



# Biology and Management of Invasive Apple Snails

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# Apple snail perivitellins, multifunctional egg proteins

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## Abstract

Egg reserves of most gastropods are accumulated surrounding the fertilised oocyte as a perivitelline fluid (PVF). Its proteins, named perivitellins, play a central role in reproduction and development, though there is little information on their structural-functional features. Studies of mollusc perivitellins are limited to *Pomacea*. A proteomic study of the eggs of *P. canaliculata* identified over 59 proteins in the PVF, most of which are of unknown function, and have not been isolated and characterised. Information on molecular structure of the most abundant perivitellins of *P. canaliculata* have shown that they possess other functions besides being storage proteins, most remarkably in defence against predation and abiotic factors. They are a cocktail containing at least neurotoxic, antinutritive and antidigestive perivitellins, with others that may provide the eggs with a bright and conspicuous colour (aposematic signal). This review compiles the current knowledge of *Pomacea* perivitellins with emphasis on the novel physiological roles they play in the reproductive biology of these gastropods that have evolved the ability to lay their eggs above the water.

**Additional keywords:** Ampullariidae, egg defences, Mollusca, *Pomacea*, predation, protein structure and function



## Introduction

During vitellogenesis the main components of the egg vitellus (lipids, proteins, carbohydrates) are synthesised either outside or inside the ovary and incorporated into primary oocytes to serve mainly as energetic and structural sources for development. In invertebrates, the major egg-yolk proteins are usually associated with lipids and/or carbohydrates, forming glycolipoproteins, complex water-soluble particles called vitellins or lipovitellins (Wallace *et al.*, 1967). Most gastropods, however, contain a limited amount of vitellus. Instead, egg reserves are accumulated surrounding the fertilised oocyte as a perivitelline fluid (PVF) (Jong-Brink *et al.*, 1983). Therefore, proteins present in the PVF have been termed perivitellins. In spite of the central role vitellins and perivitellins play in reproduction and development, there is little information on their structural features in invertebrates. Studies in molluscs are limited to the perivitellins of *Pomacea*, mostly those of *P. canaliculata*, but also of *P. scalaris* and *P. maculata* (Fig. 1). A proteomic study of the eggs of *P. canaliculata* identified over 59 proteins in the PVF of this species (Table 1), most of which are of unknown function and have not been isolated and characterised (Sun *et al.*, 2012).



**Fig. 1.** The colour of egg clutches of *Pomacea* species is provided by pigmented perivitellins, which have only been studied in the three species depicted here (perivitellin names in brackets). The conspicuous and bright colour is presumably an aposematic or warning signal. (Photos: H. Heras and M.Y. Pasquevich)

This review compiles the current knowledge of *Pomacea* perivitellins with emphasis on their structure and the novel physiological roles they play in the reproductive biology of these freshwater gastropods that have the unusual strategy of laying their eggs above water.

## Structure and functions

### *The perivitellins of Pomacea canaliculata*

#### *Structural aspects*

**BIOCHEMICAL COMPOSITION** Although the partial sequences of all the proteins present in the eggs of this species have been reported (Sun *et al.*, 2012), detailed biochemical, structural and functional information is only available for two perivitellins, named ovorubin (PcOvo) (Cheesman, 1958) and perivitellin-2 (PcPV2). They are large particles composed of a small number of subunits (oligomeric proteins). PcOvo is a 300 kDa multimer of a combination of multiple copies of three different ~30 kDa subunits, and PcPV2 is a 400 kDa octamer of four heterodimers (Frassa *et al.*, 2010). PcOvo and PcPV2 particles occur in large quantities in the PVF of *P. canaliculata* and are the most abundant perivitellins (57.0 % and 10 % of egg total protein, respectively) (Garin *et al.*, 1996; Heras & Pollero, 2002; Dreon *et al.*, 2006). They form glyco-lipoproteic complexes with variable amounts of sugars attached.

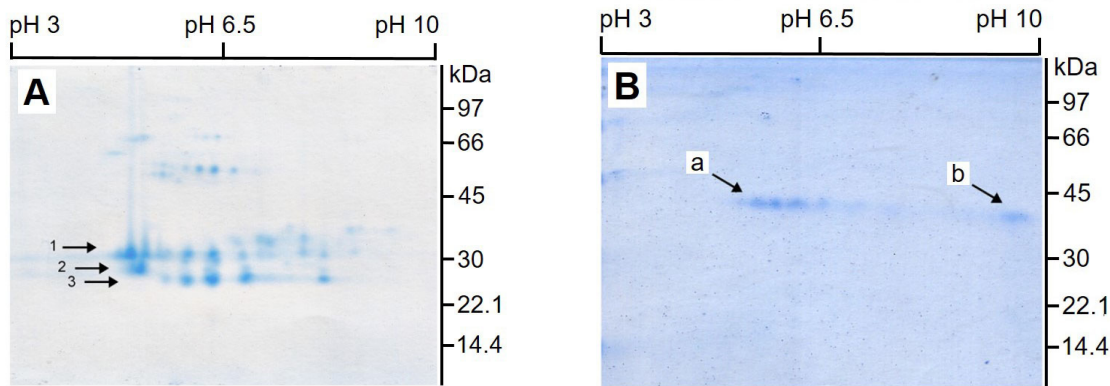
All subunits of PcOvo are highly glycosylated (17.8 % w/w carbohydrates) giving rise to glycoforms; that is, different copies of a polypeptide bear different amounts and types of oligosaccharides (Fig. 2A, C). PcPV2 contains only 2.5 % w/w carbohydrates. Mannose is the major monosaccharide in both perivitellins, but sialic acid and fucose are also present, as in other mollusc glycoproteins (Dreon *et al.*, 2004a; Ituarte *et al.*, 2010). The different glycosylation patterns of the two major perivitellins of *P. canaliculata* probably allow the differential uptake and protein targeting of these molecules observed during apple snail embryogenesis (Heras *et al.*, 1998).

These perivitellin complexes contain low but physiologically relevant amounts of lipids. PcOvo and PcPV2 lipid moieties are mainly composed of typical membrane lipids. There is also a complex fraction called PcPV3, which contains a third of all egg lipids, mainly neutral lipids and phospholipids (Garin *et al.*, 1996).

**Table 1.** Proteome of the PVF of *Pomacea canaliculata*. Proteins that have been biochemically characterized are grouped by their sequence similarity and within groups according to their abundance (highest to lowest) in the PVF.

Protein name <sup>a</sup>	Unigene /GenBank	Tissue of origin
PcOvo carotenoprotein subunits and related proteins		
PcOvo1	SSH9 / JQ818217	Albumen gland
novel protein	SSH2 / JQ818214	Albumen gland
novel protein	SSH20	Albumen gland
PcOvo2	SSH4 / JQ818215	Albumen gland
PcOvo3	SSH8 / JQ818216	Albumen gland
novel protein	SSH95 / JQ818222	Albumen gland
novel protein	SSH122	Albumen gland
ovomucoid / egg protease inhibitor	SSH140	Albumen gland
novel protein	SSH3	Albumen gland
PcPV2 neurotoxin subunits		
PcPV2 membrane attack complex and perforin (MACPF)	SSH208 / JX155861	Albumen gland
PcPV2 tachylectin-like protein	SSH115 / JX155862	Albumen gland
tachylectin-related protein	SSH218	Albumen gland
Other proteins		
cell adhesion protein	TSA:Pc109422	Extra-glandular
thioester-containing protein	TSA:Pc66440	Extra-glandular
thioester-containing protein	TSA:Pc111579	Extra-glandular
apoptosis-inducing factor	TSA:Pc123838 / JQ818223	Extra-glandular
thioester-containing protein	TSA:Pc108510	Extra-glandular
C1q domain containing protein	SSH36 / JQ818219	Albumen gland
alpha-2-macroglobulin-like protein	TSA:Pc379	Extra-glandular
novel protein	TSA:Pc54251	Extra-glandular
transferrin	TSA:Pc99555	Extra-glandular
melanotransferrin	TSA:Pc119627	Extra-glandular
melanotransferrin	TSA:Pc124918	Extra-glandular
transferrin	TSA:Pc101904	Extra-glandular
novel protein	TSA:Pc75576	Extra-glandular
novel protein	TSA:Pc46253	Extra-glandular
scavenger receptor cysteine-rich protein	TSA:Pc52282	Extra-glandular
neurotrypsin-like	SSH25	Albumen gland
FG-GAP repeat protein	TSA:Pc94087	Extra-glandular
melanotransferrin-like	TSA:Pc49292	Extra-glandular
novel protein	TSA:Pc47034	Extra-glandular
tachylectin-like protein	TSA:Pc52841	Extra-glandular
novel protein	TSA:Pc59410	Extra-glandular
novel protein	TSA:Pc65629	Extra-glandular
novel protein	SSH111	Albumen gland
novel protein	SSH6	Albumen gland
Niemann-Pick disease type C2-like (cholesterol-binding glycoprotein)	TSA:Pc29496	Extra-glandular
thioester-containing protein	TSA:Pc112854	Extra-glandular
cysteine rich transmembrane BMP regulator	TSA:Pc53884	Extra-glandular
ubiquitin	TSA:Pc86328	Extra-glandular
15-hydroxyprostaglandin dehydrogenase	TSA:Pc117840	Extra-glandular
transmembrane protease serine	TSA:Pc119142	Extra-glandular
alpha-2-macroglobulin	TSA:Pc71049	Extra-glandular
telomeric repeat-binding factor 2-interacting protein	TSA:Pc87491	Extra-glandular
beta actin	TSA:Pc17674	Extra-glandular
scavenger receptor cysteine-rich protein	SSH42	Albumen gland
peptidoglycan recognition protein SIL <sup>SF</sup>	SSH14 / JQ818218	Albumen gland
novel protein	SSH94	Albumen gland
copper/zinc superoxide dismutase (SOD)	TSA:Pc101185	Extra-glandular
kunitz-like protease inhibitor	SSH51 / JQ818220	Albumen gland
Gap-Pol polyprotein-like	TSA:Pc100845	Extra-glandular
chitotriosidase (chitinase)	TSA:Pc120886	Extra-glandular
selenium-dependent glutathione peroxidase (GPx)	TSA:Pc114721	Extra-glandular
novel protein	TSA:Pc94566	Extra-glandular
aldehyde dehydrogenase	TSA:Pc106229	Extra-glandular
calcium-binding protein	SSH86 / JQ818221	Albumen gland
aldehyde oxidase	TSA:Pc120959	Extra-glandular
lysosome-associated membrane glycoprotein 1	TSA:Pc122290	Extra-glandular
novel protein	TSA:Pc34343	Extra-glandular

<sup>a</sup> Proteins were annotated by BLAST against the NCBI Non-redundant Protein Database. A protein with no homologue in this database is listed as a novel protein. Modified from Sun *et al.* (2012).



**Fig. 2.** Two-dimensional electrophoretic analysis of PcOvo egg perivitellins highlighting the variety provided by the attachment of carbohydrates to subunits and the presence of several isoelectric point (pI) isoforms. A: PcOvo subunits: pI isoforms of the 35 kDa subunit (1), pI isoforms of the 32 kDa subunit (2), pI isoforms of the 28 kDa subunit (3). B: chemically deglycosylated PcOvo – acidic pI spots (a), basic pI spots (b). Data from Ituarte *et al.* (2010).

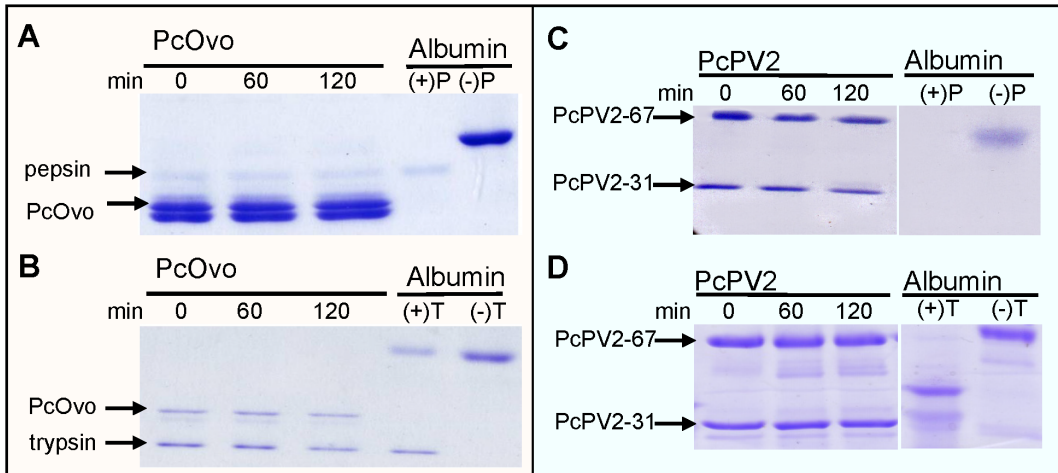
The PcOvo and PcPV3 fractions are pigmented with the carotenoid astaxanthin (Dreon *et al.*, 2004b). Astaxanthin is frequently observed in invertebrate carotenoproteins and its non-covalent binding to PcOvo is strong and specific (Dreon *et al.*, 2007). The presence of pigmented perivitellins (carotenoproteins) that provide eggs with their conspicuous colour seems to be an acquisition of most aerial egg-laying ampullariids (Heras *et al.*, 2007; Hayes *et al.*, 2009).

**STRUCTURE AND STABILITY** In spite of being essential for understanding protein function, knowledge of the structure of gastropod perivitellins is mostly limited to those of *Pomacea canaliculata*.

The cDNA sequences coding for the three PcOvo subunits show no similarity to any known sequence, though they are related among themselves (Table 1) (Dreon *et al.*, 2002, 2003; Sun *et al.*, 2012). However, the cDNA sequences of the PcPV2 subunits show that the small subunit has homology with tachylectins (sugar binding proteins), while the large subunit has high identity with a family of pore forming proteins known as membrane attack complex and perforin (MACPF) (Table 1). Both types of proteins are involved in the immune system in other animals.

Studies of the shape of native PcOvo and PcPV2 by small angle X-ray scattering (SAXS) showed that they are globular proteins with an anisometric shape of 130 x 63 Å and 130 x 44 Å, respectively (Dreon *et al.*, 2008; Frassa *et al.*, 2010).





**Fig. 3.** *In vitro* digestibility of PcPV2 and PcOvo analysed by SDS-PAGE. A, C: gastric digestion – lanes 1-3, 0, 60 and 120 min incubation, lanes 4 and 5 positive and negative controls, respectively. B, D: duodenal digestion – lanes 1-3, 0, 60 and 120 min incubation; lanes 4 and 5, positive and negative controls, respectively. Positive controls were albumin with enzyme, negative controls were albumin without enzyme. PcPV2-67 is the 67 KDa subunit, PcPV2-31 is the 31 KDa subunit. Data from Dreon *et al.* (2010, 2013).

The removal of astaxanthin does not affect the thermal or chemical structural stability of PcOvo, suggesting that it is not essential for protein stability, in contrast to most other vertebrate and invertebrate carotenoproteins (Dreon *et al.*, 2007).

PcOvo and PcPV2 are stable over a wide pH range. PcOvo shows no structural perturbations between pH 4.5 and pH 12.0, while PcPV2 is not altered between pH 4.0 and pH 10.0, a remarkably wide range for oligomeric proteins (Dreon *et al.*, 2008, 2013). PcOvo is highly stable thermally up to 100 °C (Dreon *et al.*, 2007), while PcPV2 is stable up to 60 °C (Frassa *et al.*, 2010). Both PcOvo and PcPV2 are highly resistant to the combined action of pepsin and trypsin proteases (Dreon *et al.*, 2010, 2013) (Fig. 3).

### Functions

**SOURCE OF NUTRIENTS** The major perivitellins of *P. canaliculata* are storage proteins that provide energetic and structural precursors for the developing embryo (Garín *et al.*, 1996; Heras *et al.*, 1998). In particular, PcOvo carries and stabilises astaxanthin within the PVF. Astaxanthin is a potent antioxidant that could be readily incorporated into the cytoplasmic membranes of the embryo for protection (Dreon *et al.*, 2004b) when PcOvo is taken up by the embryos at later development stages (Heras *et al.*, 1998).



*DEFENCE AGAINST PREDATION* In contrast with most eggs, which are intensely predated because of their high nutritional value, the eggs of *P. canaliculata* have only one reported predator, the fire ant *Solenopsis geminata* (see Yusa, 2001) (Fig. 4). This lack of predation is due to a suite of defences that are currently under active research. In fact, *P. canaliculata* has developed some fascinating mechanisms at the biochemical level, in which perivitellins play a central role. At present, of the 59 proteins of the PVF proteome, three proteinaceous components of the egg defences have been identified: pigmented perivitellins responsible for the warning colour of the clutches, a neurotoxin and an antinutritive/antidigestive perivitellin. Fig. 5 summarizes the role of the studied perivitellins in embryo defence.

The conspicuous reddish-pink colour of the eggs of *P. canaliculata* is provided by the pigmented perivitellins PcOvo and PcPV3, and presumably advertises to visually hunting predators the presence of egg defences, that is, it is an aposematic signal (Heras *et al.*, 2007).

PcPV2 is a potent neurotoxin that damages the spinal cord of mice, causing death within 30 hours. PcPV2 induces neuronal apoptosis and alterations in calcium homeostasis and glycan expression in the dorsal horn of the spinal cord, which may play a role in the neurological disorders it induces (Heras *et al.*, 2008; Fernández *et al.*, 2011).

*Pomacea canaliculata* is the first animal known to have a proteinaceous egg neurotoxin. If orally administered to rats at sublethal concentrations, this unusual neurotoxin, combining a lectin (presumably a delivery subunit) and a pore forming subunit (presumably the toxic moiety), is able to reach the intestine in a biologically active conformation, binding to glycocalix of enterocytes and eventually reaching general circulation (Dreon *et al.*, 2013). But PcPV2 is rather slow-acting and it seems unlikely that it could by itself account for the presence of only one predator worldwide.

In studying the structure of PcOvo, another line of defence was found. The high stability of this proteinase inhibitor under a wide range of pH and its resistance to pepsin and trypsin digestion makes it possible, as for PcPV2, to also reach the predator's intestine in a fully active form. PcOvo decreases rat growth when administered orally, probably by combining the inhibition of trypsin activity (antidigestive role) and the resistance of the protein to digestion by gut enzymes (antinutritive), thereby limiting the predator's capacity to digest egg nutrients (Dreon *et al.*, 2010) (Fig. 6). *Pomacea canaliculata* is the first animal known to have antinutritive and antidigestive compounds in its eggs.

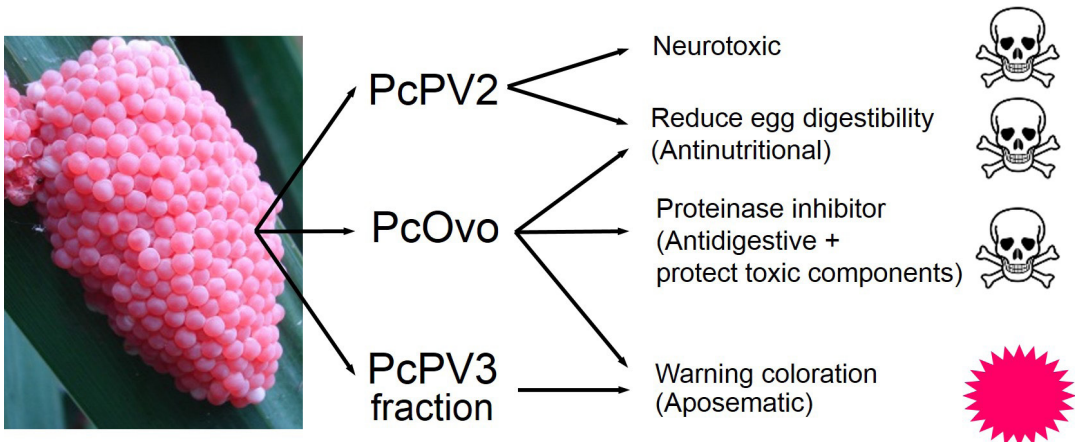


**Fig. 4.** The fire ant *Solenopsis geminata* is the only reported predator of *Pomacea canaliculata* eggs. (Photo: Y. Yusa, with permission)

**OTHER FUNCTIONS** The successful strategy of laying eggs above the water exposes them to a variety of selective challenges imposed by stressful environmental conditions such as solar radiation and desiccation that may affect embryonic development and survival of offspring (Przeslawski 2004; Przeslawski *et al.*, 2004). PcOvo exhibits functions that help the eggs cope with these harsh conditions, in addition to the roles it plays in nutrient storage and defence summarised above. For instance, the saccharide moiety of PcOvo, together with the high content of the polysaccharide galactogen in the PVF (Heras *et al.*, 1998), prevents egg desiccation.

perivitellins not only play a role as antioxidant carriers and warning signal molecules, they may also protect embryos against solar radiation by acting as filters (Dreon *et al.*, 2004b). Another perivitellin that probably complements the antioxidant role of PcOvo

In addition, the pigmented



**Fig. 5.** Components of the biochemical defence system against predation of *Pomacea canaliculata* eggs.

is a copper-zinc superoxide dismutase, a well known enzyme of the antioxidant defence system in almost all eukaryotic cells (Table 1).

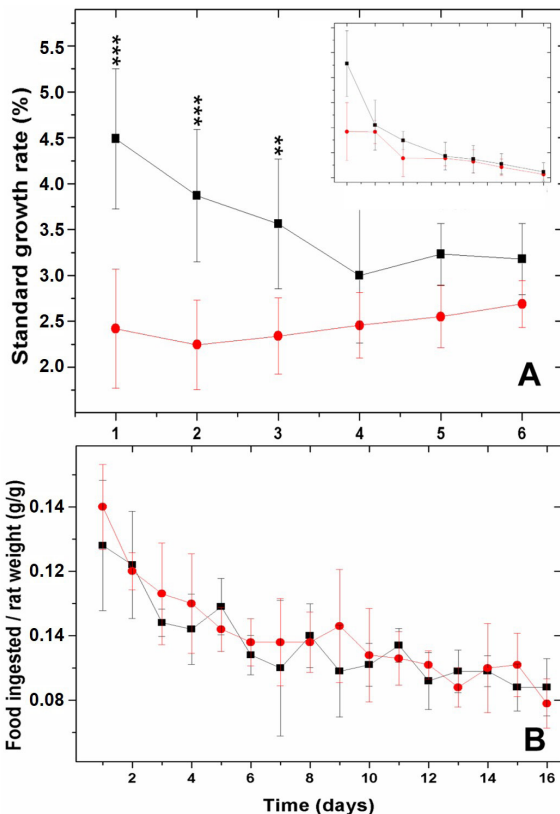
The proteome of PVF also contains several perivitellins possibly related to defence against pathogens, such as a C1q domain-containing protein, a peptidoglycan recognition protein and a chitotriosidase (Table 1). However, although PcOvo had long been assumed to defend the eggs against microbial infections (Norden, 1972), as other egg proteinase inhibitors do (Christeller, 2005), recent studies have found no bactericidal activity of either PcOvo or the whole cytosol against common bacteria like *Escherichia coli* and *Salmonella typhimurium* (Dreon *et al.*, 2010).

More than half (34) of the 59 proteins found in the PVF are of unknown function (Sun *et al.*, 2012), which highlights the need to perform a comprehensive functional characterization through biochemical studies.

### Synthesis

The synthesis of most perivitellins occurs in the albumen gland, an accessory gland of the female reproductive tract that is conspicuously reddish-pink in *P. canaliculata* and *P. maculata* (Fig. 7A). Albumen gland dry weight is mainly represented by ash,

mostly calcium carbonate, the inorganic component of the egg capsule. Calcium, stored by the labyrinth cells (Fig. 7) is largely transferred to the eggs, providing



**Fig. 6.** Effect of diets supplemented with PcOvo on the standard growth rate and food consumption of Wistar rats. A: standard growth rate during the first six days; inset: standard growth rate during 16 days showing rat adaptation to protease inhibitor (axes as in main graph); control (black square), treated (red circle); values represent the mean  $\pm$  1 SD (n = 12); \*\*\* p<0.001, \*\* p<0.01. B: food ingestion during a 16-day experiment showing that the decrease in growth rate is not due to a reduction in food intake. Data from Dreon *et al.* (2010).

an additional defence in the calcareous egg shell and later in the shell of hatchlings. Proteins are the main biochemical component, followed by lipids and carbohydrates (Cadierno, Dreon & Heras, unpublished).

Although extra-glandular synthesis of several components of the PVF was inferred using a suppressive subtractive hybridisation (SSH) cDNA library approach (Sun *et al.*, 2012), the synthesis of most perivitellins including the major components, PcOvo and PcPV2-like precursors, occurs only in the albumen secretory cells of the parenchymal mass of the albumen gland with no circulating perivitellin precursors in the haemolymph (Fig. 6) (Dreon *et al.*, 2002, 2003), in agreement with the perivitellogenic mechanism of these snails (Jong-Brink *et al.*, 1983). Ultrastructural studies describing the parenchymal mass of the albumen gland identified two major cell types, the albumen secretory cells, involved in the synthesis of perivitellins and galactogen, and the labyrinth cells, involved in the storage and delivery of the large calcium levels of the PVF (Fig. 7B-D). Immunoelectron microscopy showed that the albumen secretory cells are the only parenchymal cells involved in the synthesis of PcOvo and PcPV2 and that both proteins are packed into the same secretory granules (Fig. 7C, D) (Catalán *et al.*, 2006).

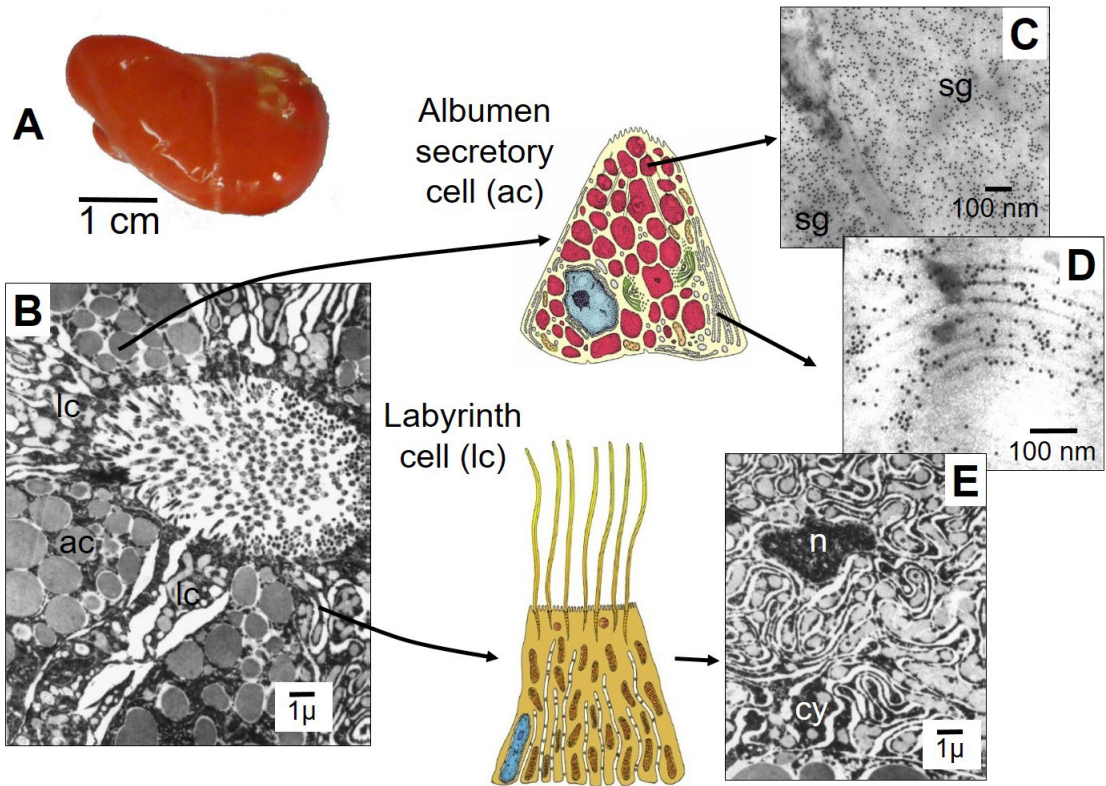
The albumen gland cytosolic fraction has a lethal effect after intraperitoneal administration in mice (Cadierno, Dreon & Heras, unpublished). The toxicity of the organ extract may be due to the presence of an active conformation of the neurotoxin PcPV2. Moreover, mice showed similar symptoms to those given purified PcPV2. This would explain the behaviour of predators, such as the snail kite (*Rostrhamus sociabilis*) and rats (*Rattus norvegicus*), which invariably discard this gland when feeding on adult female *Pomacea* spp. (Snyder & Kale, 1983; Sykes, 1987; Yusa *et al.*, 2000).

### ***The perivitellins of Pomacea scalaris and P. maculata***

The PVF of *P. maculata* resembles that of *P. canaliculata*, with two major perivitellins called PmPV1 (a carotenoprotein) and PmPV2, and a group of low molecular weight proteins called, collectively, the PmPV3 fraction (Pasquevich, Dreon & Heras, unpublished). In contrast, *P. scalaris* eggs contain two major perivitellins of which only the most abundant has been studied; this protein, named scalarin (PsSC), is a carotenoid-binding glycoprotein (Ituarte *et al.*, 2008).

The carotenoproteins of both species show several biochemical and structural similarities with PcOvo. These similarities are probably related to similarities in the reproductive strategy, since all *Pomacea* species lay eggs above the water line.





**Fig. 7.** Synthesis and storage of perivitellins in the albumen gland. A: the albumen gland has a bright and conspicuous colour given by pigmented perivitellins. B: acinus of the parenchymal mass formed by albumen secretory cells (ac) interspersed with labyrinth cells (lc). C: immunocytochemistry of an albumen secretory cell at the electron-microscope level – secretory granules (sg) clearly showing they store large amounts of PcOvo particles, as revealed by an antibody against PcOvo (dots). D: PcOvo is synthesized in the albumen secretory cell, as revealed by its presence in the rough endoplasmic reticulum of an albumen secretory cell immunolabelled with an antibody against PcOvo. E: Labyrinth cells provide the large amount of calcium found in the eggs; note the electron-dense calcium deposits that infiltrate the whole cytoplasmic matrix (cy) and the completely calcified nucleus (n); asterisks indicate mitochondria. From Catalan *et al.* (2006) and Dreon (2003). (Albumen gland photo: M.P. Cadierno)

### Structure

Both the perivitellins of *P. maculata* and *P. scalaris*, PmPV1 and PsSC, respectively, are high molecular weight glycoproteins, composed of multiple subunits.

PmPV1 is the most abundant perivitellin in *P. maculata*, representing more than 50 % of the PVF protein, and is the principal perivitellin providing the reddish-pink colour to

the eggs. It is a high molecular weight glycolipo-carotenoprotein (294 kDa) composed of five different isoelectric point (pI) isoform subunits each with mass ~30 kDa. It is highly glycosylated (13 % w/w), while the lipid component is less than 1 %.

PsSC has a molecular weight of 380 kDa, and is also composed of subunits with masses around 30 kDa, and with pIs between pH 5.0 and pH 9.0. The carbohydrate content is higher than that of PcOvo and PmPV1, 21 % w/w, and has interesting compositional features, like the presence of xylose and sialic acid. As in PcOvo, these attached sugars generate glycoforms; the multiple subunits normally observed by electrophoresis are due to the presence of glycans attached to a few different polypeptides (Figs. 2B, D) (Ituarte *et al.*, 2010).

The main cofactors of both PmPV1 and PsSC are carotenoids, which give these proteins a characteristic colour. The carotenoid fraction of PmPV1 is composed, as in PcOvo, of non-esterified astaxanthin and astaxanthin monoesters and diesters. PsSC carotenoids are also mostly composed of free astaxanthin, but instead of astaxanthin esters, an unidentified carotenoid was present. These cofactors are not essential for quaternary structure stabilisation in either PmPV1 or PsSC; this is another similarity with PcOvo, and an important difference from other invertebrate carotenoproteins. PsSC also carries phosphate groups attached to serine residues; these groups may represent a phosphorous reserve for the embryo, as has been reported for other egg proteins (Ituarte *et al.*, 2010).

Only N-terminal sequences are known for the PsSC subunits and they are very similar to the N-terminal sequences of PcOvo. No matches with other known proteins were found, probably because of the lack of gastropod egg-protein sequences in the databases (Ituarte *et al.*, 2012).

Regarding structural characteristics, PsSC is a moderately thermally stable protein, showing no important structural alterations up to 60 °C and fully unfolding only at 90 °C. It is also stable over a wide pH range (pH 2.0 to pH 10.0), denaturing only in extremely alkaline conditions (pH 12.5). PsSC is resistant to sequential enzymatic proteolysis by pepsin and trypsin (Ituarte *et al.*, 2012). These structural features of PsSC are also characteristic of PcOvo, and mark a difference between these pigmented perivitellins from *Pomacea* and other invertebrate carotenoproteins (Zagalsky *et al.*, 1990).

## Functions

Apart from its obvious role in providing nutritive molecules to the embryo, other functional aspects of PmPV1 have not yet been studied. As in PcOvo, astaxanthin cofactors are extremely labile in solution, but become protected from degradation when bound to PsSC; thus the perivitellin may be acting as a carotenoid carrier (Ituarte *et al.*, 2008). In this way pigmented perivitellins play a dual role giving pigmentation to the egg and later supplying the embryo with antioxidant molecules (Ituarte *et al.*, 2008).

In addition, PsSC showed the capacity to agglutinate red blood cells from different species, especially rabbits and humans (A and B groups). Haemagglutination is due to the capacity of the protein to recognise and bind plasma membrane carbohydrates; specificity was high towards glucosamine, galactosamine and N-acetyl galactosamine. Although, as mentioned, PsSC is structurally stable in a wide pH and temperature range, lectin activity is more sensitive: the protein was active only between pH 4.0 and pH 8.0 and at temperatures below 60 °C (Ituarte *et al.*, 2012). The presence of as yet unidentified factors that agglutinate rabbit and human erythrocytes has been reported in egg extracts of other ampullariid snails, namely *P. canaliculata* and *Pila ovata*, and in the albumen gland extracts of *Pomacea urceus* (Uhlenbruck *et al.*, 1973; Baldo & Uhlenbruck, 1974). Given the structural stability of PsSC over a wide pH range, especially at acidic pH values, and its protease resistance, it seems plausible that this perivitellin may be involved in an antinutritive defence of the eggs, like PcOvo, and as has been reported for plant lectins (Peumans & Van Damme, 1995); this remains to be explored.

The studies on PsSC and PmPV1 have shown that *Pomacea* species with aerial egg laying strategies have perivitellins with similar structural characteristics that are very different from other invertebrate carotenoproteins. Studies on perivitellins of other ampullariid species, particularly those that lay their eggs under water, will reveal if the acquisition of these peculiar proteins is exclusive to aerial eggs.

## Ecological and evolutionary implications

The shift to aerial oviposition was probably a key event in ampullariid evolution, since the derived taxa are the most speciose and widely distributed (Hayes *et al.*, 2009). Adults have developed noteworthy anatomical and physiological adaptations to leave the water during oviposition (Hayes *et al.*, 2015). Similarly, the evolutionary selective pressure

exerted on apple snail eggs by predators and by the harsh environment probably led to the acquisition of new features in their storage proteins. Data suggest that they have been co-opted into new functions, notably in embryo defences against predation. It appears that at the biochemical level, these adaptations involve a novel group of perivitellins, which in addition to being storage proteins, are multifunctional complexes constituting a suite of very efficient defences against predation and harsh environmental conditions. The presence of proteinase inhibitors/storage proteins that limit the nutritional quality of eggs as a means of defence has not been reported in the animal kingdom, but it is similar to plant defences against herbivory.

Among predator avoidance tactics, conspicuous colour advertises antipredator defence across many taxa. In this regard, *P. canaliculata* is unusual in that PcOvo provides not only the warning signal molecule but also participates in the biochemical defence. This is the only defence model reported so far that involves no trade-offs between conspicuousness and noxiousness by encoding into the same molecule both the aposematic warning signal and an antinutritive/antidigestive defence. In addition, this makes synthesis even more cost-effective because females do not need to ingest toxic prey to endow their eggs with chemical defences. Furthermore, as well as performing these multiple defence roles, PcOvo is a storage protein that is consumed at a later time by developing embryos and hatchlings (Heras *et al.*, 1998). On the whole, *P. canaliculata* egg defences appear to be a solution to allocation costs, opening new perspectives on the study of aposematism and mimicry.

The perivitellin PcPV2 is also unusual among animal toxins, as a lectin-pore-forming combination has not been reported in other species, providing the first evidence of a neurotoxic lectin in animals, and a novel function for ancient and widely distributed proteins (Dreon *et al.*, 2013). In fact, the combination of two unrelated immune polypeptides resulted in a novel protein with neurotoxic properties, a feature differing from the roles classically ascribed to either animal lectins (Vasta & Ahmed, 2008) or perforins (Rosado *et al.*, 2008). The combination of a lectin united with a toxic subunit by a disulphide bridge has only been reported in plant defences against herbivory, for example in ricin seeds (type II ribosome-inactivating protein) and in the bacterial attack neurotoxin of *Chlostridium botulinum* (“BoTox”) (Fig. 8). Comparative analyses of the evolutionary origin of the PcPV2 subunits indicate that both chains evolved separately (Dreon *et al.*, 2013).

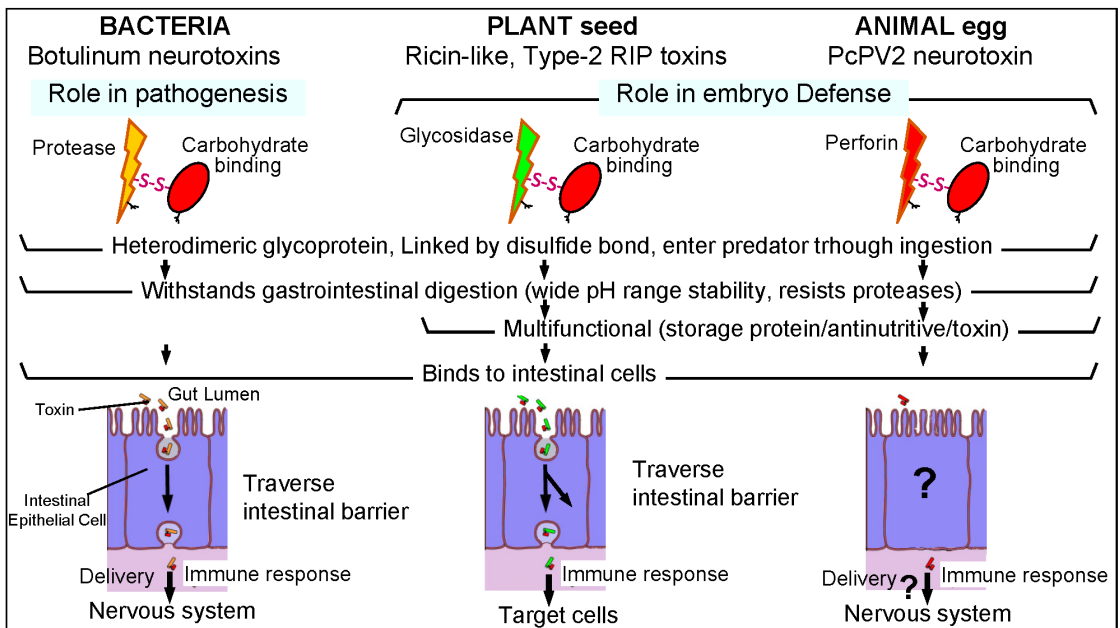
It is worth recalling that eggs and seeds are static targets and, therefore, particularly vulnerable. In this regard, it is interesting that apple snail eggs and plant seeds may both



have developed (passive) biochemical defence systems to protect their embryos as an adaptation to predation, including the preferential accumulation of toxic lectins (Peumans & Van Damme, 1995). When considering the evolution of defences, it is important to remember that something effective against one set of predators may be ineffectual against others. *Pomacea canaliculata* eggs are an exception because there is only one confirmed predator worldwide. It seems that multiple egg defences acting simultaneously would impair the acquisition of nutrients and be toxic to the predator, rendering *P. canaliculata* eggs unusually well defended.

Considering that conspicuously coloured aerial eggs are very frequent across the Ampullariidae, biochemical defences similar to those of *P. canaliculata* are probably more widespread, though more comparative work is needed to test this hypothesis.

Apple snail eggs provide an exceptional model to study the evolution of biochemical and physiological adaptations, which may have profound implications for addressing



**Fig. 8.** Similarities and differences in structure and function of the apple snail PcPV2 neurotoxin and related dichain toxic lectins from bacteria and plants. The PcPV2 toxin is composed of a combination unique in animals: a pore-forming protein (MACPF) strongly attached by disulfide bonds to a sugar-binding protein (lectin). The structures of these so called AB toxins have only been observed in the botulinum neurotoxin and in ricin-like plant seed toxins. These three toxins also share a similar mode of entry into the predator body after ingestion, as they all withstand the harsh gastrointestinal environment, bind to intestinal cells and traverse the intestinal barrier to enter general circulation. Also, similar to seed toxins, PcPV2 is not only a defensive protein but also a storage protein with antinutritive properties. Question marks indicate unknown steps. Data from Dreon *et al.* (2013).

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