Research Article

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Proteomic analysis of digestive tract peptidases and lipases from the invasive gastropod *Pomacea canaliculata*

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Abstract

Background: The invasive gastropod *Pomacea canaliculata* has received great attention in the last decades as a result of its negative impact on crops agriculture, yet knowledge of their digestive physiology remains incomplete, particularly the enzymatic breakdown of macromolecules such as proteins and lipids.

Results: Discovery proteomics revealed aspartic peptidases, cysteine peptidases, serine peptidases, metallopeptidases and threonine peptidases, as well as acid and neutral lipases and phospholipases along the digestive tract of *P. canaliculata*. Peptides specific to peptidases (139) and lipases (14) were quantified by targeted mass spectrometry. Digestion begins in the mouth via diverse salivary peptidases (nine serine peptidases; seven cysteine peptidases, one aspartic peptidase and 22 metallopeptidases) and then continues in the oesophagus (crop) via three luminal metallopeptidases (Family M12) and six serine peptidases (Family S1). Downstream, the digestive gland provides a battery of enzymes composed of aspartic peptidase (one), cysteine peptidases (nine), serine peptidases (12) and metallopeptidases (24), including aminopeptidases, carboxypeptidases and dipeptidases). The coiled gut has M1 metallopeptidases that complete the digestion of small peptides. Lipid extracellular digestion is completed by triglyceride lipases.

Conclusion: From an integrative physiological and anatomical perspective, *P. canaliculata* shows an unexpected abundance and diversity of peptidases, which participate mainly in extracellular digestion. Moreover, the previously unknown occurrence of luminal lipases from the digestive gland is reported for the first time. Salivary and digestive glands were the main tissues involved in the synthesis and secretion of these enzymes, but plausibly the few luminally exclusive peptidases are secreted by ventrolateral pouches or epithelial unicellular glands.

Supporting information may be found in the online version of this article.

Keywords: liquid chromatography-mass spectrometry; apple snails; digestive gland; salivary glands; digestive enzymes; metallopeptidase; serine peptidases; aminopeptidases; lipases

1 INTRODUCTION

The freshwater caenogastropod *Pomacea canaliculata* (Ampullariidae) is native to the Rio de la Plata basin, which extends through part of Argentina, Brazil, Uruguay and Paraguay. This snail has been listed among the 100 worst invasive species worldwide since it has colonized and adapted to different agricultural ecosystems (rice and other crops) where it was anthropogenically introduced.[1–5] Previous studies have highlighted the magnitude of the environmental impact of this invasive species based on its competition with and predation on other aquatic macroinvertebrates, and its grazing on aquatic macrophytes, algae and crops.[6–10]

The feeding mechanisms of *P. canaliculata* include the scraping of leaves, gathering of floating particles by ciliary currents using its radula (mainly in young individuals) and surface pedal collection using their propodium.[11,12] These feeding mechanisms appear also to be associated with the morphological and functional features of the gut of this snail.[11] *Pomacea canaliculata* has a gastrointestinal tract akin to other gastropods (buccal cavity,

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oesophagus, stomach, thin gut, coiled gut, and rectum) accompanied by strong jaws and radular teeth and a muscular and cuticularized gizzard.[13,14] Secretions from each salivary gland travel through a central duct that opens into the buccal cavity. After that, the partially digested food moves to the oesophagus, where it often is retained in an expanded middle portion (called the crop) associated with a pair of ventrolateral pouches.[13] The absence of a sphincter permits the movement of digestive contents from the posterior oesophagus to the stomach and *vice versa*. The stomach is partially embedded within a voluminous digestive gland and both organs are connected by two ducts, which permit the absorption and excretion of different materials.[15] The final portion of the stomach connects to the thin gut, which drains the digested food into the large coiled gut where absorption of dietary amino acids occurs after the enzymatic action of dipeptidases.[16]

The diversity, origin, and characterization of digestive enzymes have been highlighted recently in invasive ampullariids because they are essential for the digestion of different food sources of macronutrients.[7,16–22] These enzymes include a set of genetically codified enzymes, as well as enzymes plausibly secreted by symbiotic bacteria.[16,23,24] Salivary and digestive glands of *P. canaliculata* are involved in the synthesis and secretion of glycosidases (i.e. cellulases, hemicellulases, amylases, maltases, fucosidases and galactosidases).[17] The crop and the style sac are the places where the extracellular digestion of structurally diverse dietary carbohydrates occurs.[17] In addition, cellulases have been reported in the gut microbiome and bacteria associated with the gastric tissue.[2,18] Available information about digestive peptidases is more limited than that on glycosidases in *P. canaliculata*. A high specific activity of serine peptidases has been reported in the gut luminal contents of this snail when fed with a protein-rich diet.[16] Moreover, L-alanine-aminopeptidase activity has been found in the coiled gut.[16] To date, there are no reports of lipases from *P. canaliculata* or any other apple snails.

The aim of this study was to identify peptidases and lipases synthesized in the salivary and digestive glands, and the coiled gut of *P. canaliculata*, and to evaluate their presence in the crop and style sac contents. The identification and abundance of each enzyme in the digestive tract were resolved by a combination of liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) and of targeted proteomics using multiple reaction monitoring mass spectrometry (MRM-MS).

2 MATERIALS AND METHODS

2.1 Animal culture conditions

Four adult individuals (6 months old, shell length = 33–46 mm) from a cultured strain of *P. canaliculata* were used. The original stock of snails was collected at the Rosedal Lake (Palermo, Buenos Aires, Argentina).

Snails were grown under laboratory-controlled conditions as reported previously.[17] Snails were maintained in aquaria containing 6 L water at room temperature (Rt, 24–26 °C). The relative humidity was 80%. Artificial light was provided for 14 h per day. The aquarium water was changed thrice per week. Snails were fed with lettuce *ad libitum* from Monday to Friday. Diet was supplemented with fish food pellets (Peishe Car: proteins ≥40%, lipids ≥2%, crude fibre ≤3%;, Shulet SA, Las Heras, Argentina) on Thursdays and with toilet paper on Fridays.

2.2 Acclimation, sampling and processing for protein extraction

In order to explore the spatial and temporal distribution of peptidases and lipases along the gut of *P. canaliculata*, snails were sacrificed. Tissues collected included salivary (SG) and digestive glands (DG), and coiled gut (CG), and contents collected included crop (Cr) and sac style (SS).

Acclimation, sampling and protein extraction have been described previously for *P. canaliculata*.[16,17] Briefly, snails were fed *ad libitum* for 48 h and then fasted for 24 h. Animals then were isolated in a beaker (70 mL water) with fresh lettuce and three fish food pellets. The food was consumed after 90 min. Each snail was submerged in an ice-water bath (10 min) and then the shell was removed. At the beginning of the sampling, haemostatic forceps were fixed on the posterior oesophagus to prevent the passage of contents between the crop and the stomach. The Cr and SS contents were collected using a 1-mL syringe with an 18-gauge needle and then thoroughly homogenized on ice by pipetting up and down in protein extraction buffer (PEB: 100 mM Tris–HCl pH 7.4, 10 mM NaCl, 0.25% Triton X-100). The SG, DG and CG were collected in 500 µL PEB and then homogenized on ice (five cycles of 15 s) with an Ultraturrax homogenizer (IKA, Oxford, UK). Supernatants containing proteins were obtained by centrifugation at 12350×*g*. Soluble proteins were quantified by Bradford protein assay and 330 µg protein per sample were fixed in 70% ethanol and then shipped to Australia at −20 °C. Once samples arrived at the destination, they were centrifuged at 12350×*g* for 15 min at 4 °C and dried out to remove the ethanol. Samples were reconstituted in 165 µL urea buffer (8 M urea, 0.1 M Tris–HCl pH 8.5), followed by vortex (30 s, five times), and sonication (2 min); these sequential steps were repeated twice. A second Bradford protein assay then was performed to determine the protein concentration.

2.3 Sample preparation for mass spectrometry

A total 100 µg aliquots of protein were transferred to 10 kDa molecular weight cut-off filter units (Millipore; Merck, Darmstadt, Germany) and centrifuged at 20800×*g* (10 min) at RT. The filters were washed three times with 200 µL urea buffer and then centrifuged (20 800×g, 10 min); three technical replicates were prepared from each sample.

The retained proteins (at the top of the filter) were reduced with 100 mM dithiothreitol (DTT) in water for 60 min at rt and then centrifuged at 20 800×g for 10 min. After reduction, free sulfhydryl groups of proteins were alkylated by incubation with iodoacetamide (50 mM) in urea buffer for 20 min at RT followed by centrifugation at 20 800×g (10 min). The filters were washed twice with 200 µL urea buffer, and then twice with 200 µL of 50 mM ammonium bicarbonate (AmBic, pH 8.5); these washes were followed by centrifugation (20 800×g, 10 min). Protein digestion using trypsin (reconstituted in cold AmBic pH 8.5; Promega, Madison, WI, USA) was completed overnight at 37 °C at a 50:1 ratio of protein to trypsin. The filters were centrifuged at 20 800×g (10 min) at RT and then washed with 200 µL AmBic and centrifuged (20 800 g, 10 min). The obtained tryptic peptides (below the filter) were lyophilized and stored at −20 °C until analysis. The workflow in Fig. 1 summarizes the main steps of the sections 2.2 and 2.3.

2.4 Protein identification

The tryptic peptides were reconstituted in 50 µL of 1% formic acid, and aliquots (5 µL) of each sample replicate were pooled for data-dependent analysis. These samples were separated chromatographically on an Ekspert nanoLC415 (Eksigent, Dublin, CA,

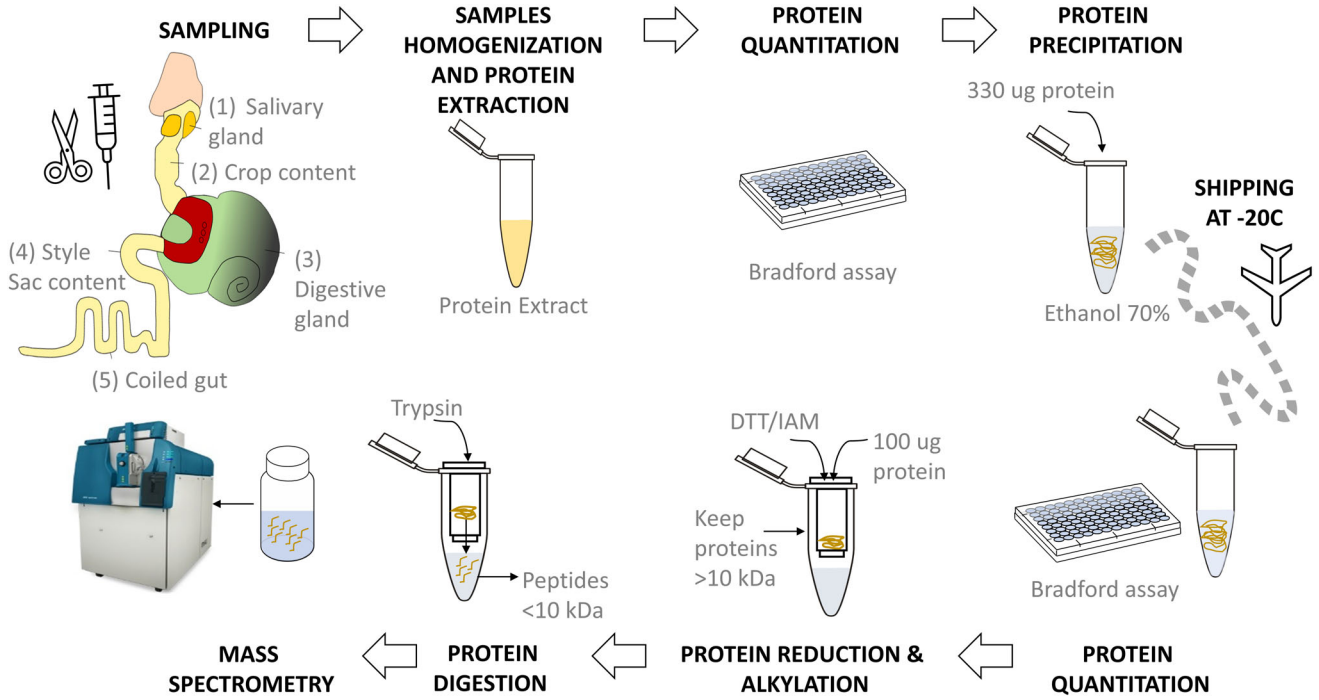

Figure 1. Workflow from sampling to MS analysis.

USA) coupled to a TripleTOF 6600 mass spectrometer (SCIEX, Redwood City, CA, USA). The detailed LC–MS acquisition parameters were described previously.[17]

Mass spectral datasets were searched against a custom database that included: (i) the Molluscan subset of Uniprot database (v2020/05; 335 960 proteins) including 21 502 translated proteins from the genome of *P. canaliculata*; (ii) the translation of 43 565 transcripts of this snail; and (iii) the common repository of adventitious proteins (cRAP) database.[2,25,26] For more information regarding the custom database of this study, the fasta file can be found in the online repository: https://doi.org/10.25919/frg0-z457.[27]

Proteins were identified using ProteinPilot™ 5.0 software (SCIEX, Framingham, MA, USA) with the Paragon Algorithm.[28] The search parameters were set as: trypsin digestion and cysteine alkylation with iodoacetamide. Proteomes included all proteins from the spectral dataset that passed the analysis of 1% global false discovery rate (FDR) threshold (i.e. proteins with at least one unique peptide with 99% peptide confidence). Peptide filtering included exclusion of unusual or missed cleavages and removal of peptides with modifications from glutamine to pyro-glutamic acid, carbamidomethyl cysteine and oxidation of methionine. Supporting Information, Table S1 shows the protein summaries for each studied content/tissue.

In order to assign a protein name, a homology search using the Blastp tool (Uniprot) was performed. Additionally, a protein family domain search algorithm of CLC Genomics Workbench (v20.0.4) using profile hidden Markov models (HMMER3) was completed to confirm the presence of peptidases (Table S2) and lipases (Table S3).[29] Each peptidase family was identified using an analysis of amino acid sequence similarities in the database MEROPS.[30] The occurrence of each enzyme is shown in Tables S2 and S3.

2.5 Targeted MS analysis

Fully tryptic peptides from peptidases and lipases (with no missed cleavages) were selected for MRM-MS. Data from precursor ion (Q1) *m/z* and fragment ion (Q3) *m/z* values were collected from protein identification analysis for developing the targeted analysis (Table S4). MRM-MS quantification was carried out in 139 peptides from 52 peptidases and 14 peptides from seven lipases. Digested peptides (5 µL) from each sample were chromatographically separated on a Nexera UHPLC system (Shimadzu, Kyoto, Japan) coupled to a QTRAP 6500 mass spectrometer (SCIEX). Three MRM transitions per peptides were monitored using a 60 s detection window for each transition and a 0.3 s cycle time. Peaks were evaluated manually to ensure co-elution, retention time (min) coordination and low signal noise, and then integrated using Skyline (MacCoss Lab Software, University of Washington in Seattle, WA, USA).[31] For each peptide, peak areas of the monitored transitions were summed and then converted mathematically into a percentage relative to the average peak area (the area sum of all sample types) for ease of data comparison. Statistical analyses were made with generalized linear mixed models (GLMMs) and Fisher's least significant difference (LSD) (Table S5) using Centurion XVI (v16.0.07; Statgraphics Technologies, Inc., The Plains, VA, USA). The significance level was fixed at $P < 0.05$.

The datasets presented in this study can be found in the online repository: https://doi.org/10.25919/frg0-z457.[27] To prevent reporting peptidases and lipases of microbial origin, datasets were searched against a protein database from Tenericutes, Firmicutes, Proteobacteria, Cyanobacteria and Bacteroidetes. Table S6 reports the motif/peptide search for the quantified peptides of the peptidases (139) and lipases (14) found in this study.

3 RESULTS

3.1 Classification and distribution of peptidases

The number of proteins identified in snail tissues (range 2537–3366) was higher than in the luminal contents (range 985–879) (Table 1). As expected, the number of tissue peptidases (98–110) was higher than those identified in the Cr and SS contents (26–32) (Fig. 2). According to their catalytic site, peptidases were

Table 1. Number of identified proteins along the digestive tract of *P. canaliculata* with 99% confidence after combining high-resolution MS/MS and database searching

Tissues & contents	IDS total proteins	IDS Uniprot Mollusca	IDS Transcriptome
Salivary gland (SG)	3351	2398	953
Crop content (Cr)	985	660	325
Digestive gland (DG)	3366	2387	979
Style sac content (SS)	879	599	280
Coiled gut (CG)	2537	1840	697

classified into aspartyl peptidase, cysteine peptidase, metallopeptidase, serine peptidase and threonine peptidase.[30] At the tissue level, metallopeptidases showed the highest number of representatives (48–56; Fig. 2), followed by serine peptidases (23–27) and cysteine peptidases (21–26). A similar distribution pattern was found in the gut contents (metallopeptidases > serine peptidases = cysteine peptidases) (Fig. 2).

A total of 168 distinctive peptidases were identified with unique accessions and sequences (Table S2): 121 derived from the Uniprot-*P. canaliculata* database, seven from the Uniprot-Mollusca database, and 40 additional identifications arising from the translated transcriptome of this gastropod. These representatives of peptidases were assigned as: (i) five aspartyl peptidases of the families A1, A22 and A28; (ii) 31 cysteine peptidases of families C1, C2, C26, C65, C69 and C78; wherein Family C1 had the greatest representation; (iii) 43 serine peptidases of families S1, S8, S9, S10, S16, S28, S33 and S51; (iv) 85 metallopeptidases of families M1, M2, M3, M4, M12, M13, M14, M16, M17, M18, M19, M20, M24, M28, M41, M48, M49, M54 and M67; and (v) four threonine peptidases from Family T3. Altogether, these enzymes often showed a higher abundance in tissues than in Cr and SS contents.

3.2 Quantification of peptidases

Peptides (139) from 52 peptidases were selected and quantified by LC-MRM-MS. Unfortunately, we were unable to quantify a peptide for each of the identified peptidases initially identified due to the criteria established for peptide quantitation: tryptic peptides with zero missed cleavages and peak intensity >5000 counts per second. Peptides selected for quantitation were subjected to homology searching within the 168 peptidases identified in the discovery proteomics; the search revealed seven peptides that were shared between peptidases and only one peptide assigned to metallopeptidase M20 (A0A2T7P6W3) matched to symbiotic Proteobacteria and Firmicutes reported previously in the gut microbiome of *P. canaliculata* (Table S6).[32–34] The relative percentage of each peptide in relation to their peptidase (classified by catalytic site) along the digestive tract is shown in Fig. 3 and Table S5. In general, the peptides of aspartyl peptidases [Family A1; Fig. 3(a)] and cysteine peptidases [Family C1; Fig. 3(b)] showed high tissue values (DG > CG > SG) and null values in both gut contents. The peptides of metallopeptidases [Fig. 3(c)] and serine peptidases [Fig. 3(d)] presented a variable distribution among tissues and contents.

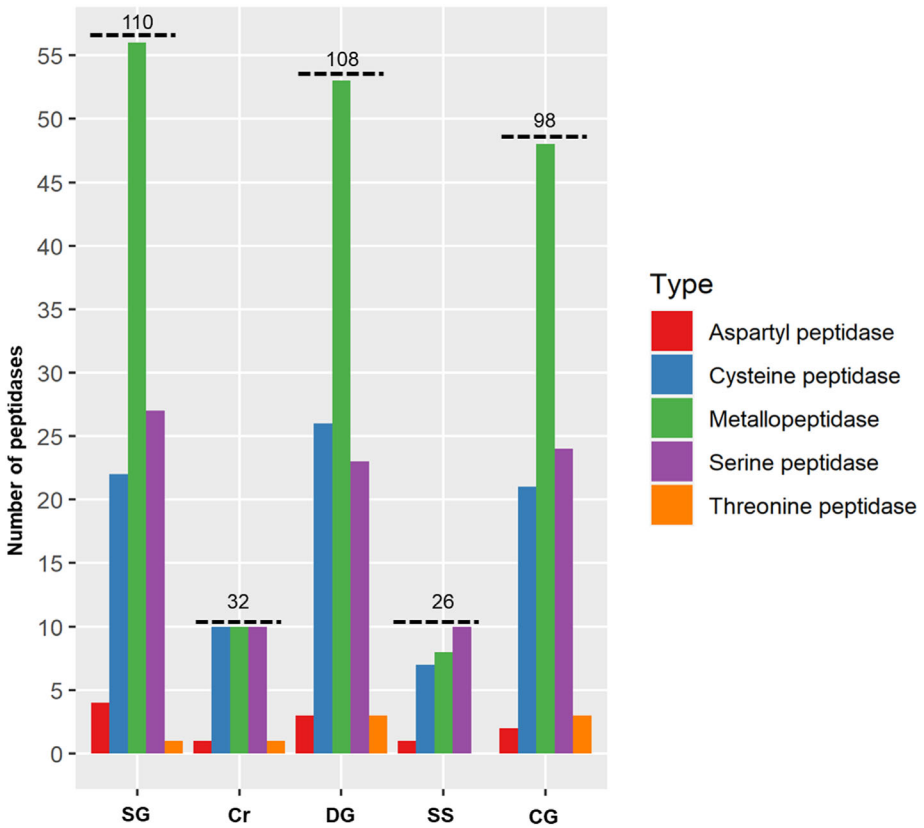

Figure 2. Total number (on dashed lines) and classification by their catalytic site (colour) of peptidases along the digestive tract of *P. canaliculata*. SG, salivary gland; Cr, crop content; DG, digestive gland; SS, style sac content; CG, coiled gut.

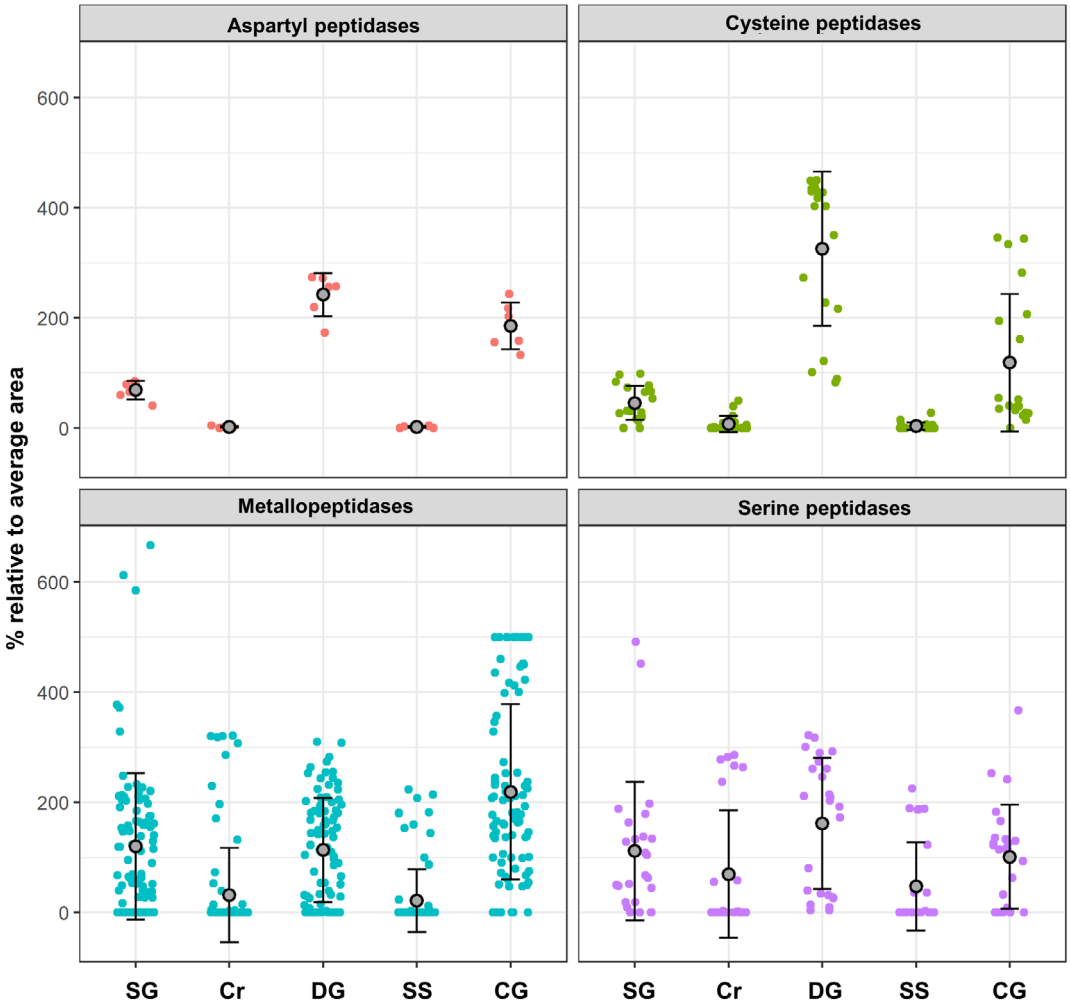

Figure 3. Distribution and abundance of peptides from four types of peptidases. The abundance of each peptide was calculated as the sum of MRM peak areas. This value was compared to the average of all measurements (i.e. 100% represents the mean area for each peptide along the gut). The circle and error bars represent the mean ± SD. SG, salivary gland; Cr, crop content; DG, digestive gland; SS, style sac content; CG, coiled gut.

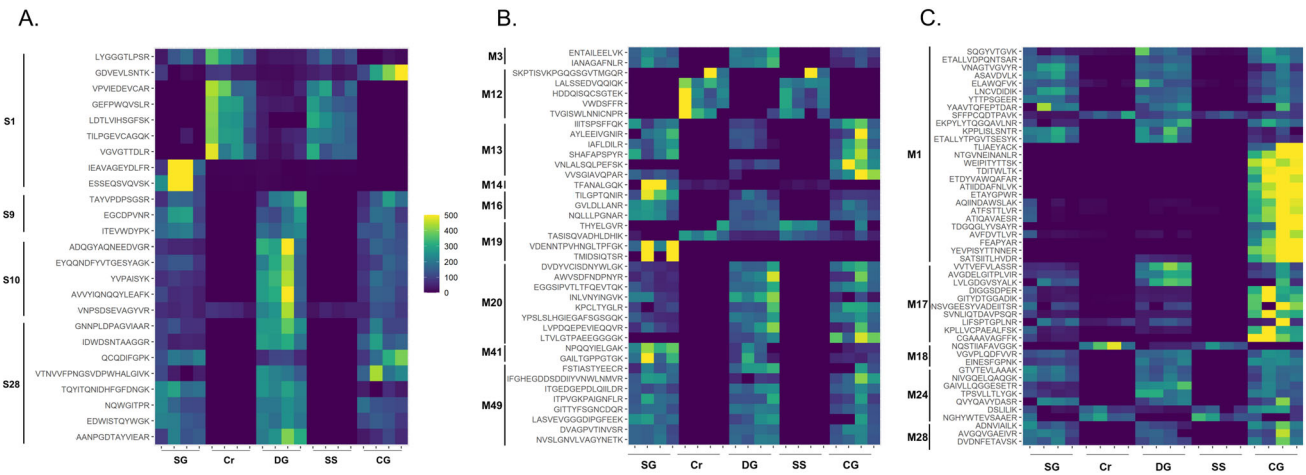

Figure 4. Distribution and abundance of peptides from different families of serine peptidases and metallopeptidases from *P. canaliculata*. Peptides were classified into families according to amino acid sequence similarities with the proteolytic enzyme database (MEROPS).[30] Peptides of serine peptidases (a), metallopeptidases (b) and (c) aminopeptidases. The abundance of each peptide was calculated as the sum of the areas of the MRM peaks. To explore the abundance of each peptide, the sum of each area throughout the intestine was taken as 100%. Dark blue to light blue, 0–200%; green to yellow, 200% to ≥500%. SG, salivary gland; Cr, crop content; DG, digestive gland; SS, style sac content; CG, coiled gut.

Heatmaps (Fig. 4) were generated to explore differences in relative abundance of serine peptidase and metallopeptidase families. A total of 12 serine peptidases [Fig. 4(a)] were classified into the families S1 (six), S9 (two), S10 (one) and S28 (three). In S1, the peptide abundances in the gut contents were higher than in the tissues; the exception was the peptidase A0A2T7NI05 (S1) whose peptides (IEAVAGEYDLFR and ESSEQSVQVSK) showed high abundance in the salivary glands and were barely detected in other tissues and contents. Peptidases from S9, S10 and S28 showed high peptide abundances in the gut tissues and they were not detected in the gut contents; the only exception was peptidase A0A2T7PAY9 (peptide VNPSDSEVAGYVR; S10), which was ubiquitously detected in the gut (tissues > contents).

Surprisingly, 30 metallopeptidases (14 families) were found using the MEROPS database [Fig. 4(b)].[30] The peptide abundance in the tissues was higher than in the contents for oligopeptidase M3 (one isoform), endopeptidases M13 (three), carboxypeptidase M14 (one), endopeptidases M16 (two), dipeptidases M19 (two), carboxypeptidase M20 (two), endopeptidase M41 (one) and dipeptidylpeptidase M49 (one). Family M12 (three isoforms) was highly abundant in the gut contents but not detected in the SG, DG or CG. A total of 14 aminopeptidases were represented in families M1 (seven), M17 (two), M18 (one), M24 (three) and M28 (one), the latter enzyme also being a putative carboxypeptidase [Fig. 4 (c)]. The tissue levels of these aminopeptidases often were higher than in the gut contents. However, aminopeptidases A0A2T7PT02 (M1), Pc19727 (M1), Pc41390 (M1), A0A2T7NGT7 (M17) and A0A2T7PCE4 (M17) in the CG had values higher than the SG and DG.

3.3 Classification and distribution of lipases

A lower number of lipases than peptidases was found [Fig. 5(a)]. A greater diversity of lipases was detected in the digestive gland (18) compared to other gastrointestinal tissues (six, seven) and contents (6–10). After a protein family search (Pfam), a total of 23 lipases with unique accessions and sequences (Table S3) were found. These included 13 phospholipases, six triglyceride lipases, two lipases/acyl hydrolases, one lipase/esterase and one lipase/alpha-beta hydrolase [Fig. 5(a)].

Quantification of five peptides from two triglyceride lipases and nine peptides from five phospholipases (Table S5) was performed. The abundance and distribution of each peptide in the gut of *P. canaliculata* is shown in Fig. 5. Triglyceride lipases [A0A2T7NR40, Pc121275; Fig. 5(b,c)] had a relatively higher abundance in the Cr and SS contents than in the DG. Phospholipases were almost exclusively found in tissues [Fig. 5(d–h)]. Contents of the phospholipases A0A2T7NRQ3, A0A2T7PI22 and Pc117932 were significantly higher in the DG than in the SG and CG [Fig. 5(d–f)]. A0A2T7NXY2 was lower in the SG than in DG and CG [Fig. 5(g)]. A0A2T7NZZ1 was significantly higher in the SG and CG than in the DG [Fig. 5(h)] and the peptide VVYPTAEDR also was detected in the Cr and SS contents.

4 DISCUSSION

It has been stated that biological invasions may be prevented by the application of physiological knowledge.[35] A focus has been placed on the digestive physiology of apple snails in recent years,[18,19,36,37] so advances in 'omics' studies could contribute towards achieving an integrated, comprehensive view of the digestive physiology of *P. canaliculata*.[2,17,25,26] In this study, we elucidated the proteome of the SG, DG and CG of *P. canaliculata*, as well as the contents of the Cr and SS, where enzymatic digestion of dietary proteins and lipids occur. The transcriptome and the genome of *P. canaliculata* allowed us to generate a specific custom database that increased the identification rate of peptidases (17%; nine of 52) and lipases (29%; two of seven).[2,25,26]

4.1 Diversity and origin of peptidases

Different parts of the digestive tract have been studied in several of gastropods treated with different food references, including representatives of four of the main subclasses of Gastropoda – Vetigastropoda, Heterobranchia, Patellogastropoda, and Caenogastropoda. *Pomacea canaliculata* is a freshwater species from the subclass Caenogastropoda, which includes ≈60% of gastropod species. It is a generalist species that consumes different trophic resources when its preferred macrophytes are absent.[12,38,39] This may include items with high protein content (48.5%), such as carrion and cannibalized eggs.[38–40] Here, a great diversity of endogenous peptidases was found in the SG, DG and CG, highlighting the importance of the digestion and assimilation processes of dietary proteins.

4.1.1 *Aspartic peptidases*

Aspartic peptidases are endopeptidases whose catalytic site is composed of two aspartic acid residues that show high specific activity at acidic pH values.[41] Cathepsin D and pepsin aspartic peptidases have been described in some gastropods.[42–44] Cathepsin D acts mainly at the acidic intracellular compartments,[42] although there are reports of enzymatic activity at the extracellular level.[45] The pepsin hydrolyses proteins at the extracellular level and acidic pH.[43,44]

This work reports for the first time five aspartic endopeptidases in ampullariids that were found via discovery proteomics. The targeted detection of enzyme A0A2T7PJD9 from Family A1 showed high levels of expression in the digestive gland of *P. canaliculata* [Fig. 2(a); Table S5] indicating a physiological digestive function mainly in acidic intracellular compartments. In gastropods, the pepsin-like aspartic peptidases have been reported in the digestive gland and viscera of phylogenetically distant species: the abalone *Haliotis fulgens* (Vetigastropoda), *Cellana radiata* (Patellogastropoda) and *Concholepas concholepas* (Caenogastropoda).[43,44,46,47]

4.1.2 *Cysteine peptidases*

These enzymes are ubiquitous in the Eukarya domain; they are characterized by the presence of cysteine at the catalytic site and high activity at acidic pH values.[41] The C1 or papain family includes endopeptidases and exopeptidases that act generally at the lysosomal level, but also may be secreted into the extracellular environment.[48–50]

Here, 31 cysteine peptidases were found. Nine members of Family C1 [Fig. 2(b)] showed high expression values at the tissue level with null or low values in the Cr and SS contents. Pc120372 and Pc95443 showed higher values in the DG, whereas A0A2T7PGN5 was high in the CG. A0A2T7PL48 was exclusively found in the DG. These expression patterns suggest that cysteine peptidases of *P. canaliculata* act primarily at the intracellular level.

Additionally, five papain/cathepsin-like enzymes (A0A2T7NPA0, A0A2T7PGR8, AA0A2T7PSJ4, A0A2T7P4F3 and A0A2T7PSJ3) were found in both tissues and gut contents. The abundance in the contents shows their additional participation in the extracellular digestion of dietary proteins, although their activity would be greater in the Cr content because *P. canaliculata* has shown an

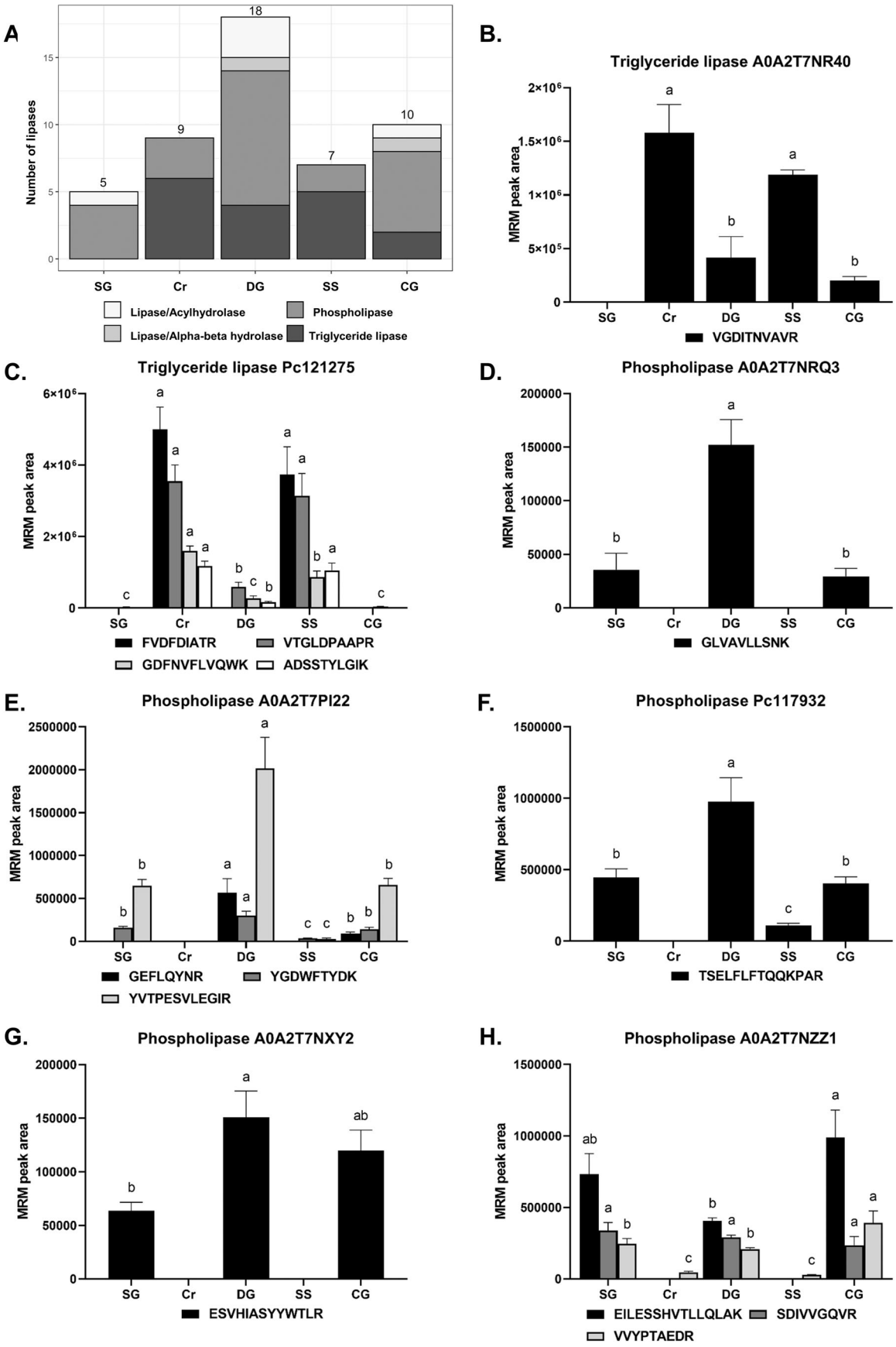

Figure 5. Distribution, classification and quantitation of peptides derived from lipases. (a) Lipases along the gastrointestinal tract of *P. canaliculata* gut. (b—h) Peptide expression patterns of lipases along the gut of *P. canaliculata*. The MRM peak area (mean ± SEM) for lipase peptides among gut tissues and contents was evaluated using multiple comparisons. Different letters indicate statistical significance. The significance level was fixed at $P < 0.05$.

aboral increase in the intestinal pH (from 5.9 to 7.4).[16] In gastropods, a cathepsin L-like protein has been described in the digestive gland of the terrestrial gastropod *Deroceras reticulatum* (Heterobranchia).[51] Also, cathepsin B and cathepsin Z have been described, respectively, in the abalones *Haliotis discus hannai* and *H. discus discus*. These cathepsins increase after a bacterial or immunogenic challenge.[52,53]

4.1.3 *Metallopeptidases*

Metallopeptidases are ubiquitous enzymes that participate in diverse physiological processes and their activity depends on the presence of divalent cations such as Mg^{2+} or Ca^{2+}.[41,54]

In this work, a large and diverse group of digestive metallopeptidases (Figs 2, 3) including 30 proteins from 14 families, were analyzed and quantified.

The SG showed a high expression of five metallopeptidases [Fig. 4(c)] (A0A2T7P840, M19; A0A2T7NJF8, M14; A0A2T7NJK8, M41; A0A2T7NTX8, M16; and A0A2T7NMX4, M16) indicating its early involvement in protein digestion. Surprisingly, three metallopeptidases (A0A2T7PWH5, A0A2T7PTG5 and A0A2T7PTH1, M12) were exclusively found at the luminal level which may indicate the participation of oesophageal ventrolateral pouches and/or unicellular glands of the digestive tract, as reported in the keyhole limpet *Megathura crenulata* (Vetigastropoda).[55] A dipeptidase (A0A2T7P9C1, M19) from the DG would function to release amino acids. The CG showed a high expression of five metallopeptidases (A0A2T7PT02, M1; Pc19727, M1; Pc41390, M1; A0A2T7NGT7, M17; AND A0A2T7PCE4, M17), which may be associated with L-alanine-aminopeptidase activity.[16] Aminopeptidases have been reported in phylogenetically distant species: the DG of *Haliotis asinine*, the mouth of *Haliotis rufescens* (Vetigastropoda), the SG of *Biomphalaria straminea*, and in the viscera of *Littorina irrorata* (Caenogastropoda).[56–59]

4.1.4 *Serine peptidases*

Barrett and Rawling proposed that the heterogeneity in the origin of serine peptidases that arose because of the incorporation of serine into catalytic centres, coupled with the independent evolution of the various 'clans,' is responsible for the higher diversity in their structure and properties.[60] The catalytic site of these enzymes is characterized by the presence of serine, histidine and aspartic acid, and they work efficiently under neutral or slightly alkaline conditions.[41] The literature supports the hypothesis that protein digestion in gastropods relies on serine peptidases such as trypsin and chymotrypsin. For example, chymotrypsin-like serine peptidases were found in samples of the intestinal, stomach, and rectal fluids of *H. rufescens* and *H. fulgens*, and in the DG of *Pecten maximus*.[43,44,61–63] Trypsin-like enzymes have been described in the stomach content of *Aplysia kurodai* (Heterobranchia) and of *H. fulgens*.[43,44,63–65]

The peptidase repertoire of *P. canaliculata* is quite peculiar because it has optimal peptidase activity at higher pH (SS = 8.5, Cr and CG = 9.5) and temperature values (30 °C for SS and 35 °C for Cr and CG).[16] These conditions differ from the physiological pH of the gut (Cr = 5.96, CG = 7.42) and overlap marginally with the thermal range of habitats occupied by this gastropod (10–30 °C).[66,67] Extracellular peptidase activity in the gut of *P. canaliculata* is dominated by serine peptidases of different molecular weights that originate in the SG and from endosymbionts hosted in the midgut gland.[16] In this work, Family S1 serine peptidases were found in the Cr and SS contents, and to a lesser extent in the SG (A0A2T7NI05 and Pc117725), DG (A0A2T7PVI9, A0A2T7PVH8, A0A2T7PRU7) and CG (A0A2T7PJS4).

Pomacea canaliculata expressed some serine peptidases of the families S9 (two), S10 (one) and S28 (three) exclusively at tissue level. According to the MEROPS database, Family S9 includes prolyl-oligopeptidases with endo and exopeptidase activities, whereas families S10 and S28 include carboxypeptidases.[30] Although the function of these enzymes is yet to be fully understood, it is possible that partially digested proteins were absorbed by endocytosis by gut epithelial cells and then hydrolyzed in acidic granular and vacuole compartments, as occur in embryos and early juveniles.[68]

It is possible that the higher structural and functional diversity of serine peptidases is influenced by the feeding state (satiated or starved) of apple snails and their ability to select among different macrophytes.[24,69] The feeding rate of macrophytes with higher nitrogen and phosphorus content positively influences eggs production and growth in *P. canaliculata*; however, these parameters may decrease when snails consume plants with high phenolic content and/or physical and chemical defences.[69] Moreover, serine peptidase diversity may support herbivore feeding based on the consumption of aquatic vascular plants containing naturally occurring antagonistic biomolecules such as plant-derived peptidase inhibitors and lectins as reported previously in herbivore insects.[70,71] Interestingly, adult apple snails ingest their own submerged egg masses (egg cannibalism). The main nutritive protein component of *P. canaliculata* eggs is the protein PcOvo, which showed high peptidase inhibitory activity and significantly affected the digestive activity in rats fed on *P. canaliculata* eggs.[39,72–75] This deleterious activity might be avoided by endocytosis and intracellular digestion by peptidases, as it occurs in embryos and early juveniles.[68] Future research could clarify whether serine peptidase diversity in apple snails may facilitate the digestion of egg proteins.

4.1.5 *Threonine peptidases*

To the best of our knowledge, this is the first report of threonine peptidases in a gastropod. In this study, Family T3 peptidases (MEROPS database) found in the DG, SG and CG of *P. canaliculata* (Table S2), fulfill intracellular functions (excepting A0S2T7PH69) as all members have a threonine residue in their catalytic site and are self-processing proteins with aminopeptidase and aminotransferase activities in their mature forms.[30] Although the physiological role of threonine peptidases of Family T3 eucaryotes is still under study, some reports suggest (a) associations with catalytic subunits of eukaryotic proteasomes where cellular protein turnover occurs, and (b) regulation of prooxidative state by preventing oxidative damage to macromolecules through the regulation of glutathione levels.[76,77]

4.2 Diversity and origin of lipases

Pomacea canaliculata consumes lipids such as fatty acids. Macrophytes can have ≤4% of fatty acids and triacylglycerols.[78–80] 80 Animal carrion has a higher nutritional quality because it has 16 different fatty acids (unsaturated 60.5%; saturated 39.5%).[40]

A total of 23 lipases were identified by global proteomic analysis and 14 peptides from seven lipases (phospholipases and triglyceride lipases) were quantified by the MRM approach (Fig. 5). Five phospholipases (A0A2T7NRQ3, A0A2T7PI22, A0A2T7NZZ1, A0A2T7NXY2 and Pc117932) were found at the tissue level, which could be involved in the signal transduction

process and intracellular digestion.[81] In the Caenogastropods *Littorina littorea* and *Buccinum undatum*, these enzymes are located on digestive and basophilic cells of their DG suggesting a participation in both intracellular and extracellular digestion.[82] However, phospholipase A2 has a key role in the reversion of logn-term memory impairment in a model of aging of *Lymnaea stagnalis* (Heterobranchia).[83]

Here, two triglyceride lipases (A0A2T7NR40, Pc121275) involved in the hydrolysis of triglyceride ester bonds were found at the luminal level indicating their participation in extracellular digestion.[84] In invertebrates, these enzymes have been described in the DG of the water flea *Daphnia magna*, the giant tiger prawn *Penaeus monodon* and the purple sea urchin *Strongylocentrotus purpuratus*, and in the DG and gonads of the pacific oyster *Crassostrea gigas*.[85–88] Interestingly, lipase activity has been described in the DG of the freshwater crustacean *Macrobrachium borelli*, a species that cohabits with *P. canaliculata*. This lipase works optimally at 40 °C and pH 8, similar to what occurs in the crab *Carcinus mediterraneus* and the shrimp *Penaeus vannamei*.[89–91]

5 CONCLUSIONS

Mass spectrometry was used to profile the digestive enzymes in the gut of *P. canaliculata*. Although this snail is mainly herbivorous, our findings revealed that is equipped with a diversity of gut enzymes capable of digesting a wide variety of materials. Our analysis revealed enzyme heterogeneity at the intraluminal level (Cr and SS), as well as functional diversity in the peptidases secreted by the SG, DG and CG. Surprisingly, we found luminal lipases originating from the DG.

Our study provides insights into the digestive machinery of *P. canaliculata* that could be a platform for future studies testing natural or synthetic molecules that could inhibit specific digestive enzymes focused on the deployment of pest management strategies. Future studies should elucidate the contribution to the digestion of putative bacterial enzymes such as those reported by Godoy *et al*., as well as others that may be found in the gut lumen.[16]

AUTHOR CONTRIBUTIONS

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in CSIRO DATA ACCESS PORTAL at https://data.csiro.au/collections/collection/Clcsiro:50676v2/Dltrue.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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