Non-digestible proteins and protease inhibitors: Implications for defense of the colored eggs of freshwater apple snails

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Abstract

Apple snails (*Pomacea* Perry, 1810*)* are successful invaders, causing ecological perturbations, economic losses and medical issues. A peculiar trait of this snail is high biological potential, related to the absence of predators of their eggs. Eggs show protease inhibitor (PI) activity, originally ascribed to PcOvo perivitellin in *Pomacea canaliculata* (Lamarck, 1822) but absent in PmPV1, the orthologoue of PcOvo, in *Pomacea maculata* Perry, 1810 eggs. As egg fluid diminishes rat growth rate, an anti-digestive effect, similar to plant defenses against herbivory, was hypothesized. However, PI activity has not been characterized in apple snail eggs. Here we identify and partially characterize *P. canaliculata* egg PI, and improve our knowledge of the quaternary structure and evolution of PcOvo. Through N-terminal, transcriptomic/proteomic sequencing and biochemical validation, we identified a Kunitz-type and a Kazal-type inhibitor which, though at low concentration in the egg, exhibit strong PI activity against trypsin, chymotrypsin, elastase and subtilisin. Additionally, we report three new subunits for the non-digestible storage protein PcOvo. They are likely products of ancient gene duplication, as their sequences exhibit moderate similarity (30%). To our knowledge, this is the first report of Kazal-type inhibition among invertebrate eggs. Inhibiting varied proteases, PI seems an efficient adaptive trait that limits predator´s capacity to digest egg nutrients.

Keywords: protease inhibitor; invasive species; snail; *Pomacea*; predation defenses; egg defenses

Introduction

The apple snail *Pomacea canaliculata* (Lamarck, 1822) is an invasive species. Among the multiple characteristics that make this snail an effective invader stands its high biological potential, as the females have high fecundity and the eggs have a high hatching success (Tamburi and Martín 2011; Gilioli et al. 2017). These eggs are laid outside the water and, despite being red and conspicuous, they have almost no predators, probably due to a set of defenses including toxins, non-nutritive and antidigestive molecules (Dreon et al. 2013; Pasquevich et al. 2017). This latter mechanism of accumulating proteins that impair digestion is of interest since, although widely studied in plant leaves and seeds, it has seldom been identified in animals. For instance, a number of seeds defend embryos against herbivory with antidigestive proteins (protease inhibitors or PIs) often in combination with antinutritive proteins (resistant to digestive proteases). The combined action of these proteins limits predator´s capacity to digest nutrients, lowering the nutritional value of seeds (Felton 2005). In animals, PIs have been reported in many eggs of both vertebrates and invertebrates (Yamashita and Konagaya 1996; Saxena and Tayyab 1997; Han et al. 2008). However, their role in eggs, unlike those in seeds, has been largely interpreted either as regulators of endogenous protease activity, or as a defense against microbial and pathogen proteinases (Benkendorff et al. 2001; Nagle et al. 2001; Wesierska et al. 2005; Han et al. 2008; Hathaway et al. 2010). In fact, their role as a chemical defense against predation in animals has only been reported in the foam nests that surround the eggs of the túngara frog *Engystomops pustulosus* (Lynch, 1970) (Fleming et al. 2009) and in the perivitelline fluid of the eggs of *Pomacea canaliculata* (Dreon et al. 2010; Dreon et al. 2013).

We focus our study in *P. canaliculata* eggs, which display a warning coloration (aposematic), and have only very few reported predators (Yusa 2001; Stevens 2015). It is known that defenses tend to be integrated by multiple noxious substances that aim at different predator targets (Ruxton et al. 2004). Indeed, *P. canaliculata* egg defenses combine antinutritive, antidigestive and neurotoxic proteins, named perivitellins (Dreon et al. 2010; Dreon et al. 2013; Dreon et al. 2014), some affecting rat intestinal morphology and absorptive surface that lead to a decreased growth rate (Dreon et al. 2014). The combination of these varied defenses is similar to plant antipredator strategies and, associated with the aposematic display, would explain the near absence of predators (Hayes et al. 2015).

Among *P. canaliculata* egg defensive perivitellins stands **PcOvo**, a non-digestible oligomeric storage protein largely accumulated in the egg (Dreon et al. 2013). In the early 1970´s, PcOvo (named ovorubin at that time) was reported to inhibit **trypsin** and the microbial proteases **takadiastase**, **pronase** and subtilisin (Norden 1972). It was assumed by this author that **PcOvo** prevented microbial growth acting as an immune molecule. However, the aposematic coloration called for a different interpretation: that PI could also be a defense against predation. Following this hypothesis, later experiments by Dreon et al (2010) reported that **PcOvo** did not prevent bacterial growth and that feeding either egg perivitelline fluid or **PcOvo** to rats diminished their growth rate; thus, the trypsin inhibition activity was linked to a plant-like antidigestive defense. Studies on plant seeds showed that effective antidigestive proteins should be active in a wide pH range and also inhibit a variety of enzymes (Terada et al. 1994). **PcOvo** is indeed stable in a wide range of pH values, although specific inhibitory activity has not been evaluated yet. Recently, PI activity was found in the eggs of a related species, *P. maculata*, but was not ascribed to **PmPV1** (Pasquevich et al. 2017), the orthologue of **PcOvo**. In addition, the

sequences of three **PcOvo** subunits were reported but no protease inhibitor domain was found (Sun et al. 2012).

Here we show that there are three additional subunits in the **PcOvo** oligomer, and that these polypeptides display interesting evolutionary relationships among them and with the carotenoprotein **PmPV1**. We found neither antiprotease motifs nor antiprotease activity in **PcOvo**. Instead, we found that antiprotease activity is confined to an up to now unexplored egg protein fraction, PV3. The present study reports two PIs belonging to the Kunitz (**PcKu**) and Kazal families (**PcKa**) which are active against diverse serine proteases (**trypsin**, **chymotrypsin**, **elastase** and **subtilisin**). We report the first Kazal-type PI among invertebrate eggs.

Methods

Ethics Statement

All studies performed with animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Council 2011) and were approved by the ‘‘Comite´ Institucional de Cuidado y Uso de Animales de Experimentación (CICUAL)’’ of the School of Medicine, UNLP (Permit No. P01-01-2016).

Protein purification

Eggs were collected from a *Pomacea canaliculata* colony reared in the laboratory at 25 °C, 12:12 h L:D photoperiod, and fed with roman lettuce *ad libitum*. Egg proteins were purified from freshly-laid egg clutches (not older than 24 h) by ultracentrifugation, ion exchange and size exclusion chromatography as previously described (Dreon et al. 2013). A workflow diagram is

presented in figure 1. Protein concentration was measured by the method of Lowry (Lowry et al. 1951). Native and SDS-PAGE for samples and molecular weight standards (GE Healthcare, Uppsala, Sweden) were performed in a Mini-Protean III System (Bio Rad, Hercules, CA) following manufacturer's instructions.

PIs were partially purified from PV3, a heterogeneous fraction obtained by density gradient ultracentrifugation (Garín et al. 1996). Several protein fractions were obtained by ion exchange chromatography using a Mono Q column (GE Healthcare) equilibrated with 20 mM Tris/HCl buffer pH 8.5 and using 1 M NaCl as eluting buffer (Dreon et al. 2003). These fractions were tested for PI capacity and positive ones were further analyzed by SDS-PAGE, blotted onto PVDF membranes and submitted for Edman N-terminal sequencing (see below).

PcOvo diversity among clutches

Individual egg masses were collected from different females. **PcOvo** (5 µg) samples (purified as stated above) were denatured in SDS-PAGE sample buffer and subjected to electrophoresis using 15 % polyacrylamide in 0.375 M BisTris gels. The gels were run in 0.05 M MOPS, 0.05 M Tris, 0.001 M EDTA, 0.1 % (p/v) at 100 v, and stained with rapid Coomassie Brilliant Blue stain (Echan and Speicher 2002). Two **PcOvo** samples were further subjected to two-dimensional electrophoresis 2DE, following a method previously described (Pasquevich et al. 2014). In short, the first dimension was carried out with immobilized pH gradient (IPG)-isoelectric focusing (IEF) in an Ettan IPGphor III (GE Healthcare) using 7 cm linear pH 3–10 Immobiline dry strips (GE Healthcare) loaded with 25µg of purified protein. For the second dimension, the IPG strips were sealed on the top of 1.5 mm thick 12% polyacrylamide gels and run at 30 mA. Gels were stained with a colloidal suspension of Coomassie Brilliant Blue R-250 (Sigma-Aldrich).

Perivitellin N-Terminal sequencing

Purified **PcOvo** subunits and HPLC fractions of PV3 were sequenced by Edman degradation at the Laboratorio Nacional de Investigación y Servicios en Péptidos y Proteínas (LANAIS-PRO, Universidad de Buenos Aires—CONICET). The system used was an Applied Biosystems 477a Protein/Peptide Sequencer interfaced with an HPLC 120 for one-line phenylthiohydantoin amino acid analysis. Sequences were deposited in GenBank (accession No: Pca61989_c2_g1).

Sequence analysis

BLASTp searches in NCBI's non-redundant database were applied to identify the Kunitz-type inhibitor (**PcKu**), and manual searches in *P. canaliculata* published egg proteome (Sun et al. 2012) were performed to identify the Kazal-type inhibitor (**PcKa**). Reciprocal best-hit BLAST was employed to find orthologues between the six **PcOvo** subunits and *P. maculata* egg protein subunits (Ip et al. 2018). Sequence alignments were performed using MAFFT (Katoh et al. 2017) with default settings. To determine the divergence of the several pairs of orthologues in the two species of snails, **PmPV1** and **PcOvo** subunits sequences were subjected to phylogenetic analysis using the Maximum Likelihood model and 1000 of bootstrap replicates were applied to construct the tree with the Jones-Taylor-Thornton (JTT) model in MEGA6 (Tamura et al. 2013). Default settings were applied for other options.

3D Homology models of **PcKu** and **PcKa** were constructed using Phyre^2 under the intensive option setting (Kelley et al. 2015). N-Glycosylation sites were predicted with Net-Glyc 1.0 (Blom et al. 2004). Secondary structure was predicted using the JPred 4 (Drozdetskiy et al. 2015) server using default parameters.

Inhibition of protease activity

To assay protease inhibition capacity, the enzymes were preincubated with the protein samples (in 0.02 M Tris/HCl buffer pH 7.0) for 5 min at 20 °C and prior to measuring activity with specific chromogenic substrates; negative controls enzymes were incubated with 0.02 M Tris/HCl buffer pH 7.0. **Trypsin** from bovine pancreas (Sigma-Adrich, #T9935) was assayed with 0.025 mM N-benzoyl-L-arginine ethyl ester (BAEE) in 0.067 M phosphate buffer pH 7.6 at 37 °C, in an enzyme:sample ratio 4 µg:15 µg, measuring absorbance at 253 nm (Schwert and Takenaka 1955). **Subtilisin** from *B. licheniformis* (Sigma-Aldrich #P5380) was also assayed with 0.025 mM BAEE in 0.067 M phosphate buffer, pH 7.6 at 50 °C in an enzyme:sample ratio 12 µg:15 µg (Schwert and Takenaka 1955). **α-Chymotrypsin** from bovine pancreas (Sigma-Aldrich #C3142) was assayed with 1.18 mM N-benzoyl-L-tyrosine ethyl ester (BTEE) in 0.08 M Tris/HCl buffer pH 7.8, 2 M $CaCl_2$ at 25 °C in an enzyme:sample ratio 3.5 µg:15 µg, measuring absorbance at 256 nm (Wirnt and Bergmeyer 1974). **Elastase** from porcine pancreas (Sigma-Aldrich #E1250) inhibition was determined with 4.4 mM N-succinyl-Ala-Ala-Ala p-nitroanilide in 0.1 M Tris/HCl buffer pH 8.0 at 25 °C in an enzyme:sample ratio 1.2 µg:15 µg, measuring absorbance at 410 nm (Bieth et al. 1974). Enzymes and substrates were from Sigma Aldrich, all measurements were performed in triplicate using an Agilent G1103A spectrophotometer (Agilent Technologies), differences between control and inhibited were tested by unpaired Student's *t*-test, using Prism v6.01.

Results

Three new PcOvo subunits but no PI sequences

Apart from the three **PcOvo** subunits already reported (Sun et al 2012), three additional polypeptides were identified by mass spectrometry and transcriptomic analysis as part of the oligomeric particle **PcOvo** (Fig. 2). These proteins, correspond to the previously reported but not identified sequences **PcOvo-4** (*AFQ23945.1*, perivitellin protein SSH95), **PcOvo-5** (*AFQ23937.1*, perivitellin protein SSH2) and **PcOvo-6** (No Pca61989_c2_g1 Ip et al. 2018) and consist of 184, 203, 181 aminoacid residues, respectively. The three new polypeptides share only between 21% to 34% sequence identity with the previously reported subunits (**PcOvo-1**, **PcOvo-2** and **PcOvo-3**) (Fig. 2), though the previously identified sequence motif (GXSWPR) is conserved, as well as the N-glycosylation site (NXS/T). Similarly, secondary structure prediction showed the six subunits share a mixed helix/strand fold, with a conserved pattern.

Interestingly, searches in NCBI non-redundant database showed that each of the six subunits has relatively high sequence similarity (81-94%) to transcripts of *P. maculata*; that correspond to the six subunits of **PmPV1**, the major perivitellin of *P. maculata* (Fig. 3) (Mu et al. 2017*b*; Pasquevich et al. 2017).

Alignments and phylogenetic analysis showed that **PcOvo-6** is more closely related to previously reported **OVO3**, while the other two (**PcOvo-4** and **PcOvo-5**) form an independent group (Supplementary Fig. S1). This large number of moderately similar sequences assembled into one oligomeric protein is intriguing. One possible explanation would be that the subunits were all structurally and functionally equivalent, which led us to evaluate if there was subunit heterogeneity/variability among individuals. An electrophoretic analysis, using of **PcOvo** purified from four egg masses coming from different females, revealed important differences in the number, relative proportion and size of the subunits among individuals (Fig. 4A arrows).

These individual differences are further analyzed by 2DE gels of those **PcOvo** samples showing most differences in SDS PAGE. A marked difference in their isoelectric point profiles was observed (Fig. 4B, arrows), indicating variations in the glycosylation pattern of the subunits, as previously reported (Ituarte et al. 2010).

Remarkably, bioinformatic analyses showed none of the 6 **PcOvo** subunits contain protease inhibitor motifs. In view of this, we screened the all fractions of the egg for PI activity (Fig. 2).

Protease inhibitors are present in PV3 fraction

First, we checked PI activity in the three major egg protein fractions: PV1 (which contains the **PcOvo** perivitellin); PV2 and PV3 (Fig. 1). **Trypsin** inhibition was detected only in fraction PV3, a heterogeneous protein fraction previously reported but not characterized (Garín et al. 1996) (Fig. 5A). This fraction also inhibited the serine proteases **α-chymotrypsin**, **elastase** and **subtilisin** (Fig. 5B).

Chromatography followed by electrophoresis of PV3 fraction indicated that it is composed mainly of 3 polypeptides. N-terminal sequencing allowed us to identify two protease inhibitors - a Kunitz-type (*AFQ23943.1*) and a Kazal-type inhibitor (SSH 140 in Sun et al. 2012).

The Kunitz-type inhibitor (**PcKu**) is 207 residues long, including a signal peptide of the first 21 residues, and three Kunitz motifs arranged in tandem (Fig. 6 A, B). Each domain shows the Kunitz-type serine protease inhibitor superfamily signature ($Fx_2GGCx_6Fx_5C$), and the six-cysteine pattern. The active sites in two of the domains have Lys and Arg residues, indicating affinity to trypsin; the central domain, however, has a Gln residue, indicating it would be inactive. Multiple sequence alignment with other members of the Kunitz family showed that the

three domains have highly conserved sequences which allowed building a 3D model of the inhibitor (Fig. 6C, D).

The Kazal-type inhibitor is a 63 residues polypeptide, **PcKa**. This polypeptide has a single Kazal motif with the six Cys residues signature (C-X_6-C-X_7-C-X_{10}-C-X_8-C-X_7-C) and bears a Leu residue at the P1 position (Fig. 7A, B). According to literature, Kazal PIs with Leu at P1 position inhibit **chymotrypsin**, **pancreatic elastase** and **subtilisin** (Rimphanitchayakit and Tassanakajon 2010); our enzyme inhibition tests confirmed all three inhibitory activities in **PcKa**. The Kazal has moderate to high sequence similarity with those of other animals (Fig. 7D) and a phylogenetic analysis showed high sequence similarity with its ortholog of *P. maculata*.

Discussion

Our understanding of the structure and role of egg chemical defenses against predation lags far behind that of defenses against pathogens. In gastropod eggs there are many reports showing the presence of defensive molecules, mostly with a role in immune protection (Benkendorff et al. 2001; Hathaway et al. 2010). Knowledge on *Pomacea canaliculata* perivitellins provides insights into putative roles for protein inhibitors and antinutritive proteins as defenses against predation. In the present study we further characterized the structure of the major antinutritive protein of the eggs, sequenced and evaluated the activity of a Kunitz-type PI and provide the first report of a Kazal-type PI in invertebrate eggs.

PcOvo subunits and potential functions in eggs

Although **PcOvo** is a physiologically, ecologically, evolutionarily (Heras et al. 2007; Dreon et al. 2008; Dreon et al. 2010; Hayes et al. 2015), and even commercially (Wu and Yang 2008) interesting protein, many aspects of its structure and putative functions remain to be clarified.

The moderate sequence similarity among the 6 **PcOvo** subunits (around 30%) found in this study indicates that gene expansion occurred early in the evolution of the species. The fact that all the paralogs retained a similar function after the expansion is not new (Zhang 2003), and may be related to the very high rate of **PcOvo** synthesis during the reproductive season, as this perivitellin is the most highly expressed transcript of the albumen gland capsule gland complex (Cadierno et al. 2018). The moderate to high divergence among paralogues may be explained through the high structural stability reported for this protein, as it is known that kinetically stable proteins may tolerate several substitutions without general functional loss (Bloom et al. 2006). Indeed, secondary structure prediction shows that the paralogues have conserved helix/strand patterns, indicating that the substitutions did not alter the basic fold of the subunits. Kinetic stability and the non-digestible properties were experimentally demonstrated in the **PcOvo** orthologue **PmPV1**, the major perivitellin of *Pomacea maculata* Perry, 1810, which, when orally administered, was able to pass unaltered through the digestive tract of mice (Pasquevich et al. 2017). On the contrary, these egg storage proteins provide a rich source of amino acids for the developing embryo (Heras et al. 1998; Koch et al. 2009).

We searched Ampubase (Ip et al. 2018) and observed that the newly found **PcOvo** subunits **PcOvo-4** and **PcOvo-6** have high similarity orthologues in *P. maculata*. Sequence similarity between these subunits and their orthologs (>90%) (Mu et al. 2017*b*; Pasquevich et al. 2017) suggests all these subunits were present already in the common ancestors of these two

species of *Pomacea*. As a whole these results further support the hypothesis that gene duplication occurred long before speciation and also suggest short speciation times in the genus (Sun et al. 2012; Pasquevich et al. 2017).

The intraspecific variability observed in the composition of **PcOvo** subunits could be associated to the dual function of the protein both as a nitrogen source for the developing embryo (Heras et al. 1998; Koch et al. 2009) and as an antinutritive (non-digestible) protein for protection against predators (Dreon et al. 2010; Pasquevich et al. 2017). Evidently, these roles of **PcOvo** do not pose a major constraint to maintain a particular amino acid sequence, nor to conserve a strict stoichiometric relationship among subunits, as was observed. However, the exact adaptive significance of this variability is still unclear.

Protease inhibitors of *P. canaliculata* eggs are small proteins

As mentioned, PIs are involved in essential biological roles including defense (Saxena and Tayyab 1997). Early reports in *P. canaliculata* showed that protease inhibition activity was associated to the large and abundant storage protein **PcOvo** (Norden 1972; Dreon et al. 2010). In the present study neither protease inhibitor activity nor inhibitor sequences were detected in **PcOvo**. This discrepancy prompted us to search for antiprotease activity in other egg proteins and found them to be in a low MW fraction of the egg fluid. The methodology employed indicates that PV3 fraction holds *P. canaliculata* egg antiprotease activity, associated to two small serine-protease inhibitors at low concentration. One was the previously reported Kunitz-type inhibitor (**PcKu**) and the other, **PcKa**, is a single domain Kazal-type polypeptide.

The Kunitz inhibitor possesses three inhibitory domains, although bioinformatic analysis of the P1 active residues suggest that only two of them would be active, while the other is inactivated

by a substitution in one of the residues of the active site (Guo et al. 2004; Ranasinghe and McManus 2013). Multi-domain Kunitz inhibitors are present in many invertebrates and often inhibit more than one protease (Rimphanitchayakit and Tassanakajon 2010); in the case of **PcKu** the presence of lysine and arginine residues in the active site, indicates that both active domains would inhibit trypsin. We wondered if the amount of this inhibitor would account for the activity of eggs against the digestive proteases of a potential predator. Assuming an egg clutch has ~300 eggs (Estebenet and Martín 2002), and that a single egg weights ~20 mg (Dreon et al. 2004), there would be an estimate of 200 µg of PV3 proteins (Heras et al. 1998) in an egg clutch. As only 15 µg PV3 was used in the inhibition assays (Fig. 5), the amount of PI of an egg clutch should be sufficient to account for their strong activity against digestive proteases.

The anti-subtilisin activity previously reported was rather puzzling, since Kunitz type inhibitors do not act on this enzyme. This was clarified by the discovery of a Kazal-type inhibitor, **PcKa**. a family of inhibitors known to inhibit **subtilisin**. This is, to our knowledge, the first report of a Kazal inhibitor in invertebrate eggs. The combined activity of these two inhibitors explained the inhibition of **subtilisin** activity, as well as the early reports of **pronase** and **takadiastase** inhibition (Norden 1972). The Kazal polypeptide contains a single inhibitory domain, which is uncommon in invertebrates where multidomain Kazal inhibitors seem to be characteristic (Rimphanitchayakit and Tassanakajon 2010). In some cases invertebrates synthesize a multi-domain polypeptide which is posttranslationally processed to render single-domain Kazal inhibitor (Rimphanitchayakit and Tassanakajon 2010); this would not be the case with apple snail **PcKa** since the mRNA already shows a single domain. The size of both PI lies within the size range reported for PIs (Ryan 1989; Walsh and Twitchell 1991; Gatehouse et al. 1998). Unlike the ubiquitous Kunitz family, the Kazal family (MEROPS I1A.

http://merops.sanger.ac.uk/cgi-bin/famsum?family=i1) is mostly represented in metazoans with a few exceptions.

These two inhibitors display an amino acid composition enriched in cysteine residues, a common feature shared by all members of these PI families. These cysteines form several disulfide bonds that confer stability to heat, pH changes, and proteolysis (Alves García et al. 2004; Teles et al. 2005). This enhanced stability was reported to allow several PIs of plants to withstand the digestive system of predators and, in fact, plant PIs are commonly described as defense-related strategies against herbivory (Joanitti et al. 2006). This seems be the case of apple snail egg inhibitors.

In vertebrates, Kunitz inhibitors play a major role in inflammatory processes while in invertebrates they are involved in a range of diverse functional roles (Ranasinghe and McManus 2013). We interpret the presence of these inhibitors as part of the egg defenses conferring predator resistance. PIs might be involved in decreasing growth rate and the intestinal morpho-physiological alterations observed in model predators fed with *P. canaliculata* perivitelline fluid (Dreon et al. 2014). However, another role regulating endogenous proteolytic activities cannot be discarded. These inhibitors are conserved in other members of the genus like the sympatric species *P. maculata* (Mu et al. 2017*a*).

As a whole, these results further extend the number of apple snail egg perivitellins involved in defenses: 1.- **PcOvo** provides warning coloration (aposematic); 2.- The massive accumulation of **PcOvo** and **PcPV2** (together >70% of total egg protein) provide non-digestible, antinutritive properties; 3.- The much less represented **PcKu** and **PcKa** provide with antiprotease activity against a wide range of digestive enzymes. Moreover, *P. canaliculata* eggs are further protected

by a neurotoxin, and a lectin-like activity affecting intestinal morpho-physiology, generating a cocktail of defenses that, to the best of our knowledge, is unique in nature.

Conclusion

Pomacea canaliculata eggs have acquired perivitellins that may lower the nutritional value of proteins and render egg less appealing to potential predators. In this study we provide evidence that this system includes low amounts of Kazal and Kunitz-type PIs that can act against a wide range of proteases. When eggs are ingested, these inhibitors would increase the half-life of the toxin **PcPV2** and other egg defensive proteins within the digestive tract, further decreasing the nutritional value of non-digestible proteins like **PcOvo**. This system seems evolutionary conserved in other members of the genus. These defenses combined are an efficient adaptive trait that limits predator´s capacity to digest egg nutrients. The success of this strategy, may be related to the invasiveness of these species.

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Figure captions

Figure 1. Workflow diagram of the experiments.

Figure 2. **Sequences of PcOvo subunits**. Multiple sequence alignment of **PcOvo** subunits, showing conserved residues, N-glycosylation sites and secondary structure prediction. **Ovorubin-1**, **Ovorubin-2** and **Ovorubin-3** reported in Sun et al. (2012); **PcOvo-4** (*AFQ23945.1*), **PcOvo-5** (*AFQ23937.1*) and **PcOvo-6** (*Pca61989_c2_g1*) this study.

Figure 3. Phylogenetic tree of PcOvo subunits and their orthologs. The tree was constructed using the Maximum Likelihood method based on the JTT matrix-based model. The percentage of trees in which the associated taxa are clustered together is shown next to the branches. Branch lengths are measured as the number of substitutions per site. The prefixes Pc and Pm indicate *Pomacea canaliculata* (Lamarck 1822) and *Pomacea maculata Perry, 1810*, respectively. **Ovorubin-1** (*AFQ23940*), **Ovorubin-2** (*AFQ23938.1*) and **Ovorubin-3** (*AFQ23939.1*) reported in Sun et al. (2012), **PcOvo-4** (*AFQ23945.1*), **PcOvo-5** (*AFQ23937.1*) and **PcOvo-6** (*Pca61989_c2_g1*) this study.

Figure 4. Differences among egg clutches in the relative proportions of PcOvo subunits. A: SDS-PAGE of **PcOvo** purified from four different egg masses (1-4); B: 2DE gel of egg masses 2 and 3. Arrows indicate major differences between samples.

Figure 5. Protease inhibition activity of major egg protein fractions of *P. canaliculata* eggs. A, Capacity to inhibit **trypsin** of the egg fractions. B, Capacity to inhibit proteases of the PV3 fraction. Control: the corresponding enzyme with buffer instead of PV3. Asterisks indicate Student's *t*-test $p<0.001$.

Figure 6. **Kunitz-type inhibitor from the apple snail eggs**. A, Complete sequence of **PcKu**. Signal peptide is in italics, the three Kunitz-type domains are in bold. B, Sequence diagram showing the three Kunitz-type domains in tandem. C, Homology model. D, Multiple sequence

alignment of the **PcKu** domain with other domains of the family, showing conserved sequence features; inhibitory active residue indicated as P1.

Figure 7. **Kazal-type inhibitor of apple snail egg**. A, Complete sequence of **PcKa**. Signal peptide is in italics, N-terminal sequence is underlined, the Kazal-type domain is in bold. B, Sequence diagram showing the putative disulfide bonds. C, Homology model. D, Multiple sequence alignment of the **PcKa** domain with other domains of the family, showing conserved sequence features; inhibitory active residue indicated as P1.

References

Alves García, V., Machado Freire, M.d.G., Novello, J.C., and Rodrigues Macedo, M.L. 2004. Trypsin inhibitor from *Poecilanthe parviflora* seeds: purification, characterization, and activity against pest proteases. The Prot. J. **23**(5): 343. doi:10.1023/B:JOPC.0000032654.67733.d5.

Benkendorff, K., Davis, A.R., and Bremner, J.B. 2001. Chemical defense in the egg masses of benthic invertebrates: An assessment of antibacterial activity in 39 mollusks and 4 polychaetes. J. Invertebr. Pathol. **78**: 109-118. doi:10.1006/jipa.2001.5047.

Bieth, J., Spiess, B., and Wermuth, C.G. 1974. The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. Biochem. Med. **11**(4): 350-357. doi:10.1016/0006-2944(74)90134-3.

Blom, N., Sicheritz-Pontén, T., Gupta, R., Gammeltoft, S., and Brunak, S. 2004. Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics, **4**(6): 1633-1649. doi:10.1002/pmic.200300771.

Bloom, J.D., Labthavikul, S.T., Otey, C.R., and Arnold, F.H. 2006. Protein stability promotes evolvability. Proc. Natl. Acad. Sci. U.S.A. **103**(15): 5869-5874. doi:10.1073/pnas.0510098103.

Cadierno, M.P., Saveanu, L., Dreon, M.S., Martin, P.R., and Heras, H. 2018. Biosynthesis in the albumen gland-capsule gland complex limits reproductive effort in the invasive Apple Snail *Pomacea canaliculata*. Biol. Bull. **235**(1): 1-11. doi:10.1086/699200.

Council, N.R. 2011. Guide for the Care and Use of Laboratory Animals. 8th ed. National Academies Press, Washington, DC. pp. 220.

Dreon, M.S., Heras, H., and Pollero, R.J. 2003. Metabolism of ovorubin, the major egg lipoprotein from the Apple Snail. Mol. Cell. Biochem. **243**(1-2): 9-14. doi:10.1023/A:1021616610241.

Dreon, M.S., 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**(12): e15059. doi:10.1371/journal.pone.0015059.

Dreon, M.S., Schinella, G., Heras, H., and Pollero, R.J. 2004. Antioxidant defense system in the Apple Snail eggs, the role of ovorubin. Arch. Biochem. Biophys. **422**(1): 1-8. doi:10.1016/j.abb.2003.11.018.

Dreon, M.S., Ituarte, S., Ceolín, M., and Heras, H. 2008. Global shape and pH stability of ovorubin, an oligomeric protein from the eggs of *Pomacea canaliculata*. FEBS J. **275**: 4522-4530. doi:10.1111/j.1742-4658.2008.06595.x.

Dreon, M.S., Fernández, P.E., Gimeno, E.J., 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**(6): e2961. doi:10.1371/journal.pntd.0002961.

Dreon, M.S., Frassa, M.V., Ceolin, M., Ituarte, S., Qiu, J.W., Sun, J., Fernández, P.E., 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**(5): e63782. doi:10.1371/journal.pone.0063782.

Drozdetskiy, A., Cole, C., Procter, J., and Barton, G.J. 2015. JPred4: a protein secondary structure prediction server. Nucleic Acids Res. **43**(W1): W389-394. doi:10.1093/nar/gkv332.

Echan, L.A., and Speicher, D.W. 2002. Protein detection in gels using fixation. Curr. Protoc. Protein Sci. **Chapter 10**: Unit 10 15. doi:10.1002/0471140864.ps1005s29.

Estebenet, A.L., and Martín, P.R. 2002. *Pomacea canaliculata* (Gastropoda: Ampullariidae): life-history traits and their plasticity. Biocell, **26**(1): 83-89. Available from http://www.ncbi.nlm.nih.gov/pubmed/12058384 [accessed.

Felton, G.W. 2005. Indigestion is a plant's best defense. Proc. Natl. Acad. Sci. U.S.A. **102**(52): 18771-18772. doi:10.1073/pnas.0509895102.

Fleming, R.I., Mackenzie, C.D., Cooper, A., and Kennedy, M.W. 2009. Foam nest components of the tungara frog: a cocktail of proteins conferring physical and biological resilience. Proc. Biol. Sci. **276**(1663): 1787-1795. doi:10.1098/rspb.2008.1939.

Garín, C.F., Heras, H., and Pollero, R.J. 1996. Lipoproteins of the egg perivitelline fluid of *Pomacea canaliculata* snails (Mollusca: Gastropoda). J. Exp. Zool. **276**: 307-314. doi:10.1002/(SICI)1097-010X(19961201)276:5<307::AID-JEZ1>3.0.CO;2-S.

Gatehouse, H.S., Christeller, J.T., Burgess, E.P., and Malone, L.A. 1998. In vivo Responses of Honey Bee Midgut Proteases to Two Protease Inhibitors from Potato. J. Insect. Physiol. **44**(2): 141-147. doi:10.1016/S0022-1910(97)00096-6.

Gilioli, G., Pasquali, S., Martin, P.R., Carlsson, N., and Mariani, L. 2017. A temperature-dependent physiologically based model for the invasive apple snail *Pomacea canaliculata*. Int. J. Biometeorol. **61**(11): 1899-1911. doi:10.1007/s00484-017-1376-3.

Guo, H.H., Choe, J., and Loeb, L.A. 2004. Protein tolerance to random amino acid change. Proc. Natl. Acad. Sci. U.S.A. **101**(25): 9205-9210. doi:10.1073/pnas.0403255101.

Han, Y.P., Yu, H.N., Yang, X.B., Rees, H.H., Liu, J.Z., and Lai, R. 2008. A serine proteinase inhibitor from frog eggs with bacteriostatic activity. Comp. Biochem. Physiol. **B 149**(1): 58-62. doi:10.1016/j.cbpb.2007.08.003.

Hathaway, J.J., Adema, C.M., Stout, B.A., Mobarak, C.D., and Loker, E.S. 2010. Identification of protein components of egg masses indicates parental investment in immunoprotection of offspring by *Biomphalaria glabrata* (Gastropoda, Mollusca). Dev. Comp. Immunol. **34**: 425-435. doi:10.1016/j.dci.2009.12.001.

Hayes, K.A., Burks, R.L., Castro-Vazquez, A., Darby, P.C., Heras, H., Martín, P.R., Qiu, J.W., Thiengo, S.C., Vega, I.A., Wada, T., Yusa, Y., Burela, S., Cadierno, M.P., Cueto, J.A., Dellagnola, F.A., Dreon, M.S., Frassa, M.V., Giraud-Billoud, M., Godoy, M.S., Ituarte, S., Koch, E., Matsukura, K., Pasquevich, M.Y., Rodriguez, C., Saveanu, L., Seuffert, M.E., Strong, E.E., Sun, J., Tamburi, N.E., Tiecher, M.J., Turner, R.L., Valentine-Darby, P.L., and Cowie, R.H. 2015. Insights from an integrated view of the biology of

apple snails (Caenogastropoda: Ampullariidae). Malacologia, **58**(1-2): 245-302. doi:10.4002/040.058.0209.

Heras, H., Garín, C.F., and Pollero, R.J. 1998. Biochemical composition and energy sources during embryo development and in early juveniles of the snail *Pomacea canaliculata* (Mollusca: Gastropoda). J. Exp. Zool. **280**: 375-383. doi:10.1002/(SICI)1097-010X(19980415)280:6<375::AID-JEZ1>3.0.CO;2-K

Heras, H., Dreon, M.S., Ituarte, S., and Pollero, R.J. 2007. Egg carotenoproteins in neotropical Ampullariidae (Gastropoda: Architaenioglossa). Comp. Biochem. Physiol. C **146**: 158-167. doi:10.1016/j.cbpc.2006.10.013.

Ip, J.C.H., Mu, H., Chen, Q., Sun, J., Ituarte, S., Heras, H., Van Bocxlaer, B., Ganmanee, M., Huang, X., and Qiu, J.W. 2018. AmpuBase: a transcriptome database for eight species of apple snails (Gastropoda: Ampullariidae). BMC Genomics, **19**(1): 179. doi:10.1186/s12864-018-4553-9.

Ituarte, S., Dreon, M.S., Pasquevich, M.Y., Fernández, P.E., and Heras, H. 2010. Carbohydrates and glycoforms of the major egg perivitellins from *Pomacea A*pple Snails (Architaenioglossa: Ampullariidae). Comp. Biochem. Physiol. B **157**: 66-72. doi:10.1016/j.cbpb.2010.05.004.

Joanitti, G.A., Freitas, S.M., and Silva, L.P. 2006. Proteinaceous protease inhibitors: Structural features and multiple functional faces. Curr. Enzym. Inhib. **2**(3): 199-217. doi:10.2174/157340806777934801.

Katoh, K., Rozewicki, J., and Yamada, K.D. 2017. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform. doi:10.1093/bib/bbx108.

Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., and Sternberg, M.J. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protoc. **10**(6): 845-858. doi:10.1038/nprot.2015.053.

Koch, E., Winik, B.C., and Castro-Vazquez, A. 2009. Development beyond the gastrula stage and digestive organogenesis in the apple-snail *Pomacea canaliculata* (Architaenioglossa, Ampullariidae). Biocell, **33**(1): 49-65. Available from http://www.ncbi.nlm.nih.gov/pubmed/19499886 [accessed.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265-275.

Mu, H., Sun, J., Heras, H., Chu, K.H., and Qiu, J.-W. 2017*a*. Dataset for the proteomic and transcriptomic analyses of perivitelline fluid proteins in *Pomacea* snail eggs. Data in Brief, **15**(Supplement C): 203-207. doi:10.1016/j.dib.2017.09.020.

Mu, H., Sun, J., Heras, H., Chu, K.H., and Qiu, J.W. 2017*b*. An integrated proteomic and transcriptomic analysis of perivitelline fluid proteins in a freshwater gastropod laying aerial eggs. J. Proteomics, **155**: 22-30. doi:10.1016/j.jprot.2017.01.006.

Nagle, G.T., de Jong-Brink, M., Painter, S.D., and Li, K.W. 2001. Structure, localization and potential role of a novel molluscan trypsin inhibitor in *Lymnaea*. Eur. J. Biochem. **268**(5): 1213-1221. doi:10.1046/j.1432-1327.2001.01972.x.

Norden, D.A. 1972. The inhibition of trypsin and some other proteases by ovorubin, a protein from the eggs of *Pomacea canaliculata*. Comp. Biochem. Physiol. B **42**(4): 569-576. doi:10.1016/0305-0491(72)90319-7.

Pasquevich, M.Y., Dreon, M.S., 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*. Comp. Biochem. Physiol. B **169**: 63-71. doi:10.1016/j.cbpb.2013.11.008.

Pasquevich, M.Y., Dreon, M.S., Qiu, J.-W., Mu, H., and Heras, H. 2017. Convergent evolution of plant and animal embryo defences by hyperstable non-digestible storage proteins. Sci. Rep. **7**(1). doi:10.1038/s41598-017-16185-9.

Ranasinghe, S., and McManus, D.P. 2013. Structure and function of invertebrate Kunitz serine protease inhibitors. Dev. Comp. Immunol. **39**(3): 219-227. doi:10.1016/j.dci.2012.10.005.

Rimphanitchayakit, V., and Tassanakajon, A. 2010. Structure and function of invertebrate Kazal-type serine proteinase inhibitors. Dev. Comp. Immunol. **34**(4): 377-386. doi:10.1016/j.dci.2009.12.004.

Ruxton, G.D., Sherratt, T.N., and Speed, M.P. 2004. Avoiding attack: The evolutionary ecology of crypsis, aposematism, and mimicry. Oxford Univ.Press, Oxford, U.K.

Ryan, C.A. 1989. Proteinase inhibitor gene families: strategies for transformation to improve plant defenses against herbivores. BioEssays : news and reviews in molecular, cellular

and developmental biology, **10**(1): 20-24. Available from http://www.ncbi.nlm.nih.gov/pubmed/2653308 [accessed.

Saxena, I., and Tayyab, S. 1997. Protein proteinase inhibitors from avian egg whites. Cell. Mol. Life Sci. **53**(1): 13-23. doi:10.1007/PL00000575.

Schwert, G.W., and Takenaka, Y. 1955. A spectrophotometric determination of trypsin and chymotrypsin. Biochim. Biophys. Acta, **16**(4): 570-575. Available from http://www.ncbi.nlm.nih.gov/pubmed/14389277 [accessed.

Stevens, M. 2015. Evolutionary Ecology: Insect Mothers Control Their Egg Colours. Curr. Biol. **25**(17): R755-757. doi:10.1016/j.cub.2015.07.010.

Sun, J., Zhang, H., Wang, H., Heras, H., Dreon, M.S., Ituarte, S., Ravasi, T., Qian, P.Y., and Qiu, J.W. 2012. First proteome of the egg perivitelline fluid of a freshwater gastropod with aerial oviposition. J. Proteome. Res **11**: 4240-4248. doi:10.1021/pr3003613.

Tamburi, N.E., and Martín, P.R. 2011. Effects of food availability on reproductive output, offspring quality and reproductive efficiency in the apple snail *Pomacea canaliculata*. Biol. Invasions, **13**(10): 2351-2360. doi:10.1007/s10530-011-0047-2.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. **30**(12): 2725-2729. doi:10.1093/molbev/mst197.

Teles, R.C., Calderon, L.A., Medrano, F.J., Barbosa, J.A., Guimaraes, B.G., Santoro, M.M., and de Freitas, S.M. 2005. pH dependence thermal stability of a chymotrypsin inhibitor

from *Schizolobium parahyba* seeds. Biophys. J. **88**(5): 3509-3517. doi:10.1529/biophysj.104.045682.

Terada, S., Fujimura, S., Kino, S., and Kimoto, E. 1994. Purification and characterization of three proteinase inhibitors from Canavalia lineata seeds. Biosci. Biotechnol. Biochem. **58**(2): 371-375. doi:10.1271/bbb.58.371.

Walsh, T.A., and Twitchell, W.P. 1991. Two Kunitz-type proteinase inhibitors from potato tubers. Plant Physiol. **97**(1): 15-18. doi:10.1104/pp.97.1.15

Wesierska, E., Saleh, Y., Trziszka, T., Kopec, W., Siewinski, M., and Korzekwa, K. 2005. Antimicrobial activity of chicken egg white cystatin. World J. Microbiol. Biotechnol. **21**(1): 59-64. Available from http://www.scopus.com/inward/record.url?eid=2-s2.0-12944263581&partnerID=40 [accessed.

Wirnt, R., and Bergmeyer, H.U. 1974. Chymotrypsin. *In* Methods of enzymatic analysis. *Edited by* H.U. Bergmeyer and K. Gawehn. Academic Press, New York. pp. 1009-1012.

Wu, Y.-L., and Yang, C.-C. 2008. Extraction of natural astaxanthin from eggs and gonads of snails. Bioptik Technology, Inc., Taiwan, US patent 435148000. p. 6pp.

Yamashita, M., and Konagaya, S. 1996. A novel cysteine protease inhibitor of the egg of chum salmon, containing a cysteine-rich thyroglobulin-like motif. J. Biol. Chem. **271**(3): 1282-1284. Available from http://www.ncbi.nlm.nih.gov/pubmed/8576113 [accessed.

Yusa, Y. 2001. Predation on eggs of the apple snail *Pomacea canaliculata* (Gastropoda: Ampullariidae) by the fire ant *Solenopsis geminata*. J. Mollus. Stud. **67**: 275-279. doi:10.1093/mollus/67.3.275.

Zhang, J. 2003. Evolution by gene duplication: An update. Trends Ecol. Evol. **18**(6): 292-298. doi:10.1016/S0169-5347(03)00033-8.

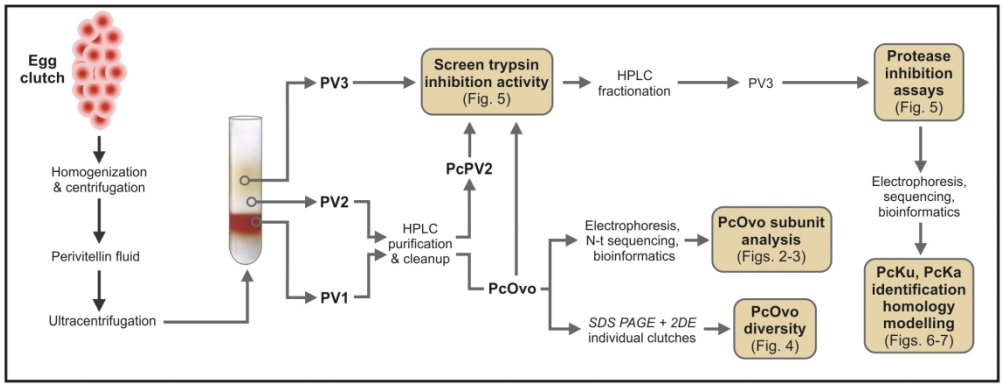

Figure 1. Workflow diagram of the experiments.

Figure 2

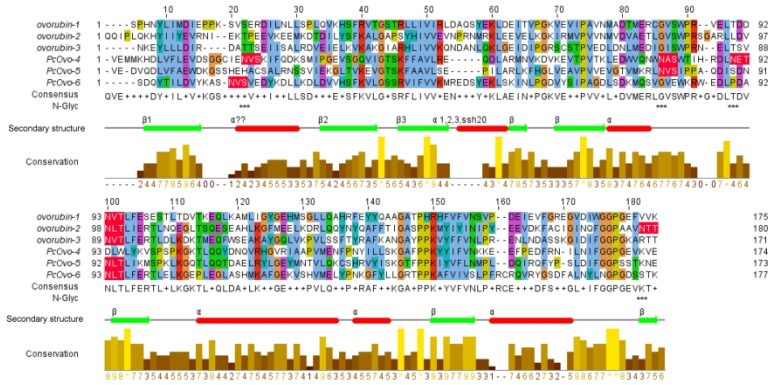

Figure 2. Sequences of PcOvo subunits. Multiple sequence alignment of PcOvo subunits, showing conserved residues, N-glycosylation sites and secondary structure prediction. ovorubin-1, ovorubin-2 and ovorubin-3 reported in Sun et al. (2012); PcOvo-4 (AFQ23945.1), PcOvo-5 (AFQ23937.1) and PcOvo-6 (Pca61989_c2_g1) this study.

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Figure 3

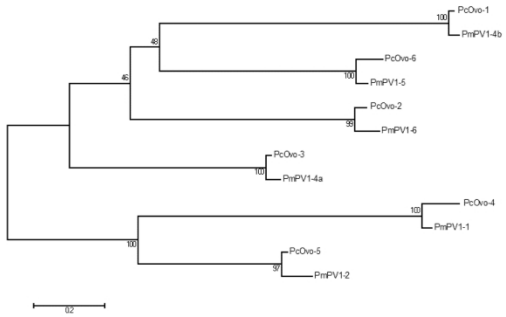

Figure 3. Phylogenetic tree of PcOvo subunits and their orthologs. The tree was constructed using the Maximum Likelihood method based on the JTT matrix-based model. The percentage of trees in which the associated taxa are clustered together is shown next to the branches. Branch lengths are measured as the number of substitutions per site. The prefixes Pc and Pm indicates Pomacea. canaliculata (Lamarck 1822) and Pomacea. mMaculata Perry, 1810, respectively. Ovorubin-1 (AFQ23940), Ovorubin-2 (AFQ23938.1) and Ovorubin-3 (AFQ23939.1) reported in Sun et al. (2012), PcOvo-4 (AFQ23945.1), PcOvo-5 (AFQ23937.1) and PcOvo-6 (Pca61989_c2_g1) this study.

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Figure 4

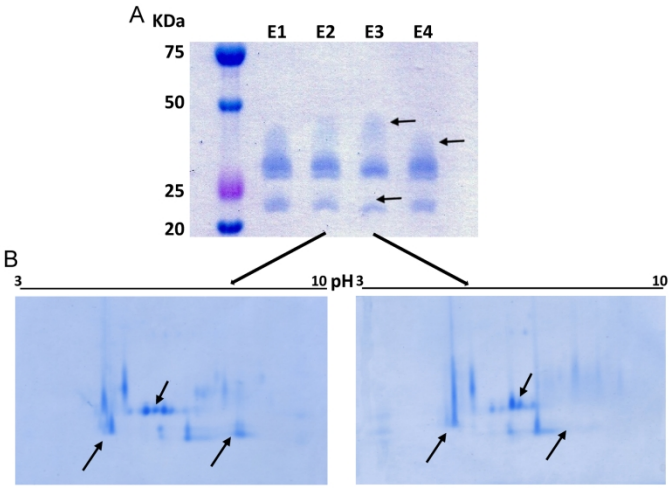

Figure 4. Differences among egg clutches in the relative proportions of PcOvo subunits. A: SDS-PAGE of PcPV1 PcOvo purified from four different egg masses (1-4); B: 2DE gel of egg masses 2 and 3. Arrows indicate major differences between samples.

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Figure 5

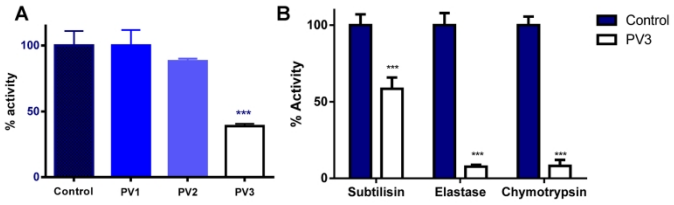

Figure 5. Protease inhibition activity of major egg protein fractions of P. canaliculata eggs. A, Fractions obtained after ultracentrifugation. B, Capacity to inhibit trypsin of the egg fractions in A. CB, Capacity to inhibit proteases of the PcPV3 fraction. Control: the corresponding enzyme with buffer instead of PcPV3. Asterisks indicate Student's t-test $p<0.001$.

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Figure 6

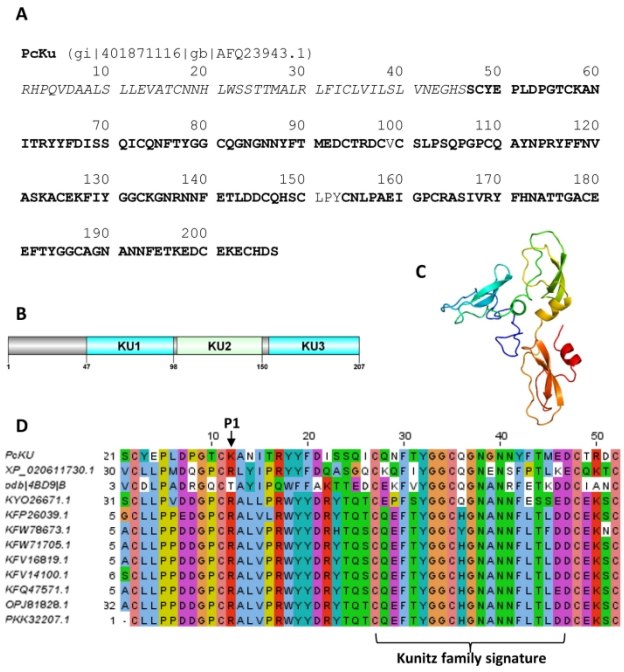

Figure 6. Kunitz- type inhibitor from the apple snail eggs. A, Complete sequence of PcKu. Signal peptide is in italics, the three Kunitz-type domains are in bold. B, Sequence diagram showing the three Kunitz-typeU domains in tandem and putative disulphide bridges. C, Homology model. D, Multiple sequence alignment of the PcKuU domains with other domains of the family, showing conserved sequence features; inhibitory active residue indicated as P1.

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Figure 7

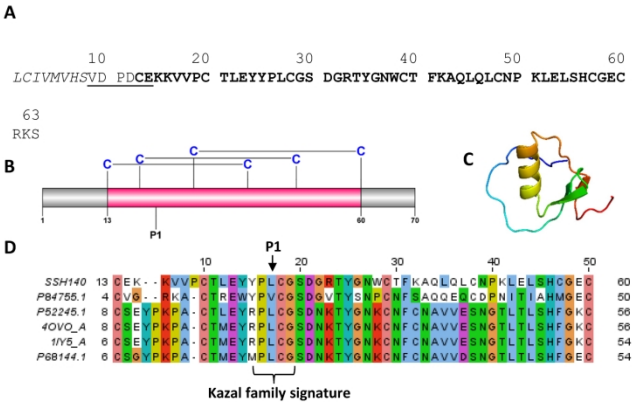

Figure 7. Kazal Kazal-type inhibitor of apple snail egg. A, Complete sequence of PcKunitzPcKa. Signal peptide is in italics, N-terminal sequence is underlined, the three KunitzKa-type domains is in bold. B, Sequence diagram showing the three KU domains in tandem and putative disulphide disulfide bridgesbonds. C, Homology model. D, Multiple sequence alignment of the PcKU PcKa domainns with other domains of the family, showing conserved sequence features; inhibitory active residue indicated as P1.

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