

RESEARCH ARTICLE

Proteolytic profile of larval developmental stages of *Penaeus vannamei*: An activity and mRNA expression approach

Liliana Rojo-Arreola^{1*}, Fernando García-Carreño², Rogelio Romero², Luis Díaz Domínguez²

1 CONACyT-Centro de Investigaciones Biológicas del Noroeste, México City, México, **2** Centro de Investigaciones Biológicas del Noroeste, México City, México

* lrojo@cibnor.mx



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Abstract

In arthropods, the cleavage of specific proteins by peptidases has pivotal roles in multiple physiological processes including oogenesis, immunity, nutrition, and parasitic infection. These enzymes are also key players in the larval development, and well-described triggers of molting and metamorphosis. In this work the peptidase complement throughout the larval development of *Penaeus vannamei* was quantified at the transcript and activity level using qPCR and fluorogenic substrates designed to be hydrolyzed by class-specific peptidases respectively, providing a detailed identification of the proteolytic repertoire in *P. vannamei* larvae. Significant changes in the peptidase activity profile were observed. During the lecithotrophic naupliar instars, the dominant peptidase activity and expression derive from cysteine peptidases, suggesting that enzymes of this class hydrolyze the protein components of yolk as the primary amino acid source. At the first feeding instar, zoea, dominant serine peptidase activity was found where trypsin activity is particularly high, supporting previous observations that during zoea the breakdown of food protein is primarily enzymatic. At decapodid stages the peptidase expression and activity is more diverse indicating that a multienzyme network achieves food digestion. Our results suggest that proteolytic enzymes fulfill specific functions during *P. vannamei* larval development.

1. Introduction

Peptidases are hydrolases that cleave peptide bonds within protein chains. They are classified according to the catalytic mechanism and the amino acids involved in substrate hydrolysis as serine, cysteine, aspartic and metallo-peptidases [1]. In arthropods, the cleavage of specific proteins by peptidases has pivotal roles in multiple physiological processes including oogenesis, immunity, metamorphosis, larval development, nutrition and parasitic invasion [2–6]. Therefore peptidases (also referred to as proteases or proteolytic enzymes), are strongly considered as targets for parasite and insect control by interfering with their activity [7].

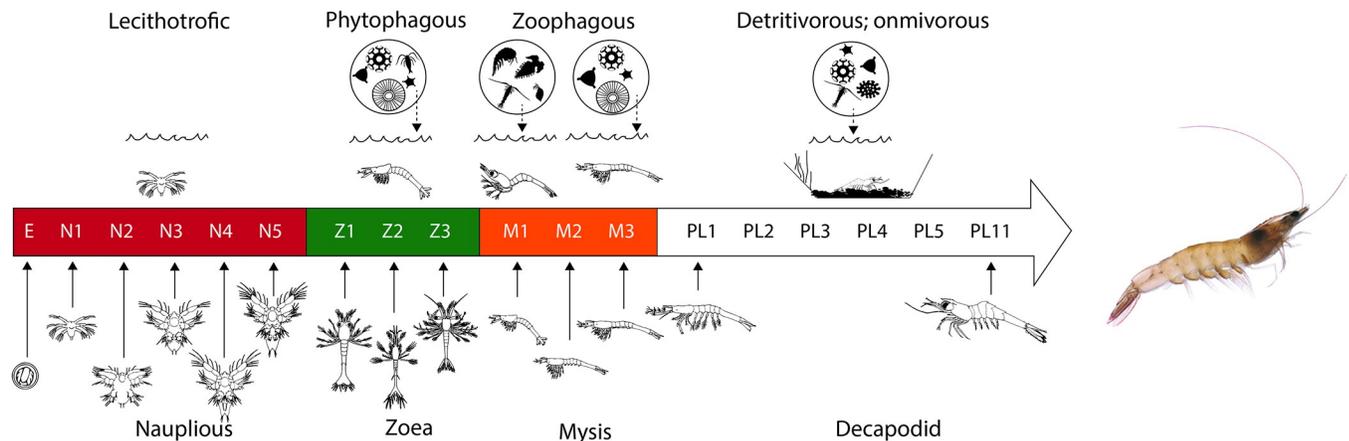


Fig 1. Larval developmental stages of *Penaeus vannamei*. Sub-stages, feeding habits and habitats are indicated.

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The relevance of proteolytic enzymes in arthropod larval development has been mostly described in economically relevant insects, for which peptidases are well-described triggers of molting and metamorphosis [5, 8], as well as partakers in fat body cell dissociation and tissue remodeling [9]. For decapod crustaceans, descriptions of peptidases in larvae are focused on enzymes participating in food digestion like trypsin and chymotrypsin [10, 11]. Profiling of expressed peptidases during larval development demonstrated a differential expression depending on the enzyme and larvae stage [12–16] suggesting peptidases as partakers in decapod larvae development.

Penaeus vannamei (syn. *Litopenaeus vannamei*) is a Penaeid shrimp distributed in tropical marine environments of the Eastern Pacific coast of North, Central, and South America. Penaeid shrimps undergo a biphasic life cycle, meaning pelagic larval stages known as nauplius, zoea, mysis followed by benthonic decapodid, juvenile and adult stages. Larvae develops gradually through a series of molts occurring within a relatively short time-frame (11–17 days depending on the temperature) [17], each stage presents anatomical, physiological and ecological adaptations that fulfill its locomotive, behavioral, and feeding habits [18, 19] (Fig 1). Albeit *P. vannamei* is a key species for the aquaculture industry, and its rearing in captivity is highly successful, descriptions of the molecular mechanisms of many important physiological processes including larvae development are rather scarce. However, proper management of natural stocks, ecology, genetics, and technification of culture methods require a profound understanding of the developmental biology of the species.

In this work, the proteolytic profile throughout larval development of *P. vannamei* was assessed by means of transcripts and activity. We quantified the gene expression of 14 annotated shrimp peptidases by qPCR. Since changes in mRNA abundance do not necessarily correlate with the corresponding mature protein products, the proteolytic activity was also assessed using fluorogenic substrates designed to be hydrolyzed by serine-, cysteine-, aspartic-, and metallo-peptidases and the specific activity was confirmed by the use of class-specific inhibitors.

2. Materials and methods

2.1. Sample collection and preservation

P. vannamei samples were donated by Larvas GranMar SA de CV, San Juan de la Costa, BCS, Mexico. Samples were obtained by filtering water from the culture ponds containing larvae at

each specific larval stage determined by microscope examination when at least 80% of the specimens reach the target developmental stage. A pool of ca 90 mg of larvae was sampled, washed with sterile marine water and collected into 1.6 mL cryotubes. Eight samples of each stage were obtained starting from embryos (8 hours post-spawning) (E), then in the larvae instars nauplius (N), zoea (Z), Mysis (M) and decapodid (D). Samples were immediately submerged into liquid nitrogen or RNAlater™ (depending on the intended analysis) for transportation and stored at -80°C.

2.2. RNA isolation, cDNA synthesis, and primer design

Total RNA was isolated from 4 replicates of 90 mg of whole larvae pools at each substage, using the Trizol™ method following the manufacturer's instructions. To remove the contaminating genomic DNA, total RNA was treated with DNase I and further purified using phenol-chloroform extraction, followed by sodium acetate and cold absolute ethanol precipitation, and 75% ethanol wash. The success of the DNase treatment was assessed by PCR, the absence of contaminating DNA resulted in no amplification bands. The concentration and purity of RNA were measured using a nano-spectrophotometer (absorbance at 260 nm and 260/280 nm ratio). The quality and integrity of the RNA were analyzed in 1% agarose gel electrophoresis.

Complementary DNA (cDNA) was synthesized from each replicate using the Reverse Transcription System kit (Promega). The reaction mixture consisted of final concentrations of 5 mM MgCl₂ (4 μL), 1X RT Buffer, 0.5 mM dNTP mixture, Ribonuclease Inhibitor RNasin (20 U), AMV Reverse Transcriptase (0.7 μL), Oligo (dT) Primer (0.5 μg), DNA-free RNA (2.3 μg) and nuclease-free water to a final volume of 20 μL. The reverse transcription reaction was done in a thermocycler for 1 h at 42°C, followed by 5 min at 95°C, and 4 min at 4°C. The cDNA was taken to a final volume of 50 μL using nuclease-free water and stored at -20°C.

2.3. Peptidase transcript selection and primer design

The selection of the peptidases for which the gene expression was quantified is based on three main criteria: the existence of reports on activity and/or transcript in *P. vannamei*; the relevance of homologous peptidases in larvae development from other Arthropods and; the curation level in the UniProt database. Only UniProt entries having experimental evidence at protein or transcript level and/or inferred by homology were selected. Sequences annotated as preliminary data derived from whole-genome shotgun experiments were not considered for the analysis. Following these criteria, 14 peptidases were selected for transcript quantification during *P. vannamei* larval development (Uniprot accession numbers in [Table 1](#)).

The design of qPCR primers was based on annotated sequences of *P. vannamei* peptidases ([Table 1](#)), each primer set was designed following the framework suggested by Bustin and Huggett [20], primer specificity was verified by Sanger sequencing and by the presence of a single peak revealed in melt curves. To determine the efficiency of each primer set, standard curves were constructed from serial dilutions of the purified PCR product as the template (from 2.5x10⁻⁴ to 2.5x10⁻⁸ ng of purified DNA).

2.4. Quantification of peptidase transcripts

The real-time polymerase chain reaction was carried out in a StepOne Real-Time PCR Systems (Applied Biosystems), using the Power SYBR Green Master Mix (Applied Biosystems). PCR reactions were performed in duplicate of the four biological replicates of each shrimp larval instars. The reaction mix included Power SYBR Green Master Mix at 1X, 0.5 mM of each primer, and the template equivalent to 34 ng of cDNA in a total volume of 10 μL. The reaction's cycling profiles were as follows: 1 cycle at 95°C for 5 min and 35 cycles at 95°C for 30 s

Table 1. List of peptidase transcripts quantified by qPCR. Uniprot or GenBank accession numbers, primer sequences, amplicon size and standard curve properties are included.

Gen/UNIPROT accession no.	Forward and reverse primer sequence (3'-5')	Ta	Amplicon size (bp)	Slope	R ²	Efficiency (%)
Cathepsin L/Q27759	F-GAGCCTCTCAGAGCAGAACC	58	159	-3.578	0.999	95.2
	R-CGACACTTACCGTCCTGAGC					
Cathepsin L2/O46153	F-GTGTACTCCGACAAGACCTG	55	105	-3.576	0.999	95.2
	R-GTTCTTGACCAGCCAGAAG					
Cathepsin B/D8X153	F-TCCACAGTAAGGGCAAGAGC	57	207	-3.455	0.996	97.4
	R-CAGGGAGCAATCTCATAGGG					
Cathepsin C/B8XGG3	F-AACTGTGGCTCCTGCTATGC	57	172	-3.618	0.997	94.5
	R-CATACCTGCCAGCGATAAGG					
Calpain B/C6KE09	F-GTCACGCCTACTCCCTTACG	54	168	-3.359	0.998	99.2
	R-ATCCCATCTCCTCCTTCTCC					
Carboxypeptidase B/Q20AS8	F-CGTGACCTACATGCTGAACG	58	163	-3.621	0.994	94.4
	R-AGGAGAACCCTGTGTCAGAGC					
Serine protease/Q6UKI3	F-CGACTGGTCACACTTTCG	52	192	-3.103	0.981	105.0
	R-TAAACACGACGTCTCTCTCC					
Trypsin 1/ Q9TY16	F-CATGAACAACCCCGATTACC	52	187	-3.723	0.999	92.8
	R-GCGAACGTTGTCATTGAAGC					
Trypsin 2/H6WSS5	F-CGACGACTTTGATAATCCAGC	58	275	-3.557	0.999	95.5
	R-AGCTGCCTCCTTCAGTGAGAGC					
Trypsin 3/O62562	F-CAGAACGACATTGACGACTCC	56	174	-3.309	0.996	100.3
	R-GTACACGCCAGGGTAGTTGG					
Chymotrypsin BI/Q00871	F-CGCCCTTCCGACTCTGCCAGC	57	142	-3.672	0.998	93.6
	R-TGCTCTTGCCGCCGGTGCCGTCG					
Chymotrypsin BII/O18488	F-GCCGCCCTCTTGACAGCG	57	143	-3.619	0.996	94.5
	R-TCCCTTACCTCCTTCGGAGTCA					
Metalloendopeptidase/Q20AS7	F-ACCATCGGAGGCAAGCAGA	57	197	-3.350	0.994	99.4
	R-TGCCAGTAGGTGCTCCTTGTTGA					
Cathepsin D/A0A3R7SR07	F-AATGGTCAGTGGACTGCAAC	57	155	-3.3	0.9962	100.5
	R-AACATCCAGGCCAATGAAGC					
Ubiquitin/A0A023H494	F-GGGAAGACCATCACCTTG	60	146	-3.113	0.994	104.8
	R-TCAGACAGAGTGGACCATC					

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each, then the annealing temperature according to [Table 1](#) for 30 s, and at 72°C for 30 s. For each primer set, a no template control (NTC) was included, for every case, such control showed no signal after 40 amplification cycles. The amplification data was collected using the StepOne Real-Time System software. The relative quantification of each peptidase gene expression was calculated using the $2^{-\Delta CT}$ method using ubiquitin as internal control since ubiquitin was the most stable reference gene in the experimental conditions analyzed, as determined with the geNorm algorithm [21].

The data matrix is represented on graphic mode using a heat map where the values are expressed in different colors to represent variations of each gene expression at each one of the larval instars. The heat map was created in the online software Heatmapper [22].

2.5. Proteolytic activity and inhibition assays

Larvae (90 mg of each instar) were thoroughly homogenized in 330 μ L of distilled water using a micro-pestle in a microtube and the cleared supernatant containing the soluble protein was

obtained by centrifugation at 10,000 g, for 10 min at 4°C. The soluble protein concentration was quantified after Bradford [23].

The proteolytic activity near the functional pH (as reported in database BRENDA-The Comprehensive Enzyme Information System [24]) was measured in four biological replicas by continuous kinetic assays using commercially available fluorogenic substrates designed to be recognized and cleaved by specific peptidases, such substrates are short synthetic peptides attached to the fluorogenic reporter 7-aminomethyl-coumarin (AMC; Ex:360 nm, Em:440 nm) or 7-methoxycoumarin-4-acetic acid (MCA; Ex:323 nm, Em:390 nm). The assays were performed at pH 3.6, 5.0 and 6.6 in citrate-phosphate buffer and at pH 8.0 in Tris-HCl buffer in the presence of 100 mM NaCl, 1mM DTT, and 0.001% Tween; for the assays at pH 8.0, 10 mM CaCl₂ was added, the substrate assay concentration was 2.5 μM.

Rates of hydrolysis of each substrate were recorded by measuring the increase of fluorescence in arbitrary units (relative fluorescence units, RFU) in black 96-well plates. A calibration curve was constructed by measuring the fluorescence of known concentrations of the fluorochrome and by plotting the RFU versus nanomoles of the molecule. Specific activity is expressed in nmol of AMC or MCA liberated per minute, per μg of protein. One unit of activity was defined as the release of 1 μmol of AMC or MCA per min per μg protein.

To confirm that the observed activity is specific for each enzyme, the larvae extract was pre-incubated for 30 min at room temperature with the countertype class-specific peptidase inhibitor; the aspartic peptidase inhibitor pepstatin A (1 μM final concentration), the cysteine peptidase inhibitor E-64 (30 μM final concentration) or the serine peptidase inhibitor PMSF (1 mM final concentration), and EDTA at 1 mM was used to chelate metal ions necessary for metallo-peptidase activity. The residual proteolytic activity was determined as described above, only the portion of the activity that resulted sensitive to the countertype inhibitor (the total activity minus the activity in the presence of inhibitor) was considered.

2.6. Statistical analysis

Data are summarized in box and whiskers plots showing the median and the min to max of four biological replicates quantified in duplicate. The statistical differences among different groups were determined by the Kruskal-Wallis test, the post hoc analysis Dunn's Multiple Comparison Test was applied and considered as statistically different when $p < 0.05$. GraphPad Prism 5 (GraphPad Software, USA) was used for statistical procedures and graph plotting.

3. Results

3.1. Peptidase gene expression profile during *P. vannamei* larval development

Patterns of gene expression of the selected peptidases throughout the larval development of *P. vannamei* are readily visualized in a heat map (Fig 2). Similar expression patterns were calculated by hierarchical clustering with Pearson's correlation similarity and average linkage distance. Three main clusters are detected (upper to lower clusters): a) peptidases expressed mainly in zoeal and mysis stages, this is a diverse group comprised by four genes encoding peptidases of the serine-, cysteine- and aspartic-classes; b) peptidases highly expressed at decapodid stages, this is the larger group comprising seven genes and includes well-characterized peptidases described as food protein-digestors as trypsin 1 and 2 [25], chymotrypsin [26] and cathepsin B [27] and; c) peptidases expressed mainly at the early naupliar instars, this group comprising the cysteine peptidases Calpain B, Cathepsin L and Cathepsin C.

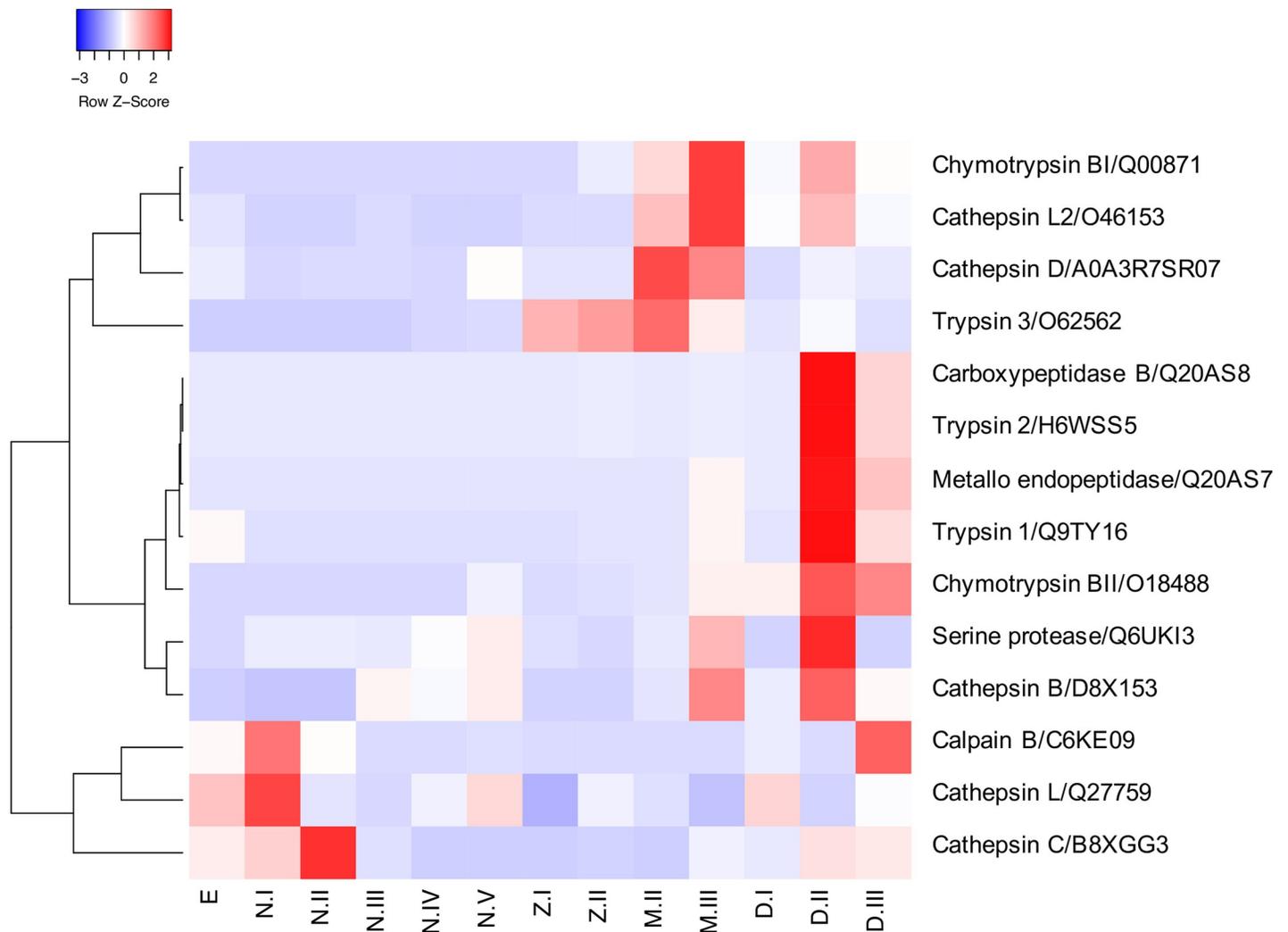


Fig 2. Peptidase gene expression patterns in the larval stages of *P. vannamei*. Heatmap colors represent relative mRNA expression as indicated in the color key. Clusters of expression patterns were calculated by hierarchical methods with Pearson's correlation similarity and average linkage distance.

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3.2. Peptidase activity profile during *P. vannamei* larval development

The proteolytic activity from the four peptidase classes was assessed. To quantify the cysteine peptidase activity, three substrates were used, Z-Phe-Arg↓AMC, Z-Leu-Arg↓AMC and Z-Val-Val-Arg↓AMC (arrow indicates the site of the scissile bond); although such substrates are recognized and efficiently cleaved by crustacean cysteine peptidases [28, 29], they might be also hydrolyzed by other non-cysteine peptidases [30], therefore only the portion of the activity that resulted sensitive to the cysteine peptidase inhibitor E-64 was considered. In agreement with the cysteine peptidase gene expression observed in the heat map (Fig 2, lower cluster), peaks of activity were observed during the early larval stages (Fig 3A). Such activity can be attributed to cathepsin L (Fig 3B), cathepsin C (Fig 3C) and calpain B (Fig 3D), since those genes showed the highest expression at naupliar stages.

Aspartic peptidase activity was assessed using the substrate MCA-Gly-Lys-Pro-Ile-Leu-Phe↓Phe-Arg-Leu-Lys(DNP)-DArg-NH₂, known to be hydrolyzed by cathepsin D and cathepsin E enzymes from diverse vertebrate and invertebrate species. A generally constant

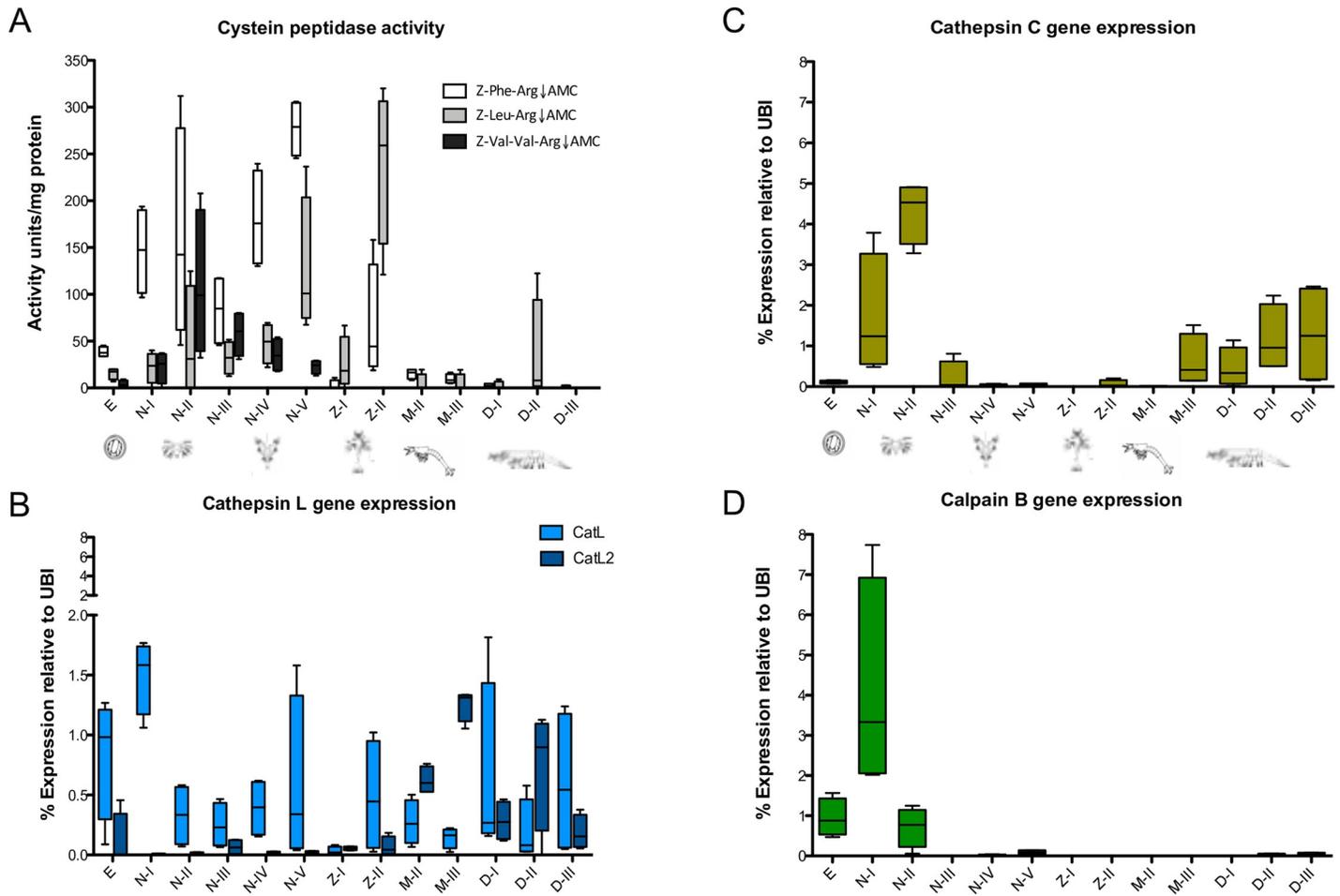


Fig 3. Cysteine peptidase gene expression and enzymatic activity throughout the larval development of *P. vannamei*. A) cysteine peptidase activity (sensitive to inhibition by E-64), B) cathepsin L and L2, C) cathepsin C, and D) calpain B gene expression.

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amount of aspartic peptidase activity was observed during the larval development with an increase at nauplii-II substage and then at zoea and mysis stages (Fig 4A). *P. vannamei* expresses at least one cathepsin D transcript (GenBank: MH171099.1; UniProt: A0A3R7SR07) and our results indicate that in general, the peak of gene expression at mysis stages (Fig 4B) match with the activity found at late zoea and mysis stages. A putative second isoform is reported in GenBank (XM_027380068) which sequence is derived from a genomic assembly (BioProject: PRJNA508983), its expression was not assessed in this work, but might be responsible for the rise in activity found in naupliar instars.

A zinc metallopeptidase expressed in the digestive tissues of *P. vannamei* has been reported [16], this enzyme belongs to the astacin family (PFAM: PF01400) and shares 59.1% of identity with *Astacus astacus* astacin, the eponym of this peptidase family that is also a digestive enzyme [31, 32]. Astacin effectively hydrolyze peptides that are bradykinin derivatives, like MCA-Arg-Pro-Pro-Gly-Phe↓Ser-Ala-Phe-Lys(DNP)-OH [33], which was used as a substrate to assess the activity of this enzyme class on developmental stages of *P. vannamei*. The activity against the metallo-peptidase substrate MCA-Arg-Pro-Lys-Pro-Tyr↓Ala-Nva-Trp-Met-Lys(DNP)-NH₂ was also assessed (Fig 4C), and effectively hydrolyzed throughout all the larval development, especially at the early stages. An increase on substrate preference was detected in later stages

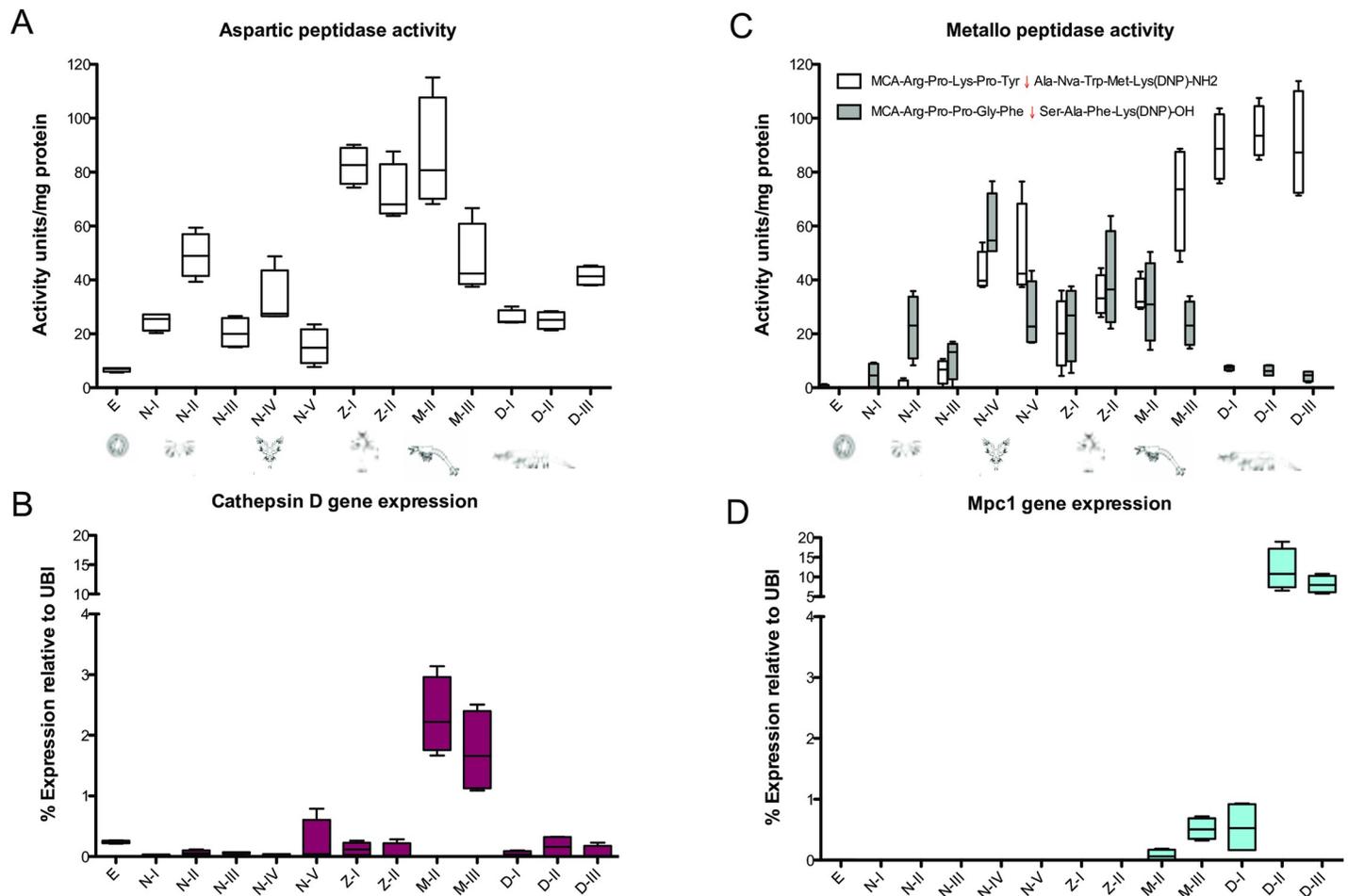


Fig 4. Aspartic and metallopeptidase gene expression and enzymatic activity throughout the larval development of *P. vannamei*. A) Aspartic peptidase activity (sensitive to inhibition by Pepstatin A), B) cathepsin D gene expression, C) Metallo peptidase activity (sensitive to inhibition by EDTA), D) Mpc1 gene expression.

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where the highest values were recorded against MCA-Arg-Pro-Lys-Pro-Tyr↓Ala-Nva-Trp-Met-Lys(DNP)-NH₂, the activity against this substrate coincides with the quantified metalloendopeptidase (UniProt: Q20AS7) gene expression at decapodid stages (Fig 4D), a late developmental stage with a fully mature digestive system.

Serine peptidases are the most-studied peptidase class in *P. vannamei*, being trypsin and chymotrypsin the predominant peptidases from the digestive tract of penaeids [34]. Boc-Phe-Ser-Arg↓AMC and Suc-Ala-Ala-Pro-Phe↓AMC were used as substrates to quantify trypsin and chymotrypsin activity respectively (Fig 5A and 5C). Activity against trypsin substrate was by far the most abundant during the larval development with the highest values recorded during the zoea and mysis instars and a decline at decapodid instars, this activity fully matches with the expression pattern of trypsin 3 gene (UniProt: O62562) (Fig 5B). Chymotrypsin BI transcripts are abundant at mysis and decapodid instars (Fig 5D) but the activity profiles are different.

4. Discussion

Peptidases are known to play key functions in larval development in arthropods. Insects are particularly well-studied taxa for which the metamorphic proteolytic repertoire has been

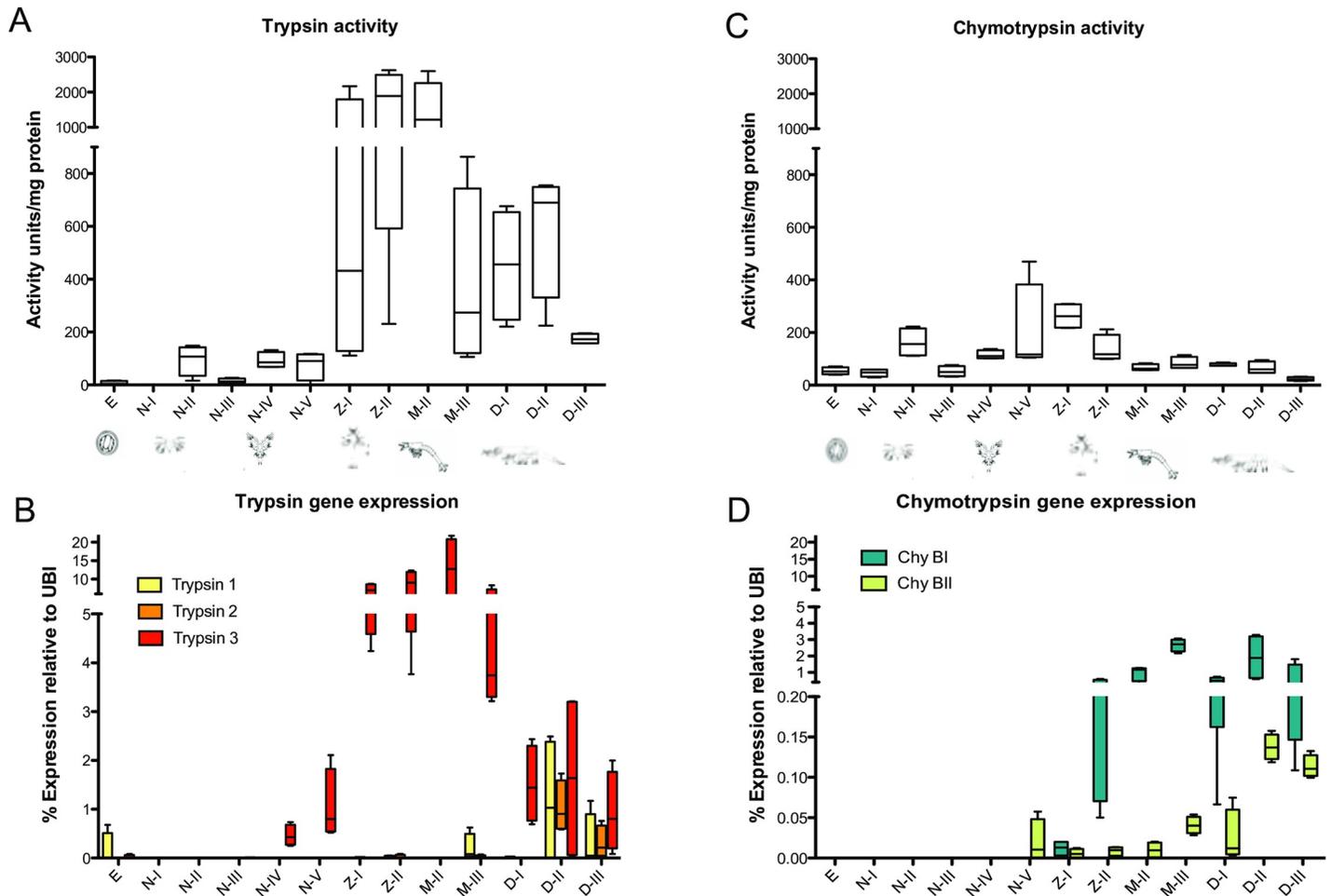


Fig 5. Serine peptidase gene expression and enzymatic activity throughout the larval development of *P. vannamei*. A) Trypsin activity (sensitive to inhibition by PMSF), B) Trypsin isoforms gene expression, C) Chymotrypsin activity (sensitive to inhibition by PMSF), and D) Chymotrypsin isoforms gene expression.

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described in detail [5, 35–40]. The present study provides a comprehensive characterization of larval proteolytic profile in both transcript and activity analysis in a crustacean, the whiteleg shrimp *P. vannamei*.

The main protein component of yolk in *P. vannamei* zygotes is vitellin, a large multi-sub-unit lipo-glyco-caroteno-protein; its degraded products are the source of amino acids and other nutrients for development in the non-feeding larvae (see Fig 1) [41], which survival depends on the nutrient reserves of the yolk [42]. *P. vannamei* cysteine peptidase activity is high at the naupliar stages (Fig 3A), we presume this activity is responsible for hydrolysis of yolk proteins, as described in other arthropods [43–45]. Cathepsin L, cathepsin C and calpain are assumed to be the main enzymes responsible of the hydrolysis of vitellin given their significantly higher expression at naupliar stages (clustered in Figs 2 and 3B–3D, respectively).

Another featured function of peptidases in insect development is the transformation and removal of cuticle components during molt. Degradation of the old cuticle is accomplished by the secretion of chitinases and peptidases into the molting space [8, 46–50]. Decapod molting cycle is one of the fundamental processes for growth and development, which involves rearrangement of muscle fibers that undergo degradation of the muscular proteins actin and myosin, leading to a muscle induced atrophy, this allows limb withdrawal from narrow openings

during ecdysis [51]. In *P. vannamei*, the formation of the exoskeleton initiates during the limb bud embryo stage [52], and is shed shortly after hatching. Several molting events accompanied by metamorphic transitions occur within a few days (11–17 days depending on the temperature), in which a new cuticle is formed and replaced cyclically at a relatively high rate to accommodate the rapidly growing larvae [42, 53, 54]. Trypsin (Fig 5A), chymotrypsin (Fig 5C), aspartic peptidase (Fig 4A) and metallo-peptidase activity (Fig 4C) were detected in all stages of larval development, suggesting a function of these peptidases in molting-related processes such as degradation and reabsorption of proteins from the cuticle, ecdysial matter, and muscle. This has been suggested for juvenile *P. vannamei* in which trypsin gene expression and activity is particularly high during juvenile pre-ecdysis stages [55, 56].

A generally sustained aspartic peptidase activity during the larval development was observed in our results (Fig 4A). In insect larvae development, cathepsin D fulfills specific functions, including yolk proteins digestion [57], fat body degradation [58], and apoptotic processes linked to insect metamorphosis [5], therefore we hypothesize that *P. vannamei* cathepsin D is the responsible of such aspartic peptidase activity. We determined the expression pattern of a *P. vannamei* cathepsin D (UniProt: A0A3R7SR07), observing a generally steady expression in all stages, with a significant increase at mysis instars (Fig 4B) that clearly matches the peak of activity at late zoea and mysis stages. During *P. vannamei* mysis instars, major growth and differentiation of hepatopancreatic lobes occur [59], based on our result we suggest that cathepsin D is synthesized in this tissue and could be involved in the larval morphogenesis of the midgut gland.

Metallo-peptidase activity was also assessed (Fig 4C), larval zinc metallopeptidase expression and activity had been reported mainly in the infective stages of helminths and insects [60, 61], but also as fat-body remodeling enzymes in insect larval stages [62]. The main *P. vannamei* metallopeptidase belongs to the astacin family and the transcript is found primarily in the digestive system [16, 63]. The expression of this enzyme was detected from late mysis stage and increasing at decapodid stages where a significant increment of activity is also observed (Fig 4D), such activity peak has been reported before [16]. *P. vannamei* also expresses a putative second isoform of metallo-peptidase that is deposited in the GenBank (XM_027379199), an astacin-like enzyme which sequence is derived from a genomic assembly (BioProject: PRJNA508983), its expression was not assessed in this work, the discrepancy in metallo-peptidase gene expression and activity might be an effect of the presence of an alternative isoform which might show a preference for MCA-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(DNP)-OH and express steadily during the larval development.

Serine peptidase activity was detected in all larval stages. Fluctuations in trypsin and chymotrypsin peptidases expression and activity during larval stages of development is documented for *P. vannamei* and other crustaceans species [12, 13, 16, 64]. Trypsin (Fig 5A) and chymotrypsin activity (Fig 5C) show a significant increase at zoea stages, trypsin is by far the dominant peptidase activity in these stages and continues through the first decapodid instar. Trypsin activity profiles are consistent with the expression pattern of Trypsin 3 (UniProt: O62562) (Fig 5B), which increase in expression at zoea instars has been reported by other authors [12, 14]. Zoea is the first feeding stage during which the gut becomes complete but the rest of the digestive organs, like the cardiac and pyloric chamber and the hepatopancreas, are still undifferentiated; the zoeal gut consists of a simple tube and two pairs of caeca that fulfill digestive and absorptive functions and are the primordium to the hepatopancreas [59, 65]. During zoea, the breakdown of larval food is primarily enzymatic [42, 66], this fact explains the general increase in serine peptidase activity detected in our assays.

Juvenile and adult *P. vannamei* midgut gland is particularly enriched with the serine peptidases trypsin and chymotrypsin, which fulfill a digestive function breaking peptide bonds of

large proteins from food into smaller peptides ready for absorption [34]. The gene expression of isoforms of trypsin and chymotrypsin was detected at decapodid stages (Fig 5B and 5C). Trypsin 1 (UniProt: Q9TY16) and 2 (UniProt: H6WSS5) are found in the digestive tract of juvenile and adult *P. vanamei* and reveal as 3 activity bands when analyzed by zymography [67]. Coincidentally, the gene expression of these enzymes was detected only at decapodid stages (Fig 5B), at which the digestive organs are fully operational [65]. In the heat map (Fig 2) Trypsin 1 and 2 show a similar expression pattern that clusters with hepatopancreatic enzymes described in other works by zymography (e.g. metallopeptidase and chymotrypsin BII) [16], reinforcing the functional characterization of this group as food protein digestors in decapodid and juvenile stages.

The fact that in our results peptidase gene expression and the corresponding activity profiles are not fully dependent during the biological conditions assessed (larval development) cannot be overlooked. Transcription and translation are generally regarded as independent processes due to the mechanism triggering them [68], a mismatch in enzyme gene expression and activity is common and almost considered the general rule [69], since several factors influence in the production of functional proteins. In the particular cases of peptidase gene expression during metamorphosis, this is a process highly controlled by hormonal changes in both insects and crustaceans [70, 71]. Some peptidases are classified as ecdysteroid-responsive genes, since they are known to contain ecdysone response elements in their promoters [72], and are under the control of the ecdysone cassette [73, 74]. At the protein level, molt has also been described to be controlled by peptidase inhibitors, and these molecules are considered regulators of the molting process [35].

The presence of different peptidases belonging to the same functional categories needs to be taken into account and is reported for decapods [75]. Peptidase variants or isoforms may be conducting the same physiological function at different stages, a phenomenon called isoform-switching that have has observed in crustacean larval stages [76]. The presence of class-specific peptidase activity in stages where the corresponding gene was not detected might be due to the presence of peptidase gene isoforms that are still to be annotated and evaluated.

In this study, we describe the mRNA expression and proteolytic activity patterns of several members of the cysteine- aspartic-, metallo- and serine-peptidases throughout the larval development of *P. vanamei*; the enzymes showed variations in expression and activity, indicating that they are controlled and play various roles in shrimp larvae development and metamorphoses like yolk degradation, cuticle transformation and degradation of unnecessary tissues. The present work contributes to a more integral perspective for understanding the developmental biology of this species, required for the technification of culture methods and proper management of natural stocks of *P. vanamei*. Further studies are required to describe in more detail the specific peptidase functions and interactions.

Supporting information

S1 Table. Distribution of gene expression data (mean, min and max) used to construct plots in Figs 3–5.

(XLSX)

S2 Table. Distribution of proteolytic activity data (mean, min and max) used to construct plots in Figs 3–5.

(XLSX)

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Author Contributions

Conceptualization: Liliana Rojo-Arreola.

Data curation: Fernando García-Carreño, Rogelio Romero, Luis Díaz Dominguez.

Formal analysis: Rogelio Romero, Luis Díaz Dominguez.

Funding acquisition: Liliana Rojo-Arreola.

Investigation: Rogelio Romero, Luis Díaz Dominguez.

Project administration: Liliana Rojo-Arreola.

Supervision: Liliana Rojo-Arreola, Fernando García-Carreño.

Writing – original draft: Liliana Rojo-Arreola.

Writing – review & editing: Liliana Rojo-Arreola, Fernando García-Carreño.

References

1. Rawlings ND, Salvesen GS. Handbook of Proteolytic Enzymes. 3rd ed. Handbook of Proteolytic Enzymes. Amsterdam: Elsevier; 2013. <https://doi.org/10.1016/C2009-1-60990-4>
2. Leyria J, Fruttero LL, Ligabue-Braun R, Defferrari MS, Arrese EL, Soulages JL, et al. DmCatD, a cathepsin D-like peptidase of the hematophagous insect *Dipetalogaster maxima* (Hemiptera: Reduviidae): Purification, bioinformatic analyses and the significance of its interaction with lipophorin in the internalization by developing oocytes. *J Insect Physiol.* 2018; 105: 28–39. <https://doi.org/10.1016/j.jinsphys.2018.01.002> PMID: 29325877
3. Ahn J-E, Zhu-Salzman K. CmCatD, a cathepsin D-like protease has a potential role in insect defense against a phytocystatin. *J Insect Physiol.* 2009; 55: 678–85. <https://doi.org/10.1016/j.jinsphys.2009.04.016> PMID: 19446566
4. Hu K-J, Leung P-C. Food digestion by cathepsin L and digestion-related rapid cell differentiation in shrimp hepatopancreas. *Comp Biochem Physiol B Biochem Mol Biol.* 2007; 146: 69–80. <https://doi.org/10.1016/j.cbpb.2006.09.010> PMID: 17208029
5. Zhong ZG, Kwang SL, Bo YK, Yong SC, Ya DW, Young MC, et al. Functional role of aspartic proteinase cathepsin D in insect metamorphosis. *BMC Dev Biol.* 2006; 6: 49. <https://doi.org/10.1186/1471-213X-6-49> PMID: 17062167
6. Dvořák J, Mashiyama ST, Braschi S, Sajid M, Knudsen GM, Hansell E, et al. Differential use of protease families for invasion by schistosome cercariae. *Biochimie.* 2008; 90: 345–358. <https://doi.org/10.1016/j.biochi.2007.08.013> PMID: 17936488
7. Sojka D, Hartmann D, Bartošová P, Dvořák J. Parasite Cathepsin D-Like Peptidases and Their Relevance as Therapeutic Targets. *Trends Parasitol.* 2016; xx: 1–16. <https://doi.org/10.1016/j.pt.2016.05.015> PMID: 27344362
8. Liu J, Shi GP, Zhang WQ, Zhang GR, Xu WH. Cathepsin L function in insect moulting: Molecular cloning and functional analysis in cotton bollworm, *Helicoverpa armigera*. *Insect Mol Biol.* 2006; 15: 823–834. <https://doi.org/10.1111/j.1365-2583.2006.00686.x> PMID: 17201774
9. Sun YX, Tang L, Wang P, Abbas MN, Tian JW, Zhu BJ, et al. Cathepsin L-like protease can regulate the process of metamorphosis and fat body dissociation in *Antheraea pernyi*. *Dev Comp Immunol.* 2018; 78: 114–123. <https://doi.org/10.1016/j.dci.2017.09.019> PMID: 28958702
10. Saborowski R, Thatje S, Calcagno JA, Lovrich GA, Anger K. Digestive enzymes in the ontogenetic stages of the southern king crab, *Lithodes santolla*. *Mar Biol.* 2006; 149: 865–873. <https://doi.org/10.1007/s00227-005-0240-x>
11. Lemos D, Hernández-Cortés MP, Navarrete A, García-Carreño FL, Phan VN. Ontogenetic variation in digestive proteinase activity of larvae and postlarvae of the pink shrimp *Farfantepenaeus paulensis*

- (Crustacea: Decapoda: Penaeidae). *Mar Biol.* 1999; 135: 653–662. <https://doi.org/10.1007/s002270050666>
12. Wei J, Zhang X, Yu Y, Li F, Xiang J. RNA-Seq reveals the dynamic and diverse features of digestive enzymes during early development of Pacific white shrimp *Litopenaeus vannamei*. *Comp Biochem Physiol Part D Genomics Proteomics.* 2014; 11: 37–44. <https://doi.org/10.1016/j.cbd.2014.07.001> PMID: [25090194](https://pubmed.ncbi.nlm.nih.gov/25090194/)
 13. Wei J, Zhang X, Yu Y, Huang H, Li F, Xiang J. Comparative transcriptomic characterization of the early development in Pacific white shrimp *Litopenaeus vannamei*. *PLoS One.* 2014; 9: 1–13. <https://doi.org/10.1371/journal.pone.0106201> PMID: [25197823](https://pubmed.ncbi.nlm.nih.gov/25197823/)
 14. Lage LPA, Plagnes-Juan E, Putrino SM, Baron F, Weissman D, Guyonvarch A, et al. Ontogenesis of metabolic gene expression in whiteleg shrimp (*Litopenaeus vannamei*): New molecular tools for programming in the future. *Aquaculture.* 2017; 479: 142–149. <https://doi.org/10.1016/J.AQUACULTURE.2017.05.030>
 15. Li Y, Hui M, Cui Z, Liu Y, Song C, Shi G. Comparative transcriptomic analysis provides insights into the molecular basis of the metamorphosis and nutrition metabolism change from zoeae to megalopae in *Eriocheir sinensis*. *Comp Biochem Physiol—Part D Genomics Proteomics.* 2015. <https://doi.org/10.1016/j.cbd.2014.10.002> PMID: [25460100](https://pubmed.ncbi.nlm.nih.gov/25460100/)
 16. Hernandez-Cortes P, Rivera-Pérez C, García-Carreño F, Martínez-Alarcón D. Proteinases during early development of the pacific whiteleg shrimp *Penaeus vannamei*. *Biol Bull.* 2017; 232: 2–11. <https://doi.org/10.1086/691381> PMID: [28445094](https://pubmed.ncbi.nlm.nih.gov/28445094/)
 17. Cook HL, Murphy MA. The Culture of Larval Penaeid Shrimp. *Trans Am Fish Soc.* 1969; 98: 751–754. [https://doi.org/10.1577/1548-8659\(1969\)98\[751:TCOLPS\]2.0.CO;2](https://doi.org/10.1577/1548-8659(1969)98[751:TCOLPS]2.0.CO;2)
 18. Poulin É, Boletzky S V., Féral JP. Combined ecological factors permit classification of developmental patterns in benthic marine invertebrates: A discussion note. *J Exp Mar Bio Ecol.* 2001; 257: 109–115. [https://doi.org/10.1016/s0022-0981\(00\)00332-4](https://doi.org/10.1016/s0022-0981(00)00332-4) PMID: [11165302](https://pubmed.ncbi.nlm.nih.gov/11165302/)
 19. Dall W, Hill BJ, Rothlisberg PC, Sharples DJ. The Biology of the Penaeidae. Dall W, Hill BJ, Rothlisberg PC, Sharples DJ, editors. *Advances in Marine Biology.* Cambridge, Massachusetts: Academic Press; 1991. [https://doi.org/10.1016/S0065-2881\(08\)60174-1](https://doi.org/10.1016/S0065-2881(08)60174-1)
 20. Bustin S, Huggett J. qPCR primer design revisited. *Biomol Detect Quantif.* 2017; 14: 19–28. <https://doi.org/10.1016/j.bdq.2017.11.001> PMID: [29201647](https://pubmed.ncbi.nlm.nih.gov/29201647/)
 21. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002; 3: research0034.1. <https://doi.org/10.1186/gb-2002-3-7-research0034> PMID: [12184808](https://pubmed.ncbi.nlm.nih.gov/12184808/)
 22. Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, et al. Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res.* 2016; 44: W147–53. <https://doi.org/10.1093/nar/gkw419> PMID: [27190236](https://pubmed.ncbi.nlm.nih.gov/27190236/)
 23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72: 248–54. Available: <http://www.ncbi.nlm.nih.gov/pubmed/942051> <https://doi.org/10.1006/abio.1976.9999> PMID: [942051](https://pubmed.ncbi.nlm.nih.gov/942051/)
 24. Scheer M, Grote A, Chang A, Schomburg I, Munaretto C, Rother M, et al. BRENDA, the enzyme information system in 2011. *Nucleic Acids Res.* 2011; 39: D670–6. <https://doi.org/10.1093/nar/gkq1089> PMID: [21062828](https://pubmed.ncbi.nlm.nih.gov/21062828/)
 25. Sainz JC, García-Carreño F, Sierra-Beltrán A, Hernández-Cortés P. Trypsin Synthesis and Storage as Zymogen in the Midgut Gland of the Shrimp *Litopenaeus Vannamei*. *J Crustac Biol.* 2004; 24: 266–273. Available: <http://dx.doi.org/10.1651/C-2423>
 26. Hernandez-Cortes P, Whitaker JR, Garcia-Carreño FL. Purification and characterization of chymotrypsin from *Penaeus vannamei* (Crustacea: Decapoda). *J Food Biochem.* 1997; 21: 497–514.
 27. Stephens A, Rojo L, Araujo-Bernal S, Garcia-Carreño F, Muhlia-Almazan A. Cathepsin B from the white shrimp *Litopenaeus vannamei*: cDNA sequence analysis, tissues-specific expression and biological activity. *Comp Biochem Physiol B Biochem Mol Biol.* 2012; 161: 32–40. <https://doi.org/10.1016/j.cbpb.2011.09.004> PMID: [21946097](https://pubmed.ncbi.nlm.nih.gov/21946097/)
 28. Aoki H, Nazmul Ahsan M, Watabe S. Heterologous expression in *Pichia pastoris* and single-step purification of a cysteine proteinase from northern shrimp. *Protein Expr Purif.* 2003; 31: 213–221. [https://doi.org/10.1016/s1046-5928\(03\)00196-7](https://doi.org/10.1016/s1046-5928(03)00196-7) PMID: [14550639](https://pubmed.ncbi.nlm.nih.gov/14550639/)
 29. Aoki H, Ahsan MN, Watabe S. Molecular and enzymatic properties of a cathepsin L-like proteinase with distinct substrate specificity from northern shrimp (*Pandalus borealis*). *J Comp Physiol B Biochem Syst Environ Physiol.* 2004; 174: 59–69. <https://doi.org/10.1007/s00360-003-0389-x> PMID: [14574613](https://pubmed.ncbi.nlm.nih.gov/14574613/)

30. Horn M, Fajtová P, Rojo Arreola L, Ulrychová L, Bartošová-Sojtková P, Franta Z, et al. Trypsin- and Chymotrypsin-like serine proteases in schistosoma mansoni—"the undiscovered country". *PLoS Negl Trop Dis*. 2014; 8: e2766. <https://doi.org/10.1371/journal.pntd.0002766> PMID: 24676141
31. Vogt G, Stöcker W, Storch V, Zwilling R. Biosynthesis of *Astacus* protease, a digestive enzyme from crayfish. *Histochemistry*. 1989; 91: 373–381. <https://doi.org/10.1007/BF00493824> PMID: 2656593
32. Geier G, Zwilling R. Cloning and characterization of a cDNA coding for *Astacus* embryonic astacin, a member of the astacin family of metalloproteases from the crayfish *Astacus astacus*. *Eur J Biochem*. 1998; 253: 796–803. <https://doi.org/10.1046/j.1432-1327.1998.2530796.x> PMID: 9654081
33. Becker-Pauly C, Barré O, Schilling O, Auf Dem Keller U, Ohler A, Broder C, et al. Proteomic analyses reveal an acidic prime side specificity for the astacin metalloprotease family reflected by physiological substrates. *Mol Cell Proteomics*. 2011; 10: 1–19. <https://doi.org/10.1074/mcp.M111.009233> PMID: 21693781
34. Navarrete del Toro MA, García-Carreño F, Córdova-Murueta J. Comparison of digestive proteinases in three penaeids. *Aquaculture*. 2011; 317: 99–106.
35. Liu H-W, Wang L-L, Tang X, Dong Z-M, Guo P-C, Zhao D-C, et al. Proteomic analysis of *Bombyx mori* molting fluid: Insights into the molting process. *J Proteomics*. 2018; 173: 115–125. <https://doi.org/10.1016/j.jprot.2017.11.027> PMID: 29197581
36. Borges-Veloso A, Saboia-Vahia L, Cuervo P, Pires RC, Britto C, Fernandes N, et al. Proteolytic profiling and comparative analyses of active trypsin-like serine peptidases in preimaginal stages of *Culex quinquefasciatus*. *Parasites and Vectors*. 2012; 5: 1–11. <https://doi.org/10.1186/1756-3305-5-1> PMID: 22212459
37. Martynov AG, Elpidina EN, Perkin L, Oppert B. Functional analysis of C1 family cysteine peptidases in the larval gut of *Tenebrio molitor* and *Tribolium castaneum*. *BMC Genomics*. 2015; 16: 75. <https://doi.org/10.1186/s12864-015-1306-x> PMID: 25757364
38. Qu M, Ma L, Chen P, Yang Q. Proteomic analysis of insect molting fluid with a focus on enzymes involved in chitin degradation. *J Proteome Res*. 2014; 13: 2931–2940. <https://doi.org/10.1021/pr5000957> PMID: 24779478
39. Perkin L, Elpidina EN, Oppert B. Expression patterns of cysteine peptidase genes across the *Tribolium castaneum* life cycle provide clues to biological function. *PeerJ*. 2016; 4: e1581. <https://doi.org/10.7717/peerj.1581> PMID: 26819843
40. Saikhedkar N, Summanwar A, Joshi R, Giri A. Cathepsins of lepidopteran insects: Aspects and prospects. *Insect Biochem Mol Biol*. 2015; 64: 51–59. <https://doi.org/10.1016/j.ibmb.2015.07.005> PMID: 26210259
41. García-Orozco KD, Vargas-Albores F, Sotelo-Mundo RR, Yepiz-Plascencia G. Molecular characterization of vitellin from the ovaries of the white shrimp *Penaeus (Litopenaeus) vannamei*. *Comp Biochem Physiol—B Biochem Mol Biol*. 2002; 133: 361–369. [https://doi.org/10.1016/s1096-4959\(02\)00152-5](https://doi.org/10.1016/s1096-4959(02)00152-5) PMID: 12431404
42. Anger K. *The Biology of Decapod Crustacean Larvae*. Crustacean Issues. Lisse, The Netherlands: A. A. Balkema; 2001. [https://doi.org/10.1651/0278-0372\(2005\)025](https://doi.org/10.1651/0278-0372(2005)025)
43. Cho WL, Tsao SM, Hays AR, Walter R, Chen JS, Snigirevskaya ES, et al. Mosquito cathepsin B-like protease involved in embryonic degradation of vitellin is produced as a latent extraovarian precursor. *J Biol Chem*. 1999; 274: 13311–13321. <https://doi.org/10.1074/jbc.274.19.13311> PMID: 10224092
44. Zhang TT, Qiu ZX, Li Y, Wang WY, Li MM, Guo P, et al. The mRNA expression and enzymatic activity of three enzymes during embryonic development of the hard tick *Haemaphysalis longicornis*. *Parasites and Vectors*. 2019; 12: 1–8. <https://doi.org/10.1186/s13071-018-3256-z> PMID: 30606222
45. Estrela A, Seixas A, Termignoni C. A cysteine endopeptidase from tick (*Rhipicephalus (Boophilus) microplus*) larvae with vitellin digestion activity. *Comp Biochem Physiol—B Biochem Mol Biol*. 2007; 148: 410–416. <https://doi.org/10.1016/j.cbpb.2007.07.009> PMID: 17765577
46. Liu H-W, Wang L-L, Meng Z, Tang X, Li Y-S, Xia Q-Y, et al. A clip domain serine protease involved in moulting in the silkworm, *Bombyx mori*: cloning, characterization, expression patterns and functional analysis. *Insect Mol Biol*. 2017; 26: 507–521. <https://doi.org/10.1111/imb.12312> PMID: 28597953
47. Liu H, Heng J, Wang L, Tang X, Guo P, Li Y, et al. Identification, characterization, and expression analysis of clip-domain serine protease genes in the silkworm, *Bombyx mori*. *Dev Comp Immunol*. 2020; 105: 103584. <https://doi.org/10.1016/j.dci.2019.103584> PMID: 31863792
48. Broehan G, Arakane Y, Beeman RW, Kramer KJ, Muthukrishnan S, Merzendorfer H. Chymotrypsin-like peptidases from *Tribolium castaneum*: a role in molting revealed by RNA interference. *Insect Biochem Mol Biol*. 2010; 40: 274–83. <https://doi.org/10.1016/j.ibmb.2009.10.009> PMID: 19897036

49. Wei Z, Yin Y, Zhang B, Wang Z, Peng G, Cao Y, et al. Cloning of a novel protease required for the molting of *Locusta migratoria manilensis*. *Dev Growth Differ*. 2007; 49: 611–621. <https://doi.org/10.1111/j.1440-169X.2007.00957.x> PMID: 17716305
50. Chikate YR, Dawkar V V., Barbole RS, Tilak P V., Gupta VS, Giri AP. RNAi of selected candidate genes interrupts growth and development of *Helicoverpa armigera*. *Pestic Biochem Physiol*. 2016; 133: 44–51. <https://doi.org/10.1016/j.pestbp.2016.03.006> PMID: 27742360
51. de Oliveira Cesar JR, Zhao B, Malecha S, Ako H, Yang J. Morphological and biochemical changes in the muscle of the marine shrimp *Litopenaeus vannamei* during the molt cycle. *Aquaculture*. 2006; 261: 688–694. <https://doi.org/10.1016/J.AQUACULTURE.2006.08.003>
52. Gao Y, Wei J, Yuan J, Zhang X, Li F, Xiang J. Transcriptome analysis on the exoskeleton formation in early developmental stages and reconstruction scenario in growth-moulting in *Litopenaeus vannamei*. *Sci Rep*. 2017; 7: 1098. <https://doi.org/10.1038/s41598-017-01220-6> PMID: 28439089
53. Anger K. Contributions of larval biology to crustacean research: a review Contributions of larval biology to crustacean research: a review. *Invertebr Reprod Dev*. 2010; 49: 175–205.
54. Mrak P, Bogataj U, Štrus J, Žnidaršič N. Cuticle morphogenesis in crustacean embryonic and postembryonic stages. *Arthropod Struct Dev*. 2017; 46: 77–95. <https://doi.org/10.1016/j.asd.2016.11.001> PMID: 27816526
55. Gao Y, Zhang X, Wei J, Sun X, Yuan J, Li F, et al. Whole transcriptome analysis provides insights into molecular mechanisms for molting in *Litopenaeus vannamei*. *PLoS One*. 2015; 10: 1–26. <https://doi.org/10.1371/journal.pone.0144350> PMID: 26650402
56. Muhlia-Almazán A, García-Carreño FL. Influence of molting and starvation on the synthesis of proteolytic enzymes in the midgut gland of the white shrimp *Penaeus vannamei*. *Comp Biochem Physiol—B Biochem Mol Biol*. 2002; 133: 383–394. [https://doi.org/10.1016/s1096-4959\(02\)00163-x](https://doi.org/10.1016/s1096-4959(02)00163-x) PMID: 12431406
57. Fialho E, Nakamura A, Juliano L, Masuda H, Silva-Neto MAC. Cathepsin D-mediated yolk protein degradation is blocked by acid phosphatase inhibitors. *Arch Biochem Biophys*. 2005; 436: 246–253. <https://doi.org/10.1016/j.abb.2005.01.005> PMID: 15797237
58. Kang T, Jin R, Zhang Y, Wan H, Lee KS, Jin BR, et al. Functional characterization of the aspartic proteinase cathepsin D in the beet armyworm (*Spodoptera exigua*). *Gene*. 2017; 617: 1–7. <https://doi.org/10.1016/j.gene.2017.03.035> PMID: 28351737
59. Vogt G. Functional cytology of the hepatopancreas of decapod crustaceans. *J Morphol*. 2019; 280: 1405–1444. <https://doi.org/10.1002/jmor.21040> PMID: 31298794
60. Alipour H, Raz A, Zakeri S, Djadid ND. Molecular characterization of matrix metalloproteinase-1 (MMP-1) in *Lucilia sericata* larvae for potential therapeutic applications. *Electron J Biotechnol*. 2017; 29: 47–56. <https://doi.org/10.1016/J.EJBT.2017.06.007>
61. Hambrook JR, Kaboré AL, Pila EA, Hanington PC. A metalloprotease produced by larval *Schistosoma mansoni* facilitates infection establishment and maintenance in the snail host by interfering with immune cell function. Jolly ER, editor. *PLOS Pathog*. 2018; 14: e1007393. <https://doi.org/10.1371/journal.ppat.1007393> PMID: 30372490
62. Jia Q, Liu Y, Liu H, Li S. Mmp1 and Mmp2 cooperatively induce *Drosophila* fat body cell dissociation with distinct roles. *Sci Rep*. 2014; 4: 7535. <https://doi.org/10.1038/srep07535> PMID: 25520167
63. Rojo-Arreola L, Choquet C, Cordova-Murueta J, García-Carreño F. The protease-based compensatory mechanism to minimize the effect of dietary Soybean Trypsin Inhibitor in *Litopenaeus vannamei*. *Aquaculture*. 2019; 500: 18–23. <https://doi.org/10.1016/J.AQUACULTURE.2018.10.002>
64. Andrés M, Gisbert E, Díaz M, Moyano FJ, Estévez A, Rotllant G. Ontogenetic changes in digestive enzymatic capacities of the spider crab, *Maja brachydactyla* (Decapoda: Majidae). *J Exp Mar Bio Ecol*. 2010. <https://doi.org/10.1016/j.jembe.2010.02.003> PMID: 20526380
65. Lovett D, Felder D. Ontogeny of Gut Morphology in the White Shrimp *Penaeus setiferus*. *J Morphol*. 1989; 272: 253–272.
66. Lovett DL, Felder DL. Ontogenetic Changes in Enzyme Distribution and Midgut Function in Developmental Stages of *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). *Biol Bull*. 1990; 178: 160–174. <https://doi.org/10.2307/1541974> PMID: 29314934
67. Sainz JC, García-Carreño FL, Hernández-Cortés P. *Penaeus vannamei* isotrypsins: Purification and characterization. *Comp Biochem Physiol—B Biochem Mol Biol*. 2004; 138: 155–162. <https://doi.org/10.1016/j.cbpc.2004.03.002> PMID: 15193270
68. Slobodin B, Han R, Calderone V, Vrieling JAF, Loayza-Puch F, Elkou R, et al. Transcription Impacts the Efficiency of mRNA Translation via Co-transcriptional N6-adenosine Methylation. *Cell*. 2017; 169: 326–337. <https://doi.org/10.1016/j.cell.2017.03.031> PMID: 28388414

69. Glanemann C, Loos A, Gorret N, Willis LB, O'Brien XM, Lessard PA, et al. Disparity between changes in mRNA abundance and enzyme activity in *Corynebacterium glutamicum*: Implications for DNA microarray analysis. *Appl Microbiol Biotechnol*. 2003; 61: 61–68. <https://doi.org/10.1007/s00253-002-1191-5> PMID: 12658516
70. Brandão A dos S, do Amaral JôB, Rezende-Teixeira P, Hartfelder K, Siviero F, Machado-Santelli GM. Cell death and tissue reorganization in *Rhynchosciara americana* (Sciaridae: Diptera) metamorphosis and their relation to molting hormone titers. *Arthropod Struct Dev*. 2014; 43: 511–522. <https://doi.org/10.1016/j.asd.2014.05.001> PMID: 24943875
71. Cheong SPS, Huang J, Bendena WG, Tobe SS, Hui JHL. Evolution of Ecdysis and Metamorphosis in Arthropods: The Rise of Regulation of Juvenile Hormone. *Integr Comp Biol*. 2015; 55: 878–890. <https://doi.org/10.1093/icb/icv066> PMID: 26105594
72. Yu J, Wu FY, Zou FM, Jia JQ, Wang SP, Zhang GZ, et al. Identification of ecdysone response elements (EcREs) in the *Bombyx mori* cathepsin D promoter. *Biochem Biophys Res Commun*. 2012; 425: 113–118. <https://doi.org/10.1016/j.bbrc.2012.07.068> PMID: 22828514
73. Cai XY, Yu J, Yu HY, Liu YW, Fang Y, Ren ZX, et al. Core promoter regulates the expression of cathepsin B gene in the fat body of *Bombyx mori*. *Gene*. 2014; 542: 232–239. <https://doi.org/10.1016/j.gene.2014.03.012> PMID: 24630970
74. Guo S-Y, Wu W-M, Li S-Y, Liu Y, Ruan ZF, Ye M-Q, et al. 20-Hydroxyecdysone-upregulated proteases involved in *Bombyx* larval fat body destruction. *Insect Mol Biol*. 2018. <https://doi.org/10.1111/imb.12511> PMID: 29888823
75. Rojo L, Muhlia-Almazan A, Saborowski R, Garcia-Carretero F. Aspartic Cathepsin D Endopeptidase Contributes to Extracellular Digestion in Clawed Lobsters *Homarus americanus* and *Homarus gammarus*. *Mar Biotechnol*. 2010; 12: 696–707. <https://doi.org/10.1007/s10126-010-9257-3> PMID: 20169386
76. Havird JC, Santos SR. Developmental transcriptomics of the hawaiian anchialine shrimp *Halocaridina rubra* holthuis, 1963 (Crustacea: Atyidae). *Integr Comp Biol*. 2016; 56: 1170–1182. <https://doi.org/10.1093/icb/icw003> PMID: 27400978