371/journal.pone.0015059.

Dussourd DE, Ubik K, Harvis C, Resch J, Meinwald J, and Eisner T. 1988. Biparental defensive endowment of eggs with acquired plant alkaloid in the moth *Utetheisa ornatrix*. *Proceedings of the National Academy of Sciences U S A* 85:5992-5996.

Fasulo V, Zhang Z, Price E, Chediack J, Karasov W, and Caviedes-Vidal E. 2013. Paracellular absorption in laboratory mice: Molecule size-dependent but low capacity. *Comparative biochemistry and physiology Part A, Molecular & integrative physiology* 164:71-76. DOI: 10.1016/j.cbpa.2012.09.008.

Fleming RI, Mackenzie CD, Cooper A, and Kennedy MW. 2009. Foam nest components of the túngara frog: a cocktail of proteins conferring physical and biological resilience. *Proceedings of the Royal Society B: Biological Sciences* 276:1787-1795. DOI: 10.1098/rspb.2008.1939.

Garin CF, Heras H, and Pollero RJ. 1996. Lipoproteins of the egg perivitellin fluid of *Pomacea canaliculata* snails (Mollusca: Gastropoda). *Journal of Experimental Zoology* 276:307-314.
Gavhane YN, and Yadav AV. 2012. Loss of orally administered drugs in GI tract. *Saudi Pharmaceutical Journal* : SPJ 20:331-344. DOI: 10.1016/j.jsps.2012.03.005.

Giglio ML, Ituarte S, Pasquevich MY, and Heras H. 2016. The eggs of the apple snail *Pomacea maculata* are defended by indigestible polysaccharides and toxic proteins. *Canadian Journal of Zoology* 94:777-785. DOI: 10.1139/cjz-2016-0049.

Hayes KA, Burks RL, Castro-Vazquez A, Darby PC, Heras H, Martín PR, Qiu JW, Thiengo SC, Vega IA, Wada T, Yusa Y, Burela S, Cadierno MP, Cueto JA, Dellagnola FA, Dreon MS, Frassa MV, Giraud-Billoud M, Godoy MS, Ituarte S, Koch E, Matsukura K, Pasquevich MY, Rodriguez C, Saveanu L, Seuffert ME, Strong EE, Sun J, Tamburi NE, Tiecher MJ, Turner RL, Valentine-Darby PL, and Cowie RH. 2015. Insights from an integrated view of the biology of apple snails (Caenogastropoda: Ampullariidae). *Malacologia* 58:245-302. DOI: 10.4002/040.058.0209.

Hayes KA, Cowie RH, Jorgensen A, Schultheis R, Albrecht C, and Thiengo SC. 2009a. Molluscan models in evolutionary biology: Apple snails (Gastropoda: Ampullariidae) as a system for addressing fundamental questions. *Amer Malacol Bull* 27:47-58.

Hayes KA, Cowie RH, and Thiengo SC. 2009b. A global phylogeny of apple snails: Gondwanan origin, generic relationships, and the influence of outgroup choice (Caenogastropoda: Ampullariidae). *Biological Journal of the Linnean Society* 98:61-76.

Hayes KA, Cowie RH, Thiengo SC, and Strong EE. 2012. Comparing apples with apples: Clarifying the identities of two highly invasive Neotropical Ampullariidae (Caenogastropoda). *Zoological Journal of the Linnean Society* 166:723-753. DOI: 10.1111/j.1096-3642.2012.00867.x.
Heras H, Dreon MS, Ituarte S, and Pollero RJ. 2007. Egg carotenoproteins in neotropical Ampullariidae (Gastropoda: Architaenioglossa). *Comparative Biochemistry and Physiology C* 146:158-167. DOI: 10.1016/j.cbpc.2006.10.013.

Heras H, Frassa MV, Fernández PE, Galosi CM, Gimeno EJ, and Dreon MS. 2008. First egg protein with a neurotoxic effect on mice. *Toxicon* 52:481-488. DOI: 10.1016/j.toxicon.2008.06.022.

Heras H, Garín CF, and Pollero RJ. 1998. Biochemical composition and energy sources during embryo development and in early juveniles of the snail *Pomacea canaliculata* (Mollusca: Gastropoda). *Journal of Experimental Zoology* 280:375-383.

Ishiguro M, Harada H, Ichiki O, Sekine I, Nishimori I, and Kikutani M. 1984. Effects of ricin, a protein toxin, on glucose absorption by rat small intestine. (Biochemical studies on oral toxicity of ricin. II). *Chemical and Pharmaceutical Bulletin* 32:7.

Ituarte S, Dreon MS, Ceolin M, and Heras H. 2012. Agglutinating activity and structural characterization of scalarin, the major egg protein of the snail *Pomacea scalaris* (d'Orbigny, 1832). *PLoS ONE* 7:e50115. DOI: 10.1371/journal.pone.0050115.

Kisielinski K, Willis S, Prescher A, Klosterhalfen B, and Schumpelick V. 2002. A simple new method to calculate small intestine absorptive surface in the rat. *Clinical and Experimental Medicine* 2:131-135. DOI: 10.1007/s102380200018.

Lavin S, McWhorter T, and Karasov W. 2007. Mechanistic bases for differences in passive absorption. *The Journal of Experimental Biology* 210:2754-2764. DOI: 10.1242/jeb.006114.

Linderoth A, Prykhod'ko O, Ahrén B, Fak F, Pierzynowski SG, and Westrom BR. 2006. Binding and the effect of the red kidney bean lectin, phytohaemagglutinin, in the gastrointestinal
tract of suckling rats. *British Journal of Nutrition* 95:105-115. DOI: 10.1079/BJN20051612.

Martínez del Río C. 1990. Dietary, Phylogenetic, and Ecological Correlates of Intestinal Sucrase and Maltase Activity in Birds. *Physiological Zoology* 63:987-1011.

National Research Council. 2011. *Guide for care and use of laboratory animals*. Washington: Academic Press.

Pan YT, Ghidoni J, and Elbein AD. 1993. The Effects of Castanospermine and Swainsonine on the Activity and Synthesis of Intestinal Sucrase. *Archives of Biochemistry and Biophysics* 303:134-144. DOI: 10.1006/abbi.1993.1264

Pappenheimer JRaR, K.Z. 1987. Contribution of solvent drag through intercellular junctions to absorption of nutrients by the small intestine of the rat. *Journal of Membrane Biology* 100:123-136.

Pasquevich MY, Dreon MS, and Heras H. 2014. The major egg reserve protein from the invasive apple snail *Pomacea maculata* is a complex carotenoprotein related to those of *Pomacea canaliculata* and *Pomacea scalaris*. *Comparative Biochemistry and Physiology B* 169 B:63-71. DOI: 10.1016/j.cbpb.2013.11.008

Pasquevich MY, Dreon MS, Qiu J-W, Mu H, and Heras H. 2017. Convergent evolution of plant and animal embryo defences by hyperstable non-digestible storage proteins. *Scientific Reports* 7. DOI: 10.1038/s41598-017-16185-9

Peumans WJ, and Van Damme EJ. 1995. Lectins as plant defense proteins. *Plant Physiology* 109:347-352.
Price ER, Rott KH, Caviedes-Vidal E, and Karasov WH. 2014. Paracellular nutrient absorption is higher in bats than rodents: integrating from intact animals to the molecular level. *Journal of Experimental Biology* 217:3483-3492. DOI: 10.1242/jeb.105619

Sadowski DC, and Meddings JB. 1993. Luminal nutrients alter tight-junction permeability in the rat jejunum: an in vivo perfusion model. *Canadian Journal of Physiology and Pharmacology* 71:835-839.

Secor SM. 2005a. Evolutionary and Cellular Mechanisms Regulating Intestinal Performance of Amphibians and Reptiles. *Integrative and Comparative Biology* 45:282-294.

Secor SM. 2005b. Physiological responses to feeding, fasting and estivation for anurans. *Journal of Experimental Biology* 208:2595-2609. DOI: 10.1242/jeb.01659

Thaysen EH, and Thaysen JH. 1949. Morphological changes in the gastrointestinal tract of the white rat following inanition. *Acta Pathologica Microbiologica Scandinavica* 26:370-380.

Immons J, Chang ET, Wang J-Y, and Rao JN. 2012. Polyamines and Gut Mucosal Homeostasis. *J Gastrointest Digest Sys* S7:001-009.DOI: 0.4172/jgds.S7-001.

Yusa Y, Sugiura N, and Ichinose K. 2000. Predation on the apple snail, *Pomacea canaliculata* (Ampullariidae), by the Norway rat, *Rattus norvegicus*, in the field. *Veliger* 43:349-353.
Figure 1 (on next page)

Effect of *P. canaliculata* perivitellin fluid on mice small intestine morphology.

Histology of mice fed on a diet without (A,D) or with (B,C,E,F) perivitellin fluid of eggs of *P. canaliculata*, each dose contained 0.56 mg protein. P: proximal section. M: medial section. * fused, “tongue-like” villi. Bar 100 µm.
Changes in small intestine mucosal absorptive surface of mice without (control) or gavaged with perivitellin fluid

Bars represent the mucosal-to-serosal amplification ratio (M), a measure of the absorptive surface of the small intestine of mice without (control) or gavaged with perivitellin fluid, each dose contained 0.56 mg protein. (A) Proximal and (B) medial portion of the intestine.
Disaccharidase activities of the three portions of the small intestine of mice with or without orally administered perivitellin fluid.

Sucrase (A) and maltase (B) activity means ± 1 s.e.m. Control (orange bars), one- (blue bars) and four-doses (green bars) treated mice groups (9 individuals per group) with egg perivitellin fluid. Each dose contained 0.56 mg protein.
Changes in the intestinal permeability mice effected by the perivitellin fluid of eggs of *Pomacea canaliculata*.

Clearance of L-arabinose (A) and L-proline absorption per nominal intestinal surface area (B) after 2-h intestinal perfusion in control mice (9 individuals) and those that received either one (9 individuals) or four (8 individuals) doses of perivitellin fluid of eggs of *P. canaliculata*, and percentage contribution of the paracellular (white bars) and transcellular (yellow bars) pathways of L-proline to the total intestinal absorption (C), using L-arabinose absorption as paracellular absorption reference. Bars represent means ± 1 s.e.m. and bars sharing letters indicate no statistically significant differences ($P > 0.05$).
