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In silico analysis and effects of environmental salinity in the expression and activity of digestive α-amylase and trypsins from the euryhaline crab *Neohelice granulata*

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In silico analysis and effects of environmental salinity in the expression and activity of digestive α-amylase and trypsins from the euryhaline crab *Neohelice granulata*

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Abstract

Studies on molecular characteristics and modulation of expression of α-amylase and trypsin in the hepatopancreas of intertidal euryhaline crabs are lacking. In this work, we cloned and studied by *in silico* approaches the characteristics of cDNA sequences for α-amylase and two trypsins isoforms, as well as the effect of environmental salinity on gene expression and protein activities in hepatopancreas of *Neohelice granulata* (Dana,1852), as a good invertebrate model species. The cDNA sequence of α-amylase is 1,637 bp long, encoding 459 amino acid residues. Trypsin 1 and 2 are 689 bp and 1,174 bp long, encoding 204 and 151 amino acid residues, respectively. Multiple sequence alignment of deduced protein sequences revealed the presence of conserved motifs found in other invertebrates. In crabs acclimated at 37 psu (hypo-regulation), α-amylase mRNA level and total pancreatic amylase activity were higher than at 10 psu (hyper-regulation) and 35 psu (osmoconformation). Trypsin 1 mRNA levels increased at 37 psu while trypsin 2 levels decreased at 10 and 37 psu. Total trypsin activity was similar in all salinities. Our results showed a differential modulation of α-amylase and trypsins expression and total amylase activity by salinity acclimation, suggesting the occurrence of distinct mechanisms of regulation at different levels that could lead to digestive adjustments in relation to hyper- and/or hypo-regulation.

Keywords: α-amylase, trypsin, cloning, digestive flexibility, hepatopancreas, *Neohelice granulata*.

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57 **Introduction**

58 Phenotypic flexibility involves changes in attributes at different levels (from molecular to organisms), in
59 response to variations to external stimuli. These reversible changes can increase probability of survival
60 particularly in heterogeneous habitats (Piersma and Drent 2003; Pfenning et al. 2010; Kelly et al. 2012).
61 Digestive enzymes play an essential physiological role, since they are a link between ingestion,
62 absorption and assimilation of nutrients. Therefore, modulation of digestive enzymes activities can lead to
63 digestive adjustments for facing variations in key environmental conditions (del Valle et al. 2004, 2006;
64 Karasov et al. 2011; Karasov and Douglas 2013).
65
66 Euryhaline crabs inhabiting the intertidal zone of estuaries and coastal lagoons face abrupt and broad
67 changes in environmental salinity (McNamara and Faria 2012; Romano and Zeng 2012). It is known that
68 biochemical adaptation to environmental salinity involves adjustments in branchial and extrabranchial
69 tissues (Pinoni and López Mañanes 2008, 2009; Michiels et al. 2013, 2015; Pinoni et al. 2013, 2015).
70 However, adjustments at different levels of biological organization (molecular, biochemical and
71 physiological) in the hepatopancreas still need to be elucidated (McNamara and Faria 2012; Romano and
72 Zeng 2012; Pinoni et al. 2013, 2015). In decapod crustaceans, the hepatopancreas is the main site of
73 digestive enzymes synthesis as well as digestion, absorption and storage of nutrients (Muhlia-Almazán
74 and Garcia-Carreño 2003; Zeng et al. 2010; Alexandre et al. 2014). In some species, the distinct
75 regulation of specific digestive enzymes activities in the hepatopancreas constitutes one of the responses
76 to different environmental salinities (Romano and Zeng 2012; Michiels et al. 2013, 2015; Pinoni et al.
77 2013, 2015; Wang et al. 2013). However, integrative studies on the modulation, at the biochemical and
78 molecular levels (i.e. gene expression), of key digestive enzymes such as α-amylase and trypsin upon
79 acclimation to different salinities are lacking.
80
81 α-Amylases (α -1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) have a central physiological importance in
82 all animals due to their role in the initial steps of the digestion of key glycogenic substrates (i.e. starch and
83 glycogen) (Singh et al. 2014; Date et al. 2015; Peng et al. 2015; Tiwari et al. 2015). Amylase activity was
84 detected in the hepatopancreas of various decapod crustaceans (Le Moullac et al. 1997; Perera et al.
85 2008*a*, 2008*b*; Aragón-Axomulco et al. 2012; Rodríguez-Viera et al. 2014; among others). Trypsin (EC
86 3.4.21.4) represents the main proteolytic enzyme in the hepatopancreas of decapod crustaceans (Johnson

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et al. 2002; Lemos et al. 2000). The high activities of amylase and trypsin found in the hepatopancreas have been associated with the presence of several isoforms, especially for trypsin (Muhlia-Almazán et al. 2008; Perera et al. 2008*a*, 2008*b*; 2015; Rodríguez-Viera et al. 2016). However, the knowledge about these enzymes in the hepatopancreas of euryhaline crabs is still scarce (Asaro et al. 2011; Lancia et al. 2013; Pinoni et al. 2013, 2015). In this context, the knowledge on how low and high salinities can affect gene expression of α-amylase and trypsin in hepatopancreas has not been previously addressed.

Neohelice granulata (Dana, 1852) is an intertidal euryhaline burrowing crab, serving as animal model for biochemical, physiological and ecological research (Spivak 2010). *N. granulata* is found on intertidal areas of the South-Western Atlantic from the south of Brazil to the north of Patagonia, Argentina (Spivak 1997; Iribarne et al. 2003; Luppi et al. 2013). *N. granulata* is a dominant crab species in Mar Chiquita coastal lagoon (Argentina) in which successfully occupies the complete intertidal area and faces abrupt and highly variable shifts in salinity (from 2 to 47 psu) (Spivak et al. 1994; Fanjul et al. 2008; Luppi et al. 2013). Biochemical adaptation to low and high salinity implies a metabolic reorganization to cope with the energy requirements caused by osmoregulatory processes. The differential modulation of specific digestive enzymes activities in euryhaline crabs such as *N. granulata* would be an important response in this reorganization through the potential enhancement of available metabolites (Romano and Zheng 2012; Pinoni et al. 2013; Michiels et al. 2015). In this context, the modulation of the expression at both mRNA and protein activity levels of α-amylase and trypsin in the hepatopancreas, as key components of digestion processes, could be part of the responses to salinity in this invertebrate species. The aim of this work was to study the effect of low and high salinities on the mRNA expression and total activity of α-amylase and trypsin in hepatopancreas of *N. granulata*. In this context, the cloning and characterization of one α-amylase and two trypsins cDNA sequence isoforms were initially carried out from hepatopancreas and then their mRNA levels were determined by quantitative real time PCR (qPCR) and total corresponding activities in hepatopancreas of individuals acclimated to different salinities.

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Materials and Methods

Animal collection and maintenance

Adult male crabs (carapace width greater than 2.5 cm) were captured from the mudflat area of Mar Chiquita coastal lagoon (37°32′–37°45′S; 57°19′–57°26′W, Argentina). All individuals collected were in intermolt (Drach and Tchergovtzeff 1967). Crabs were transported to the laboratory on the day of collection. Salinity was determined in practical salinity units (psu) in all cases. Animals were distributed in three different experimental groups (n = 7-8 individuals per condition) and submitted to the following environmental salinities: i) dilute seawater (10 psu), ii) seawater (35 psu), and iii) concentrated seawater (37 psu) for 10 days (López Mañanes et al. 2000; Michiels et al. 2015). Diluted and concentrated seawater preparation and the maintenance of crabs in aquaria were done as previously described (Michiels et al. 2015). Crabs were fed three times a week using commercial food (55 % carbohydrates, 31 % protein, 5 % fat, 2 % fiber, Tetra Pond, Koi vibrance, Germany) (0.07 g per individual) and starved 120 h before sampling. We have previously shown that in males of *N. granulata* digestive and metabolic adjustments occurred up to 120 h after feeding (Méndez et al. 2011, 2012; Pinoni et al. 2015; unpublished results). Under the experimental conditions used, no differences in the feeding behavior were observed and no mortality occurred. This research project was done following the regulations and statements of Ethics Committee CICUAL (OCA 1499/12) FCEyN Universidad Nacional de Mar del Plata.

Sampling procedures

Animals were put on ice for about 20 min to cryoanesthesize. A sample of hemolymph was taken for osmolality determination as described below. The hepatopancreas was immediately excised, weighed and biopsies of approximately 30-40 mg were placed in tubes containing 1 mL RNAlater® (Ambion®), maintained for 96 h at 4 ºC and then stored at -20 ºC until used for RNA extraction. The remaining of the hepatopancreas was homogenized in 50 mM Tris/HCl pH 7.4 (4 mL g^{-1} of tissue) (CAT homogenizer x 120, tool T10) on ice and centrifuged at 10,000 × g for 15 min at 4 °C (Sorvall, rotor SS-34) as previously described (Michiels et al. 2015). The supernatant was fractionated into 200 µL aliquots and stored at −20 °C until biochemical analyses.

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147 **Hemolymph osmolality**

148 Hemolymph extraction (about 500 µL) from the intrabranchial sinus and osmolality determination were
149 done as previously described by Michiels et al. (2015). Osmolality was determined in the plasma obtained
150 by centrifugation of hemolymph 3 min at 2,000 × g (IEC-Centra 7R, refrigerated). Both hemolymph and
151 experimental water osmolality (mOsm kg^{-1}) were measured with a cryoscopic osmometer (Osmomat 030,
152 Gonotec).
153
154 **Cloning and mRNA expression**

155 First, a set of degenerate primers (Table 1) was designed according to the sequences of cDNA most
156 highly conserved between different crustaceans, insects, birds and fish species for α-amylase (as
157 described by Rodríguez-Viera et al. 2016), trypsin (as described by Perera et al. 2010b), and β-actin (as
158 described by Baldisserotto et al. 2014) for partial cloning. Primers were synthesized by IDT® (Integrated
159 DNA Technologies). Total RNA was isolated from hepatopancreas using a NucleoSpin® RNA II kit
160 (Macherey-Nagel) with an appropriate volume of RA1 according to the weight of the tissue, and the on-
161 column RNase-free DNase digestion according to manufacturer's protocol. The amount of RNA was
162 spectrophotometrically measured at 260 nm with a BioPhotometer Plus (Eppendorf), and its quality was
163 determined in an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies). After
164 reverse transcription to obtain the first-strand cDNA (SuperScript III, Life Technologies™), PCR
165 amplifications were performed with the proofreading VELOCITY DNA Polymerase (BIOLINE) and
166 samples were cycled (98 ºC, 5 min; [98 ºC, 30 s; 65–55 ºC in touchdown, 30 s; 72 ºC, 1 min] × 35 cycles;
167 72 ºC, 10 min). PCR products were visualized in 1 % agarose gel electrophoresis using GelRed™
168 (Biotium) as the stain. PCR products of the expected size were excised from the gel, purified with the
169 NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), and cloned on *Escherichia*
170 *coli* cells (Top 10, Invitrogen™) using the pJET1.2/blunt cloning vector of the CloneJET PCR Cloning
171 Kit (Thermo Scientific, Waltham, MA, USA). Several clones for putative α-amylase, trypsin and β-actin
172 cDNAs were sequenced in both strands using pJET1.2 Forward and Reverse sequencing primers, by the
173 dideoxy method in StabVida sequencing company (Caparica, Portugal). All kits were used according to
174 manufacturer's instructions. The sequence homology for all the clones was confirmed by blast using the
175 NCBI website (http://www.ncbi.nlm.nih.gov/). ClustalW (http://www.ebi.ac.uk/clustalw/) was used for

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fragment assemblage. Homology analysis of putative protein sequences was carried out with blast at the NCBI website. ClustalO was used for protein alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/).

After sequencing and checking the partial sequences by NCBI blastn, specific forward primers were designed in the fragments previously cloned at two or three different positions (Table 1) and used in combination with specific reverse primers provided in the 3´ Rapid Amplification of cDNA Ends (FirstChoice® RLM-RACE kit, Life Technologies™) to amplify the 3´ ends. The clones obtained were fully sequenced in both strands, sequence homology of all genes was confirmed by NCBI blastn and blastx, and phylogenetic analyses were performed (see below). Finally, the obtained sequences were used to design specific primers for their use in quantitative PCR (qPCR).

For this purpose, total RNA isolation, quantification and the assessment of quality were performed as previously described. Only samples with an RNA integrity number (RIN) above 8.5, which was indicative of clean and intact RNA, were used for analysis. First, different amounts of cDNA, synthesized using the qScript™ cDNA Synthesis Kit (Quanta BioSciences), were applied in triplicate (six serial 1/2 dilutions from 10 ng to 0.325 ng per reaction) to check the assay linearity and the amplification efficiency for each one of the designed specific pair of primers (Table 2). Although the assay was linear along the six serial dilutions (α-amylase: $r^2 = 0.972$, efficiency (E) = 0.99; trypsin 1: $r^2 = 0.999$, $E = 0.94$; trypsin 2: $r^2 = 0.997$, $E = 0.99$; β-actin: $r^2 = 0.991$, $E = 1.01$), 10 ng of cDNA was used in each qPCR reaction. Finally, each reaction mixture (10 µL) contained 4 µL of template (10 ng), 0.5 µL of each specific forward and reverse primer at their respective final concentration (Table 1), and 5 µL of PerfeCTa SYBR® Green FastMix™ (Quanta BioSciences). Reactions were conducted in Hard-Shell® PCR Plates 96-well WHT/CLR plates (Bio-Rad) covered with adhesive Microseal® 'B' seal films (Bio-Rad). The thermocycling procedures were performed in a CFX Connect Real-Time system (Bio-Rad). The PCR thermal profile was as follows: 95 ºC, 10 min; [95 ºC, 30 s; 60 ºC, 45 s] × 40 cycles; melting curve [60–95 ºC, 20 min], 95 ºC, 15 s. β- actin was used as the internal reference gene due to its low variability (less than 0.20 C_T, with no differences detected between experimental groups) under our experimental conditions. Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

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206 **Phylogenetic analysis**

207 A phylogenetic analysis of all putative translated sequences was performed using MEGA7 software
208 (Kumar et al. 2016) with the Neighbor-Joining algorithm based on amino acid differences (p-distances)
209 and pairwise deletions. The reliability of the tree was assessed with the bootstrap method (1,000
210 replicates). Amino acid sequences were retrieved from the NCBI protein database
211 (www.ncbi.nlm.nih.gov/pubmed, accessed in October 2015).

212

213 **Biochemical assay**

214 α-Amylase activity was determined using the method described by Biesiot and Capuzzo (1990) with
215 some modifications (Asaro et al. 2011). Briefly, amylase activity was assayed in a reaction mixture with
216 15 mg mL^{-1} starch in 50 mM phosphate buffer (pH 5.2) at 30 °C (Asaro et al. 2011). An aliquot of the
217 corresponding sample (which falls in the linearity zone on activity vs. protein concentration plot) was
218 added to the reaction mixture and incubated for 15 minutes. Then, 1.5 mL of dinitrosalicylic acid reagent
219 (DNS) (Miller 1959) was added, and after incubation for 10 min at 100 °C assay tubes were cooled in ice.
220 The amount of released maltose was determined by reading the absorbance at 540 nm (ZL5000 PLUS,
221 Zeltec). α-Amylase activity was expressed as μg released maltose min^{-1} mg^{-1} of protein.

222

223 Trypsin activity was determined using N_{α}-Benzoyl-D,L-arginine 4-nitroanilide (BAPNA) as substrate
224 according to Ezquerra et al. (1997) with some modifications (Michiels 2015). In short, trypsin activity
225 was determined in a reaction medium containing 1.23 mM BAPNA in 50 mM Tris-HCl (pH 7.4) at
226 45 °C. An aliquot of the corresponding sample (which falls in the linearity zone on activity vs. protein
227 concentration plot) was added and incubated for 15 min. After this, 250 μL of KOH were added to stop
228 the reaction. Trypsin activity was expressed as μmol min^{-1} mg^{-1} protein.

229

230 Total amount of proteins was determined by the method described by Bradford (1976) using bovine
231 serum albumin as standard.

232

233 **Statistical analysis**

234 Data were statistically analyzed by using the software Sigma 3.0 for Windows, which automatically
235 performs previous test of equality of variances and normality. Either a parametric analysis (one-way

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ANOVA) or a nonparametric analysis (Kruskal–Wallis) was used to estimate the statistical significance at $P < 0.05$. A posteriori test to ANOVA (Holm–Sidak method) was used to identify differences.

Results

Characteristics of cDNA sequences

The partial α-amylase, trypsin 1, trypsin 2 and β-actin cDNAs of euryhaline crab *N. granulata* were identified and submitted to GenBank (accession nos. **KU531567, KU531568, KU531569** and **KU531570**, respectively). Sequencing revealed partial cDNAs to be 1,637 bp for α-amylase, 689 bp for trypsin 1, 1,174 bp for trypsin 2 and 349 bp for β-actin.

The obtained partial nucleotide and deduced amino acid sequence of *N. granulata* α-amylase are shown in Figure 1. The termination codon (taa) is at position 1,320 followed by the 3′ untranslated region. The sequence encodes 439 amino acid residues and includes three potential O-glycosylation sites (S^{10}, S^{17} and T^{70}). In addition, nine out of ten cysteine residues observed in the amylase protein of other arthropods are present in the cloned partial sequence. Figure 2 shows a protein alignment between different crustaceans α-amylases using ClustalO version 1.2.1. The alignment shows a high degree of conservation, especially in the amino acid residues that participated in the catalytic triad (D^{141}, E^{178} and D^{242}) and calcium ion binding site (N^{44} and D^{111}). The comparison of α-amylase protein sequence of *N. granulata* to other crustacean species shows a high identity (67.51-77.40 %) (Table 3).

The partial nucleotide and deduced amino acid sequence of *N. granulata* trypsin 1 and 2 isoforms are presented in Figures 3 and 4, respectively. For trypsin 1 (Fig. 3), the termination codon (taa) is at position 613 followed by the 3′ untranslated region containing a polyadenilation site (aataaa) at position 656. The partial sequence encodes 204 amino acid residues and includes the conserved amino acids that participate in the catalytic triad (H^{17}-D^{68}-S^{160}), the substrate-binding pocket (D^{154}-S^{175}-G^{177}), which determines the substrate specificity of trypsin, and the conserved motif with catalyzing site (CQGDSGGP) at position 156-163. For trypsin 2 (Fig. 4), and similarly to isoform 1, the sequence presents the termination codon (taa) at position 456 followed by the 3′untranslated region containing a polyadenilation sites (aataaa) at position 497 and 1,065. The sequence encodes 151 amino acid residues and includes part of the catalytic triad (D^{16}-S^{107}), the substrate-binding pocket (D^{101}-S^{122}-G^{124}) and the conserved motif for the catalyzing

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site (CQGDSGGP) at position 103-110. Partial amino acid sequences of trypsin 1 and trypsin 2 present nine and six out of ten cysteine residues. In addition, the multiple protein alignment between different crab trypsins shows a high degree of conservation in amino acid residues involved in the catalytic triad (HDS), in the residues D, S and G determining the substrate specificity, and in the motif of catalyzing-site (CQGDSGGP) (Fig. 5). Moreover, the deduced amino acid sequence of both isoforms of trypsin from *N. granulata* shows a high identity (55.2-88.89 %) with other crab trypsins (Table 4).

The partial coding and predicted amino acid sequence of β-actin from hepatopancreas of *N. granulata* is shown in Figure 6. The sequence presents the termination codon (tga) at position 327 and encodes 108 amino acid residues. The deduced amino acid sequence of β-actin from *N. granulata* is very similar to that from other crabs, such as *Scylla serrata*, *S. paramamosain* and *Eriochieir sinesis* (98 % identity in all cases, data not shown).

Phylogenetic analysis

A phylogenetic tree was constructed with the deduced amino acid sequences to determine the relatedness of *N. granulata* α-amylase with other invertebrates (mollusks, crustaceans, insects and arachnids) amylases using the Neighbor-Joining analysis. The α-amylase of the bacterium *Streptococcus equinus* was used as an out-group. The obtained tree showed that animal amylases were divided into three major groups, one group comprising insects and crustaceans amylases, including *N. granulata*, a second group containing mollusks, whereas the third group grouped arachnids separated from the rest (Fig. 7).

Moreover, a phylogenetic tree of arthropod trypsin amino acid sequences was obtained using the Neighbor-Joining analysis (Fig. 8). The trypsin of the bacterium *S. equinus* was used as an out-group. Two main groups were distinguished, one with crustacean and arachnid trypsins, and the other group including principally trypsins from arachnids allergen type 3 and insects. The close relationship among trypsins from Penaeidae and from Caridea and Euphausiacea has been previously evidenced (Shi et al. 2009; Perera et al. 2010*a*).

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297 **Salinity effect on α-amylase and trypsin**

298 Hemolymph osmolality in individuals acclimated to 10 and 37 psu was significantly higher or lower than
299 that of the external environment, respectively, while no differences were detected at 35 psu (Table 5).
300
301 α-Amylase gene expression and total α-amylase activity showed a similar pattern of change respect to
302 environmental salinity (Fig. 9a and 9b). Individuals exposed to high salinity (37 psu) exhibited higher
303 levels than those acclimated to 10 or 35 psu, with no statistical differences between 10 and 35 psu.
304
305 Both trypsins (1 and 2) presented different patterns of gene expression respect to environmental
306 salinities. Trypsin 1 mRNA expression level was enhanced (about 341 %) in individuals acclimated to 37
307 psu respect to 10 or 35 psu (Fig.10a). However, trypsin 2 mRNA level decreased (about 67.5 %) in
308 individuals acclimated to low (10 psu) and high (37 psu) environmental salinities respect to 35 psu (Fig.
309 10b). On the other hand, no differences were found between total trypsin activity in hepatopancreas of *N.*
310 *granulata* individuals acclimated to different environmental salinities (Fig. 10c).
311
312 **Discussion**

313 Reports on sequencing of α-amylase from euryhaline crabs are lacking, while cloning of trypsins has
314 been done in a few species (Rudenskaya et al. 2004; Hettermann et al. 2008; Jing et al. 2011). We carried
315 out the cloning and determined characteristics of cDNA sequences for α-amylase and two trypsins
316 isoforms from the hepatopancreas of the intertidal euryhaline crab *Neohelice granulata,* a model species
317 for biochemical, physiological and ecological studies. A partial 1,637 bp long cDNA sequence for α-
318 amylase encoding 459 amino acid residues was obtained (Fig. 1). α-Amylase sequence in the shrimp
319 *Penaeus vannamei* consists of approximately 1,600 bp and is highly conserved (Van Wormhoudt and
320 Sellos 1996). Multiple alignment of deduced protein sequences (Fig. 2) revealed that α-amylase from *N.*
321 *granulata* has conserved motifs, such as calcium chloride sites, substrate recognition sites and catalytic
322 sites (Asp, Glu and Asp) that are commonly found in animal amylases (Bezerra et al. 2014; Peng et al.
323 2015). In addition, nine out of the ten characteristics cysteine residues were observed in the partial amino
324 acid sequence of *N. granulata*, which enable five disulfide bridges (Rodríguez-Viera et al. 2016). All
325 these regions have been proposed to be essentials for the function of α-amylases (Kuriki and Imanaka
326 1999; Kumari et al. 2012). The phylogenetic analysis based on the protein sequences of different groups

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of invertebrates (crustaceans, insects, mollusks and arachnids) (Fig. 7) showed that α-amylases from crustaceans are closer to the insects group and further from mollusks and arachnids, contrary to that observed by Sellos and Van Wormhoudt (2002). Moreover, the phylogenetic analysis also showed a high conservation within the group of crustaceans, as suggested by Rodríguez-Viera et al. (2016), as well as that the α-amylase from *N. granulata* appeared in a monophyletic group within the crustacean group.

On the other hand, our results revealed the predicted amino acid sequence for trypsins 1 and 2 in hepatopancreas of *N. granulata* (Figs. 3 and 4). Partial trypsin 1 is 689 bp long, whereas the isoform 2 is 1,174 bp long, encoding 204 and 151 amino acid residues respectively (Figs. 3 and 4). Nine and six cysteine residues in the partial amino acid sequence for trypsin 1 and trypsin 2, respectively, were found (Fig. 3 and 4) in positions agreeing with those found in other crab trypsins (Fig.5). The amino acid sequences of both trypsins 1 and 2 were similar to those of other crabs, displaying common features of crustacean trypsins (Fig. 5). The conserved residues His, Asp, and Ser, which form the active site, as well as Asp, Ser and Gly, which determine the substrate binding site, were all present (Muhlia-Almazán et al. 2008). The phylogenetic analysis of trypsin proteins from several crustaceans (Fig. 8) showed that two isoforms from hepatopancreas of *N. granulata* belongs to the group of brachyura and both isoforms are close to other trypsin isoforms from the crab species *Eriocheir sinensis*.

Variations in α-amylase and trypsin gene expression and/or activity in hepatopancreas of several crustaceans have been associated with different environmental and physiological factors (Le Moullac et al. 1997; Perera et al. 2008*a*, 2008*b*, 2012; Michiels 2015; Peng et al. 2015). Since digestive enzymes are a link between ingestion and absorption their regulation can result in a distinct availability of energy substrates (e.g. glucose and amino acids) upon osmoregulatory conditions (Li et al. 2008; Perera et al. 2012; Romano and Zeng 2012; Wang et al. 2014). The higher α-amylase mRNA level and the corresponding increase (about 216 %) of total α-amylase activity (Fig. 9) in hepatopancreas of *N. granulata* acclimated to 37 psu, conditions at which this crab hypo-regulates (Table 5) compared to 35 psu (osmoconforming condition, Table 5), suggests that modulation at the molecular level (i.e. gene expression) of α-amylase is one of the responses to high salinity underlying hypoosmotic regulation. Whether the hepatopancreas total α-amylase activity enhancement observed at 37 psu is also related to modulation at other levels (i.e. translational and/or post-translational) cannot be discarded. In the shrimp

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357 *Penaeus vannamei* (Le Moullac et al. 1997) a transcriptional regulation of α-amylase by casein level was
358 reported. An ecdysteroid-responsive amylase gene was identified in hepatopancreas of the red crayfish
359 *Procambarus clarkii*, which plays a role in the developmental process of this species (Peng et al. 2015).
360 Even so, the mechanistic cause of how the increased mRNA level and total α-amylase activity in
361 hepatopancreas of *N. granulata* acclimated to higher salinity are linked with a higher availability of
362 glucose to maintain glucose homeostasis under hypo-regulation requires further investigation. Hyper-
363 and hypo-osmoregulation in crabs require different digestive and metabolic adjustments (McNamara and
364 Faria 2012; Romano and Zeng 2012; Pinoni et al. 2013; Michiels et al. 2013, 2015). The fact that no
365 changes occurred at either mRNA level or total α-amylase activity, in hepatopancreas of *N. granulata*
366 acclimated to 10 psu (hyper-regulation condition) (Fig. 9), supports the idea that different and specific
367 adjustments at molecular level are occurring in relation to osmoregulatory condition.
368
369 In several species, regulation of trypsin is displayed at different levels, including transcription and
370 translation (Muhlia-Almazán et al. 2008; Perera et al. 2015). Perera et al. (2012) showed that different
371 isoforms of trypsin from the lobster *Panulirus argus* are transcriptionally regulated by diet. In *Daphnia*
372 *magna*, differences in trypsin isoforms expression occur in response to protease inhibitors in the diet
373 (Schwarzenberger et al. 2010). The adaptive value of polymorphisms of trypsins are not fully assessed in
374 crustaceans due to the lack of experimental evidence (Perera et al. 2015). In the lobster, *P. argus*,
375 efficiency in protein digestion appears to depend on phenotypes of present isozymes (Perera et al. 2010*a*,
376 2012). The results of this work showed the distinct effect of low and high salinity acclimation on mRNA
377 level of trypsins 1 and 2 in hepatopancreas of *N. granulata* (Fig. 10a and b). Similar to α-amylase, the
378 mRNA enhancement in trypsin 1 at high salinity (37 psu) with no changes in low salinity (10 psu)
379 (Fig.10a) further supports the occurrence of specific and different regulation of carbohydrate and protein
380 metabolism upon hyper and hypo-regulatory conditions. On the other hand, the decreased expression of
381 trypsin 2 in both low (10 psu) and high (37 psu) salinities (Fig. 10b) suggests the occurrence of specific
382 mechanisms of regulation of trypsin isoforms at the transcriptional level depending on environmental
383 salinities. To our knowledge, this is the first work to show distinct responses at mRNA level of trypsin
384 isoforms to environmental salinity in crustacean decapods. Furthermore, the differential regulation of
385 trypsin 1 and 2 expressions in low and high salinity suggests the possible diverse physiological roles of
386 trypsin isoforms in biochemical adaptation to salinity. Since total trypsin activity was not affected in any

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387 case (Fig. 10c), qualitative changes (i.e. different isoforms accounting for total trypsin activity) could be
388 occurring in low and high environmental salinities. In this sense, trypsin isoforms in the lobster *P. argus*
389 have distinct catalytic properties and/or specificity (i.e. different digestion efficiency) (Perera et al.
390 2010*b*), which can give an approach about this interesting environmental regulation.
391
392 In summary, our results showed a differential modulation of α-amylase and trypsins expression as well as
393 total activity by low and high salinities, suggesting the occurrence of distinct mechanisms of regulation at
394 different levels (i.e. molecular, biochemical and physiological) for these enzymes, which could lead to
395 digestive adjustments in carbohydrate and protein digestion in relation to hyper/hypo-regulation. We have
396 demonstrated that carbohydrate digestive capacities of the crab *N. granulata* change according to the
397 environmental salinity, whereas protein digestive capacity is orchestrated by different isoforms of trypsin
398 at mRNA level to acquire a homeostatic load, probably as a result of different metabolic requirements.
399 We further demonstrate that these changes involve both quantitative and qualitative (i.e. isoform
400 differences) changes in gene expression of at least α-amylase and trypsin enzymes.
401
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597 **Table 1.** Nucleotide sequence of degenerate primers designed for molecular identification of α-amylase,
598 trypsin and β-actin partial cDNA sequences, as well as specific primers used for 3′- Rapid Amplification
599 of cDNA Ends (RACE).
600

Degenerate primers	Nucleotide sequence	Size amplified	
α-amy Fw_1	5′-ATBGTSCAYYTBTTYGARTGG-3′	1,250 bp	
α-amy Rw_1	5′-TVACVNBCTTBCCMGTGCA-3′		
α-amy Fw_2	5′-GGCCDTGGTGGGARMGDTAC-3′	1,155 bp	
α-amy Rw_2	5′-CCRGARATSACRTCRCAGTA-3′		
α-amy Fw_3	5′-GCYDSCAARCACATGTGGCC-3′	550 bp	
α-amy Rw_3	5′-TTNCGGAADNBVACCATGTT-3′		
tryp Fw_1	5′-CCAARATCATCCARCACGARG-3′	284 bp	
tryp Rv_1	5′-AGTCACCCTGGCANGMGTC-3′		
tryp Fw_2	5′-TTCTGCGGHGCBTCCATCTACA-3′	132 bp	
tryp Rv_2	5′-CYTCGTGYTGGATGATYTTGG-3′		
β-act Fw_1	5'-ACCACAGCYGARMGKGAAAT-3'	293 bp	
β-act Rv_1	5'-TCCKGTCWGCRATGCCAGGGT-3'		
Primers for 3′RACE	**Nucleotide sequence**	**Position**	**Direction**
Ng α-Amyelong3′Fw1	5′-TCCGGGGACATTGAGAACTAC-3′	266-287	Forward
Ng α-Amyelong 3′ Fw2	5′-CAACTCAAGTGGCTGGTCAAC-3′	645-665	Forward
Ng α-Amyelong 3′Fw3	5'-CCCCCTCACGACGATAGTTAC-3′	906-926	Forward
Ng Tryelong3′Fw1	5′-AAACAATACGCCGGGTTCACCC-3′	18-39	Forward
Ng Tryelong3′ Fw2	5' -TGACCTTCAACACCTTCGTT-3′	78-98	Forward
Ng Trypelong3′ Fw3	5′-GAGAACTGGGCCGTGTGTGCC-3′	25-45	Forward
Ng Trypelong 3′ Fw4	5′-TTGGACGTTSTTRAGGGT-3′	115-132	Forward
Ng Trypelong3′ Fw5	5′-CTGYGWYCRGSKRGRGSACT-3′	145-164	Forward
Ng Trypelong3′Fw6	5′-CYSWCTRTCCTTCAKKTTGT-3′	80-98	Forward
Ng q PCR β-act Fw1	5′-CCTCCTCCCTTGAGAAGTCC-3'	94-113	Forward
Ng q PCR β-act Fw2	5'-ATTCCAGCCTTCCTTCTTGG-3′	179-198	Forward

601
602

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603 **Table 2.** Nucleotide sequence of specific primers designed for qPCR analysis and size amplified by each
604 pair of primers.
605

qPCR	Nucleotide sequence	Primer concentration	Size amplified
Ng qPCR α-Amy Fw	5′-TCCGGGGACATTGAGAACTAC-3′	600 nM	136 bp
Ng qPCR α-Amy Rv	5′-CGTACCCGATCAGATTGTTCA-3′		
Ng qPCR Tryp 1 Fw	5′-CCCTTCTCCAACTGTCCTCC-3′	400 nM	145 bp
Ng qPCR Tryp 1Rv	5′-TGAAGGATAGAGGGAGTGCT-3′		
Ng qPCR Tryp 2 Fw	5′-GCGAAGTTTACGGAGAAGAAG-3′	400 nM	176 bp
Ng qPCR Tryp 2 Rv	5′-GGCGTACACGCCGGGGCTGTC-3′		
Ng qPCR β-act Fw	5'-ATTCCAGCCTTCCTTCTTGG-3′	600 nM	105 bp
Ng qPCR β-act Rv	5′-TTTGCAAACAGGTCCTTCCT-3′		

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608 **Table 3.** Percentage of identity for the amino acid sequences of the α-amylase from four crustacean
609 species and *N. granulata* derived from pairwise nucleotide and amino acid sequence alignment using
610 ClustalO.
611

	N. granulata
N. granulata	100
M. japonicus	77.4
L. vanammei	75.57
P. argus	70.57
D. pulex	67.51

612
613

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614 **Table 4** Percentage of identity for the amino acid sequences of two trypsin isoforms from
615 several crabs and *N. granulata.* Derived from pairwise nucleotide and amino acid
616 sequence alignment using ClustalO.
617

618

	N. granulata1	***N. granulata2***
N. granulata1	100	88.30
N. granulata2	88.30	100
E. sinensis1	84.21	75.42
E. sinensis2	88.89	74.58
E. sinensis3	55.22	76.27
S. paramamosain	77.19	56.00
P. trituberculatus1	71.35	66.10
P. trituberculatus2	71.35	66.10
P. trituberculatus3	71.35	65.25
C.japonica1	69.41	66.67
C. japonica2	68.42	66.10
C. japonica3	73.68	65.25
P. pelagicus	55.22	55.33
P. camtschaticus	67.65	66.23

619

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620 **Table 5**. Osmolality (mOsmol kg^{-1}) in external environment and *N. granulata* hemolymph of individuals
621 acclimated to different environmental salinity (10, 35 and 37 psu).
622

10 psu		**35 psu**		**37 psu**	
Environment	Hemolymph	Environment	Hemolymph	Environment	Hemolymph
255 ± 15	646 ± 30*	813 ± 41	869 ± 33	974 ± 33	899 ± 19*

623 *Significantly different from the corresponding concentration of the external environment (t-test, $P <$
624 0.05). Data are the mean ± S.E.M. n = 7-8.
625
626

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627 **Legends**

628

629 **Fig. 1.** Nucleotide and deduced amino acid sequence of *N. granulata* α-amylase cDNA. The deduced
630 amino acid sequence is displayed in bold capital letters above the nucleotide sequence. Stop codon (taa) is
631 represented in bold, italic and underlined. Potential O-glycosylation sites are shaded in grey. The cysteine
632 residues are shaded in black.

633

634 **Fig. 2.** Comparison of α-amylase amino acid sequences of different crustaceans species using Clustal
635 Omega. The conserved amino acids that participate in the catalytic triad are marked in grey boxes.
636 Calcium ion binding site is marked in black boxes. The cysteine residues are marked with a triangle on
637 the column. *Marsupenaeus japonicas* (**AHN91844.1**), *Litopenaeus vannamei* (**AIJ02083.1**), *Panulirus*
638 *argus* (**CDU84835.1**), *Daphnia pulex* (**EFX81580.1**). The symbols indicate: identity (*), conserved
639 substitutions (:), and semi-conserved substitutions (.).

640

641 **Fig. 3.** Nucleotide and deduced amino acid sequence of *N. granulata* trypsin 1 cDNA. The deduced
642 amino acid sequence is displayed in bold capital letters above nucleotide sequence. Stop codon (taa) and
643 consensus polyadenylation signal (aataaa) are represented in bold, italic and underlined. The conserved
644 amino acids that participate in the catalytic triad (His, Asp, Ser) are shaded in grey. The amino acids that
645 participate in the substrate-binding pocket (Asp, Ser, Gly) are shaded in black. The conservative motif of
646 catalyzing-site is boxed. The cysteine residues are marked with a circle.

647

648 **Fig. 4.** Nucleotide and deduced amino acid sequence of *N. granulata* trypsin 2 cDNA. The deduced
649 amino acid sequence is displayed in bold capital letters above nucleotide sequence. Stop codon (taa) and
650 consensus polyadenylation signal (aataaa) are represented in bold, italic and underlined. Two of the three
651 amino acids that participate in the catalytic triad (Asp, Ser) are shaded in grey. The amino acids that
652 participate in the substrate-binding pocket (Asp, Ser, Gly) are shaded in black. The conservative motif of
653 catalyzing-site is boxed. The cysteine residues are marked with a circle.

654

655

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Fig. 5. Alignment of protein sequences of crab trypsins using Clustal Omega. The conserved amino acids that participate in the catalytic triad (His,Asp,Ser) are shaded in grey. The amino acids that participate in the substrate-binding pocket (Asp, Ser, Gly) are marked in bold with an open arrow on the column. The conserved motif for catalyzing-site is boxed. The cysteine residues are marked with a triangle on the column. Amino acid differences between isoforms *of N. granulata* are indicated in black boxes. *Sylla paramamosain* (**ADB55592**), *Eriocheir sinensis* 1 (**ABQ02519.1**), *E. sinensis* 2 (**ABQ02520.1**), *E. sinensis* 3 (**ABQ02521.1**), *Portunus trituberculatus* 1 (**ABQ02537.1**), *P. trituberculatus* 2 (**ABQ02538.1**), *P. trituberculatus* 3 (**ABQ02539.1**), *Charybdis japonica* 1 (**ABQ02512.1**), *C. japonica* 2 (**ABQ02513.1**), *C. japonica* 3 (**ABQ02514.1**), *Portunus pelagicus* (**ABM65758.1**), *Paralithodes camtschaticus* (**AAL67442.1**). The symbols indicate: identity (*), conserved substitutions (:), and semi-conserved substitutions (.).

Fig. 6. Nucleotide and deduced amino acid sequences of *N. granulata* β-actin cDNA. The deduced amino acid sequence is displayed in bold capital letters and above nucleotide sequence. Stop codon (tga) is represented in bold, italic and underlined.

Fig. 7. Phylogenetic tree using the deduced amino acid sequences of α-amylase from several crustaceans, including the euryhaline crab *N. granulata*, as well as insects, mollusks, arachnids, and one out-group using Neighbor-Joining analysis and based on amino acid difference (p-distance). Reliability of the tree was assessed by bootstrapping (1,000 replicates). Species and accession numbers are shown in the tree. Deduced sequence from *N. granulata* is marked by a black triangle.

Fig. 8. Phylogenetic tree using the deduced amino acid sequences of trypsin from several crustaceans, including the euryhaline crab *N. granulata*, arachnids, insects, and one out-group using Neighbor-Joining analysis and based on amino acid differences (p-distance). Reliability of the tree was assessed by bootstrapping (1,000 replicates)**.** Species and accession numbers are shown in the tree. Deduced sequence from *N. granulata* are marked by black triangles. Some arachnids allergen type 3 are included in the tree (3a) given their similarities with trypsinogens.

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685 **Fig. 9.** Gene expression (a) and activity of α-amylase (b) in hepatopancreas of *N. granulata* acclimated to
686 different environmental salinities (10, 35 and 37 psu). Different letters indicate statistically significant
687 differences ($P < 0.05$). Data are expressed as mean ± SE for 6-8 individuals.
688
689 **Fig. 10.** Gene expression of trypsin 1 (a) and trypsin 2 (b), as well as total trypsin activity of trypsin (c) in
690 hepatopancreas of *N. granulata* individuals acclimated to different environmental salinities (10, 35 and 37
691 psu). Different letters indicate statistically significant differences ($P < 0.05$). Data are showed as mean ±
692 SE for 5–7 individuals.

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Figure 1

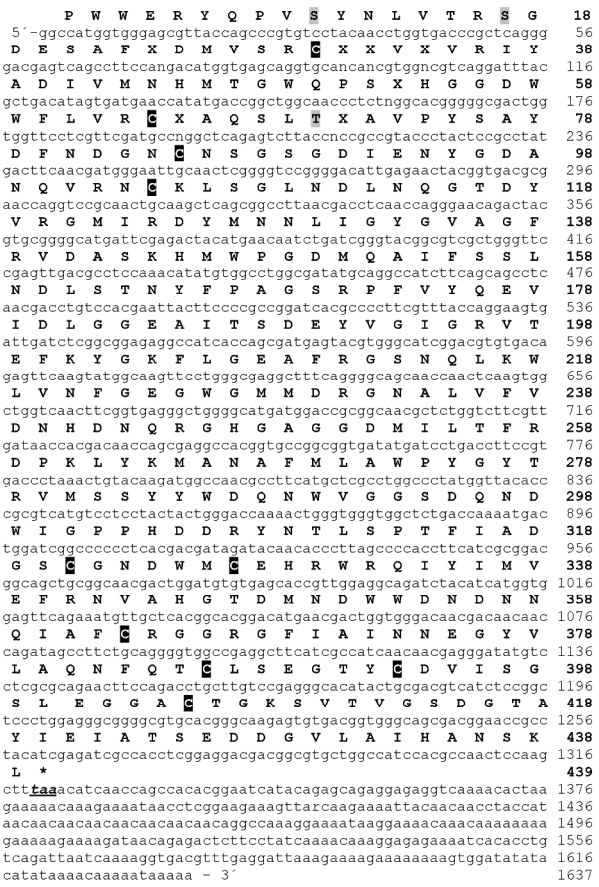

Figure 1

215x279mm (300 x 300 DPI)

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Figure 2

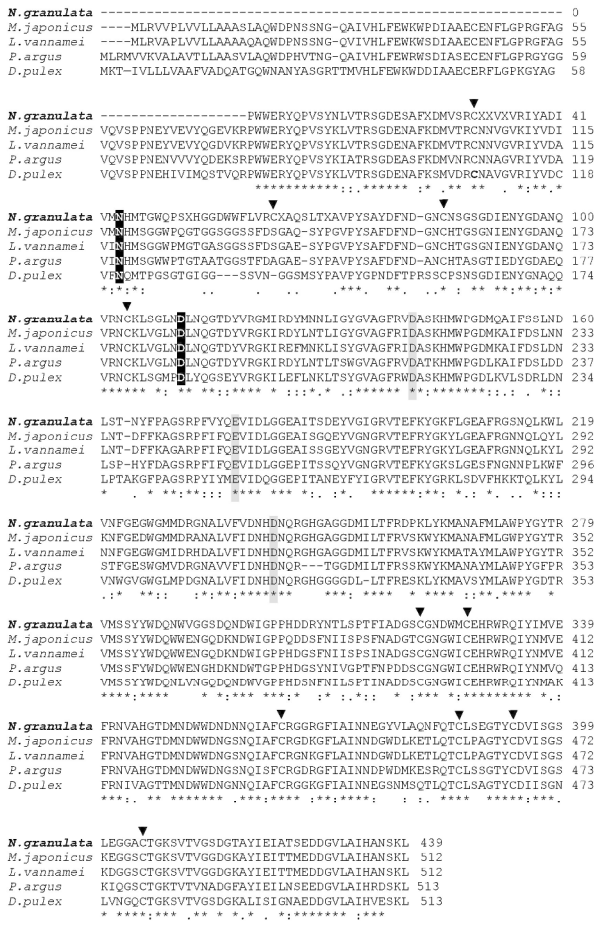

Figure 2

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Figure 3

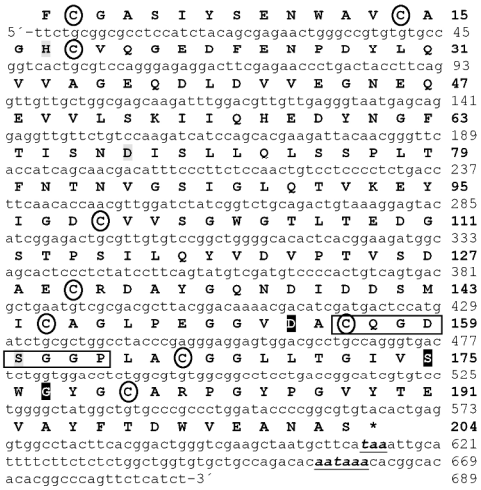

Figure 3

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Figure 4

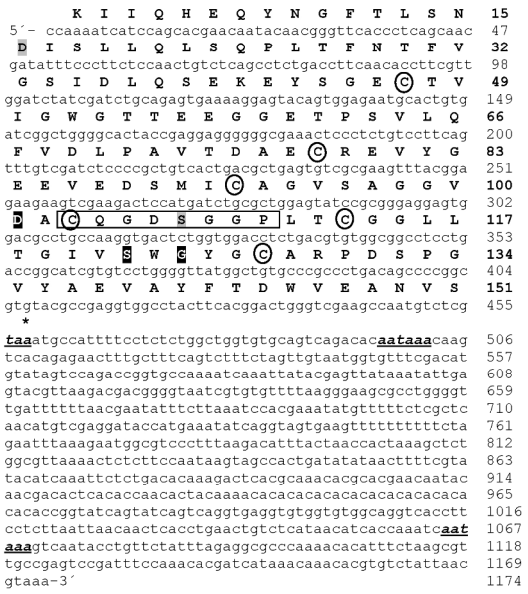

Figure 4

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Figure 5

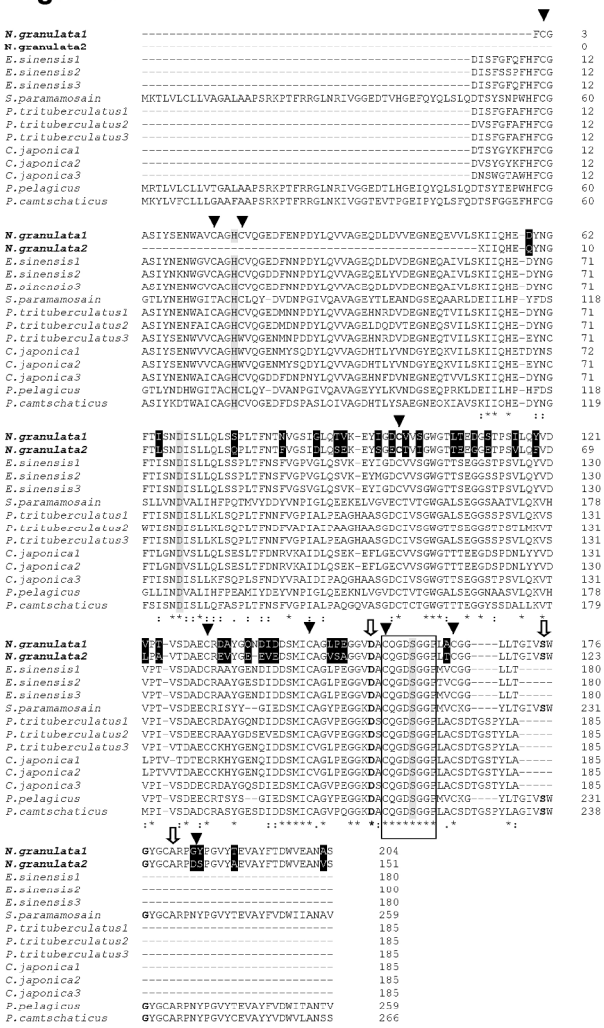

Figure 5

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Figure 6

```
          T  A  E  R  E  I  V  R  D  I  K  E  K  L  C  Y  V  A   18
 5´- ccacagctgagagggaaatcgtgcgcgatatcaaggagaagctttgctatgtcgct   56
 L  D  F  E  S  E  M  N  V  A  A  A  S  S  S  L  E  K  S  Y    38
cttgacttcgaaagtgagatgaacgtggctgctgcctcctcctcccttgagaagtcctac   116
 E  L  P  D  G  Q  V  I  T  I  G  N  E  R  F  R  A  P  E  S   58
gagctgcccgatggtcaggtcatcaccatcggcaacgagcgcttccgtgcaccagaatct   176
 L  F  Q  P  S  F  L  G  M  E  S  V  G  I  H  E  T  V  Y  N   78
ctattccagccttccttcttgggtatggaatctgttggcattcacgagaccgtctacaac   236
 S  I  M  R  C  D  I  D  I  R  K  D  L  F  A  N  N  V  M  S   98
tccataatgagatgcgacattgacatcaggaaggacctgtttgcaaacaacgtaatgtct   296
 G  G  T  T  M  Y  P  G  I  A  *                             108
ggcggcaccaccatgtaccctggcatcgca***tga***ccggatctgcggaaaaattg-3`   349
```

Figure 6

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Figure 7

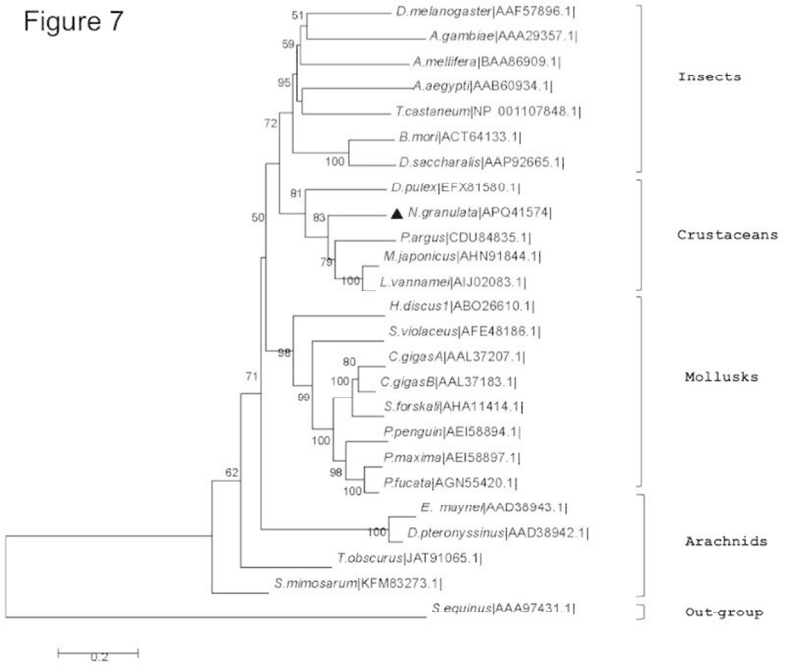

Figure 7

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Figure 8

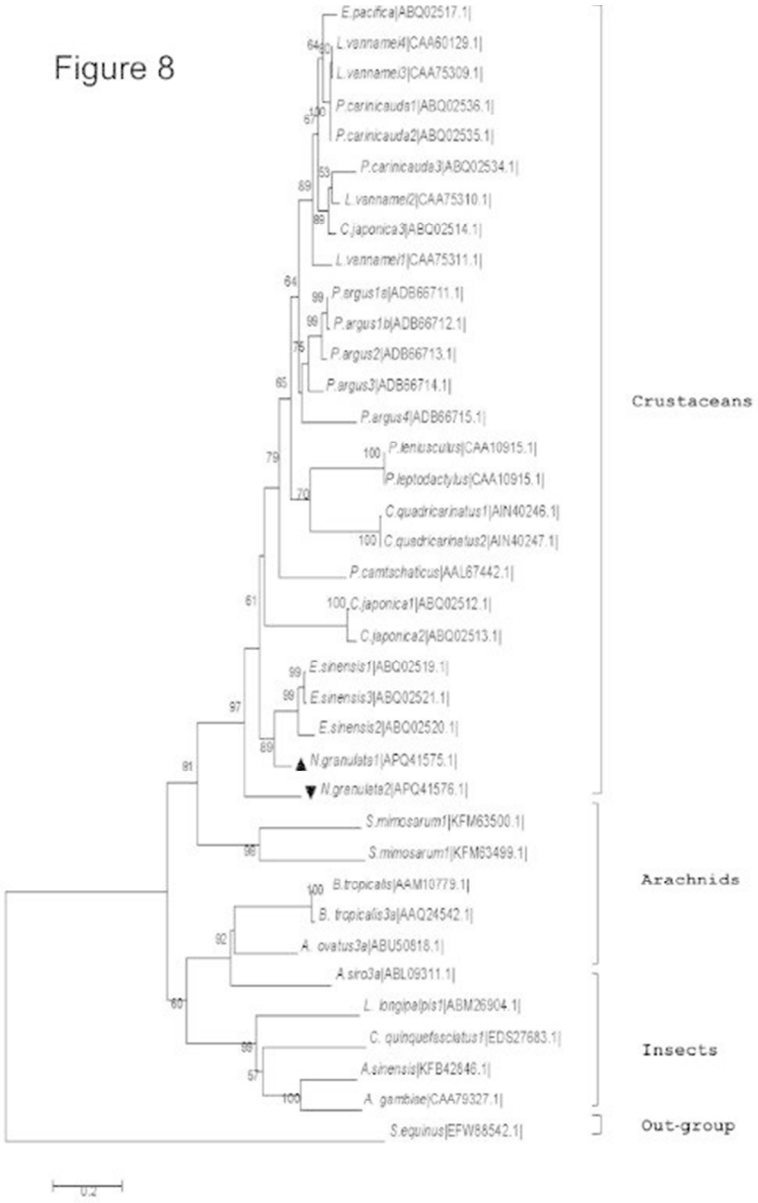

Figure 8

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Figure 9

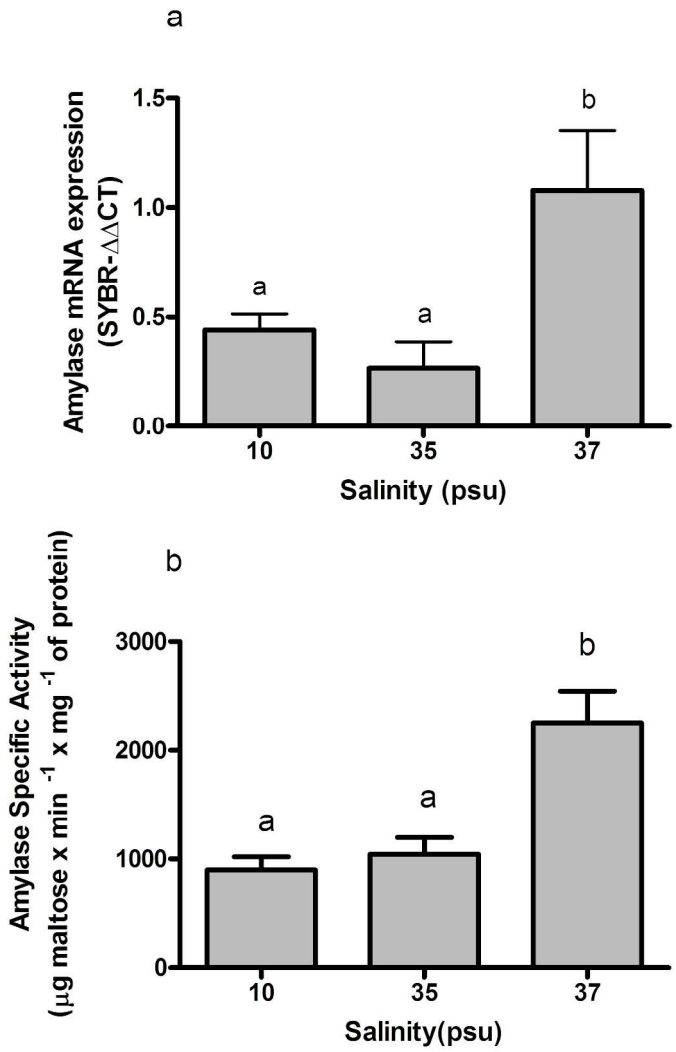

Figure 9

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Figure 10

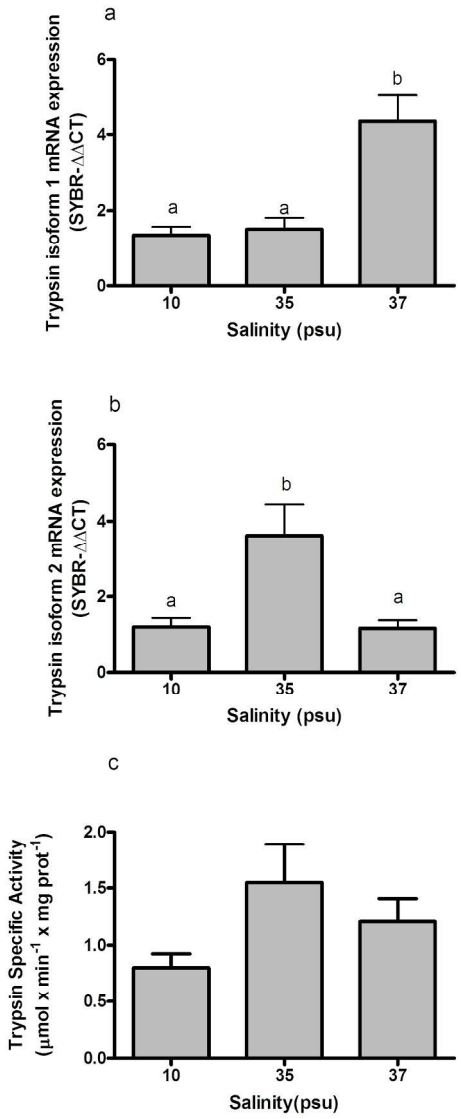

Figure 10

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