1	In silico analysis and effects of environmental salinity in the expression and activity of digestive α -
2	amylase and trypsins from the euryhaline crab Neohelice granulata
3	
4	Antonela Asaro ^{1#} Juan Antonio Martos-Sitcha ^{2,3 #‡} Conzalo Martínez-Rodríguez ² Juan Miguel
5	Managua ³ Alaiandua Antonia Lánar Mažanaa ^{1*}
5	Mancera, Alejandra Antonia Lopez Mananes
6	
7	
8	1 Instituto de Investigaciones Marinas y Costeras (IIMyC), Consejo Nacional de Investigaciones
9	Científicas y Técnicas (CONICET)-Universidad Nacional de Mar del Plata, Funes 3250, Mar del Plata,
10	Argentina.
11	2 Instituto de Ciencias Marinas de Andalucía (ICMAN), Consejo Superior de Investigaciones Científicas
12	(CSIC), E-11519 Puerto Real (Cádiz), Spain.
13	3 Department of Biology, Faculty of Marine and Environmental Sciences, Campus de Excelencia
14	Internacional del Mar (CEI-MAR). University of Cádiz. 11510 Puerto Real (Cádiz). Spain.
15	[‡] Current address: Nutrigenomics and Eich Growth Endogrinology Group. Institute of Aqueculture Torre
15	Current address. Nutrigenomics and Fish Growth Endocrinology Group, institute of Aquacuture Torre
16	de la Sal, Consejo Superior de Investigaciones Científicas (IATS-CSIC), Ribera de Cabanes, E-12595
17	Castellón, Spain.
18	
19	# Both authors contributed equally to the study
20	Total number: 22 pages, 5 tables, 10 graphs.
21 22	Running headline: Amylase and trypsin in Neohelice granulata
23	*Corresponding author:
24	Dra. Alejandra López Mañanes
25	Instituto de Investigaciones Marinas y Costeras (IIMyC) Consejo Nacional de Investigaciones
26	Científicas y Técnicas (CONICET)-Universidad Nacional de Mar del Plata, Argentina.
27	Funes 3250
28	7600 Mar del Plata, Argentina
29	Tel. +54-223-4753554
30	mananes@mdp.edu.ar
31	

- 32 In silico analysis and effects of environmental salinity in the expression and activity of digestive
- 33 α-amylase and trypsins from the euryhaline crab *Neohelice granulata*
- 34

35

36 A. Asaro, J. A. Martos-Sitcha, G. Martínez-Rodríguez, J. M. Mancera, A. A. López Mañanes

- 37
- 38 Abstract

39

40 Studies on molecular characteristics and modulation of expression of α -amylase and trypsin in the 41 hepatopancreas of intertidal euryhaline crabs are lacking. In this work, we cloned and studied by in silico 42 approaches the characteristics of cDNA sequences for α -amylase and two trypsins isoforms, as well as the 43 effect of environmental salinity on gene expression and protein activities in hepatopancreas of Neohelice 44 granulata (Dana, 1852), as a good invertebrate model species. The cDNA sequence of α -amylase is 1,637 45 bp long, encoding 459 amino acid residues. Trypsin 1 and 2 are 689 bp and 1,174 bp long, encoding 204 46 and 151 amino acid residues, respectively. Multiple sequence alignment of deduced protein sequences 47 revealed the presence of conserved motifs found in other invertebrates. In crabs acclimated at 37 psu 48 (hypo-regulation), α -amylase mRNA level and total pancreatic amylase activity were higher than at 10 49 psu (hyper-regulation) and 35 psu (osmoconformation). Trypsin 1 mRNA levels increased at 37 psu while 50 trypsin 2 levels decreased at 10 and 37 psu. Total trypsin activity was similar in all salinities. Our results 51 showed a differential modulation of α -amylase and trypsins expression and total amylase activity by 52 salinity acclimation, suggesting the occurrence of distinct mechanisms of regulation at different levels 53 that could lead to digestive adjustments in relation to hyper- and/or hypo-regulation.

54

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

57 Introduction

Phenotypic flexibility involves changes in attributes at different levels (from molecular to organisms), in response to variations to external stimuli. These reversible changes can increase probability of survival particularly in heterogeneous habitats (Piersma and Drent 2003; Pfenning et al. 2010; Kelly et al. 2012). Digestive enzymes play an essential physiological role, since they are a link between ingestion, absorption and assimilation of nutrients. Therefore, modulation of digestive enzymes activities can lead to digestive adjustments for facing variations in key environmental conditions (del Valle et al. 2004, 2006; 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

α-Amylases (α -1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) have a central physiological importance in
all animals due to their role in the initial steps of the digestion of key glycogenic substrates (i.e. starch and
glycogen) (Singh et al. 2014; Date et al. 2015; Peng et al. 2015; Tiwari et al. 2015). Amylase activity was
detected in the hepatopancreas of various decapod crustaceans (Le Moullac et al. 1997; Perera et al.
2008*a*, 2008*b*; Aragón-Axomulco et al. 2012; Rodríguez-Viera et al. 2014; among others). Trypsin (EC
3.4.21.4) represents the main proteolytic enzyme in the hepatopancreas of decapod crustaceans (Johnson

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.

94 Neohelice granulata (Dana, 1852) is an intertidal euryhaline burrowing crab, serving as animal model for 95 biochemical, physiological and ecological research (Spivak 2010). N. granulata is found on intertidal 96 areas of the South-Western Atlantic from the south of Brazil to the north of Patagonia, Argentina (Spivak 97 1997; Iribarne et al. 2003; Luppi et al. 2013). N. granulata is a dominant crab species in Mar Chiquita 98 coastal lagoon (Argentina) in which successfully occupies the complete intertidal area and faces abrupt 99 and highly variable shifts in salinity (from 2 to 47 psu) (Spivak et al. 1994; Fanjul et al. 2008; Luppi et al. 100 2013). Biochemical adaptation to low and high salinity implies a metabolic reorganization to cope with 101 the energy requirements caused by osmoregulatory processes. The differential modulation of specific 102 digestive enzymes activities in euryhaline crabs such as N. granulata would be an important response in 103 this reorganization through the potential enhancement of available metabolites (Romano and Zheng 2012; 104 Pinoni et al. 2013; Michiels et al. 2015). In this context, the modulation of the expression at both mRNA 105 and protein activity levels of α -amylase and trypsin in the hepatopancreas, as key components of 106 digestion processes, could be part of the responses to salinity in this invertebrate species. The aim of this 107 work was to study the effect of low and high salinities on the mRNA expression and total activity of α -108 amylase and trypsin in hepatopancreas of N. granulata. In this context, the cloning and characterization of 109 one α -amylase and two trypsins cDNA sequence isoforms were initially carried out from hepatopancreas 110 and then their mRNA levels were determined by quantitative real time PCR (qPCR) and total 111 corresponding activities in hepatopancreas of individuals acclimated to different salinities.

- 112
- 113
- 114
- 115
- 116

117 Materials and Methods

118

119 Animal collection and maintenance

120 Adult male crabs (carapace width greater than 2.5 cm) were captured from the mudflat area of Mar 121 Chiquita coastal lagoon (37°32'-37°45'S; 57°19'-57°26'W, Argentina). All individuals collected were in 122 intermolt (Drach and Tchergovtzeff 1967). Crabs were transported to the laboratory on the day of 123 collection. Salinity was determined in practical salinity units (psu) in all cases. Animals were distributed 124 in three different experimental groups (n = 7-8 individuals per condition) and submitted to the following 125 environmental salinities: i) dilute seawater (10 psu), ii) seawater (35 psu), and iii) concentrated seawater 126 (37 psu) for 10 days (López Mañanes et al. 2000; Michiels et al. 2015). Diluted and concentrated 127 seawater preparation and the maintenance of crabs in aquaria were done as previously described 128 (Michiels et al. 2015). Crabs were fed three times a week using commercial food (55 % carbohydrates, 129 31 % protein, 5 % fat, 2 % fiber, Tetra Pond, Koi vibrance, Germany) (0.07 g per individual) and starved 130 120 h before sampling. We have previously shown that in males of N. granulata digestive and metabolic 131 adjustments occurred up to 120 h after feeding (Méndez et al. 2011, 2012; Pinoni et al. 2015; 132 unpublished results). Under the experimental conditions used, no differences in the feeding behavior 133 were observed and no mortality occurred. This research project was done following the regulations and 134 statements of Ethics Committee CICUAL (OCA 1499/12) FCEyN Universidad Nacional de Mar del 135 Plata.

136

137 Sampling procedures

138 Animals were put on ice for about 20 min to cryoanesthesize. A sample of hemolymph was taken for 139 osmolality determination as described below. The hepatopancreas was immediately excised, weighed and 140 biopsies of approximately 30-40 mg were placed in tubes containing 1 mL RNAlater® (Ambion®), 141 maintained for 96 h at 4 °C and then stored at -20 °C until used for RNA extraction. The remaining of the 142 hepatopancreas was homogenized in 50 mM Tris/HCl pH 7.4 (4 mL g⁻¹ of tissue) (CAT homogenizer x 143 120, tool T10) on ice and centrifuged at $10,000 \times g$ for 15 min at 4 °C (Sorvall, rotor ss-34) as previously 144 described (Michiels et al. 2015). The supernatant was fractionated into 200 µL aliquots and stored at 145 -20 °C until biochemical analyses.

147 Hemolymph osmolality

Hemolymph extraction (about 500 μ L) from the intrabranchial sinus and osmolality determination were done as previously described by Michiels et al. (2015). Osmolality was determined in the plasma obtained by centrifugation of hemolymph 3 min at 2,000 × g (IEC-Centra 7R, refrigerated). Both hemolymph and experimental water osmolality (mOsm kg⁻¹) were measured with a cryoscopic osmometer (Osmomat 030, 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 described by Baldisserotto et al. 2014) for partial cloning. Primers were synthesized by IDT® (Integrated 158 DNA Technologies). Total RNA was isolated from hepatopancreas using a NucleoSpin[®] RNA II kit 159 160 (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 °C, 5 min; [98 °C, 30 s; 65–55 °C in touchdown, 30 s; 72 °C, 1 min] × 35 cycles; 167 72 °C, 10 min). PCR products were visualized in 1 % agarose gel electrophoresis using GelRedTM 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, InvitrogenTM) 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

176 fragment assemblage. Homology analysis of putative protein sequences was carried out with blast at the

177 NCBI website. ClustalO was used for protein alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/).

178

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 TechnologiesTM) 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).

186

187 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 188 189 of clean and intact RNA, were used for analysis. First, different amounts of cDNA, synthesized using the 190 qScript[™] cDNA Synthesis Kit (Quanta BioSciences), were applied in triplicate (six serial 1/2 dilutions 191 from 10 ng to 0.325 ng per reaction) to check the assay linearity and the amplification efficiency for each 192 one of the designed specific pair of primers (Table 2). Although the assay was linear along the six serial 193 dilutions (α -amylase: $r^2 = 0.972$, efficiency (E) = 0.99; trypsin 1: $r^2 = 0.999$, E = 0.94; trypsin 2: $r^2 = 0.94$; trypsin 3: $r^2 = 0.94$ 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, 194 195 each reaction mixture (10 μ L) contained 4 μ L of template (10 ng), 0.5 μ L of each specific forward and 196 reverse primer at their respective final concentration (Table 1), and 5 µL of PerfeCTa SYBR[®] Green 197 FastMixTM (Quanta BioSciences). Reactions were conducted in Hard-Shell[®] PCR Plates 96-well 198 WHT/CLR plates (Bio-Rad) covered with adhesive Microseal® 'B' seal films (Bio-Rad). The 199 thermocycling procedures were performed in a CFX Connect Real-Time system (Bio-Rad). The PCR 200 thermal profile was as follows: 95 °C, 10 min; [95 °C, 30 s; 60 °C, 45 s] × 40 cycles; melting curve [60– 201 95 °C, 20 min], 95 °C, 15 s. β- actin was used as the internal reference gene due to its low variability (less 202 than 0.20 C_{T_x} with no differences detected between experimental groups) under our experimental 203 conditions. Relative gene quantification was performed using the $\triangle \triangle C_T$ method (Livak and Schmittgen 204 2001).

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 the NCBI protein database from 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

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

229

Total amount of proteins was determined by the method described by Bradford (1976) using bovineserum 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

236	ANOVA) or a nonparametric analysis (Kruskal-Wallis) was used to estimate the statistical significance at
237	P < 0.05. A posteriori test to ANOVA (Holm–Sidak method) was used to identify differences.
238	
239	Results
240	
241 242	Characteristics of cDNA sequences The partial α -amylase, trypsin 1, trypsin 2 and β -actin cDNAs of euryhaline crab <i>N. granulata</i> were
243	identified and submitted to GenBank (accession nos. KU531567, KU531568, KU531569 and KU531570,
244	respectively). Sequencing revealed partial cDNAs to be 1,637 bp for α -amylase, 689 bp for trypsin 1,

245 1,174 bp for trypsin 2 and 349 bp for β -actin.

246

247 The obtained partial nucleotide and deduced amino acid sequence of N. granulata α -amylase are shown 248 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 (S¹⁰, S¹⁷ and 249 250 T^{70}). In addition, nine out of ten cysteine residues observed in the amylase protein of other arthropods are 251 present in the cloned partial sequence. Figure 2 shows a protein alignment between different crustaceans 252 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 253 254 binding site (N⁴⁴ and D¹¹¹). The comparison of α -amylase protein sequence of N. granulata to other 255 crustacean species shows a high identity (67.51-77.40 %) (Table 3).

256

257 The partial nucleotide and deduced amino acid sequence of N. granulata trypsin 1 and 2 isoforms are 258 presented in Figures 3 and 4, respectively. For trypsin 1 (Fig. 3), the termination codon (taa) is at position 259 613 followed by the 3' untranslated region containing a polyadenilation site (aataaa) at position 656. The 260 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 261 262 substrate specificity of trypsin, and the conserved motif with catalyzing site (COGDSGGP) at position 263 156-163. For trypsin 2 (Fig. 4), and similarly to isoform 1, the sequence presents the termination codon 264 (taa) at position 456 followed by the 3'untranslated region containing a polyadenilation sites (aataaa) at 265 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 266

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 catalyzingsite (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).

273

274 The partial coding and predicted amino acid sequence of β -actin from hepatopancreas of *N. granulata* is 275 shown in Figure 6. The sequence presents the termination codon (tga) at position 327 and encodes 108 276 amino acid residues. The deduced amino acid sequence of β -actin from *N. granulata* is very similar to 277 that from other crabs, such as *Scylla serrata*, *S. paramamosain* and *Eriochieir sinesis* (98 % identity in 278 all cases, data not shown).

279

280 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).

287

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*).

294

295

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

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

Both trypsins (1 and 2) presented different patterns of gene expression respect to environmental salinities. Trypsin 1 mRNA expression level was enhanced (about 341 %) in individuals acclimated to 37 psu respect to 10 or 35 psu (Fig.10a). However, trypsin 2 mRNA level decreased (about 67.5 %) in individuals acclimated to low (10 psu) and high (37 psu) environmental salinities respect to 35 psu (Fig. 10b). On the other hand, no differences were found between total trypsin activity in hepatopancreas of *N*. *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 327 of invertebrates (crustaceans, insects, mollusks and arachnids) (Fig. 7) showed that α -amylases from 328 crustaceans are closer to the insects group and further from mollusks and arachnids, contrary to that 329 observed by Sellos and Van Wormhoudt (2002). Moreover, the phylogenetic analysis also showed a high 330 conservation within the group of crustaceans, as suggested by Rodríguez-Viera et al. (2016), as well as 331 that the α -amylase from *N. granulata* appeared in a monophyletic group within the crustacean group.

332

333 On the other hand, our results revealed the predicted amino acid sequence for trypsins 1 and 2 in 334 hepatopancreas of N. granulata (Figs. 3 and 4). Partial trypsin 1 is 689 bp long, whereas the isoform 2 is 335 1,174 bp long, encoding 204 and 151 amino acid residues respectively (Figs. 3 and 4). Nine and six 336 cysteine residues in the partial amino acid sequence for trypsin 1 and trypsin 2, respectively, were found 337 (Fig. 3 and 4) in positions agreeing with those found in other crab trypsins (Fig.5). The amino acid 338 sequences of both trypsins 1 and 2 were similar to those of other crabs, displaying common features of 339 crustacean trypsins (Fig. 5). The conserved residues His, Asp, and Ser, which form the active site, as well 340 as Asp, Ser and Gly, which determine the substrate binding site, were all present (Muhlia-Almazán et al. 341 2008). The phylogenetic analysis of trypsin proteins from several crustaceans (Fig. 8) showed that two 342 isoforms from hepatopancreas of N. granulata belongs to the group of brachvura and both isoforms are 343 close to other trypsin isoforms from the crab species Eriocheir sinensis.

344

345 Variations in α -amylase and trypsin gene expression and/or activity in hepatopancreas of several 346 crustaceans have been associated with different environmental and physiological factors (Le Moullac et 347 al. 1997; Perera et al. 2008a, 2008b, 2012; Michiels 2015; Peng et al. 2015). Since digestive enzymes are 348 a link between ingestion and absorption their regulation can result in a distinct availability of energy 349 substrates (e.g. glucose and amino acids) upon osmoregulatory conditions (Li et al. 2008; Perera et al. 350 2012; Romano and Zeng 2012; Wang et al. 2014). The higher α -amylase mRNA level and the 351 corresponding increase (about 216 %) of total α -amylase activity (Fig. 9) in hepatopancreas of N. 352 granulata acclimated to 37 psu, conditions at which this crab hypo-regulates (Table 5) compared to 35 353 psu (osmoconforming condition, Table 5), suggests that modulation at the molecular level (i.e. gene 354 expression) of α -amylase is one of the responses to high salinity underlying hypoosmotic regulation. 355 Whether the hepatopancreas total α -amylase activity enhancement observed at 37 psu is also related to 356 modulation at other levels (i.e. translational and/or post-translational) cannot be discarded. In the shrimp 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. 2010a, 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

case (Fig. 10c), qualitative changes (i.e. different isoforms accounting for total trypsin activity) could be
occurring in low and high environmental salinities. In this sense, trypsin isoforms in the lobster *P. argus*have distinct catalytic properties and/or specificity (i.e. different digestion efficiency) (Perera et al.
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

402 Acknowledgments. The authors wish to thank Mrs. María Francisca Osta and Mr. Juan José Blanco for
403 their excellent technical assistance. This study was partly supported by grant AGL2013-48835-C2-1-R
404 (Ministerio de Economía y Competitividad, Spain) to JMM and from the University of Mar del Plata
405 (EXA787/16, 15/E738) and Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET)
406 (PIP N° 112 201301 00009 CO), Argentina. A. Asaro was supported by a scholarhip from AUIP in the
407 program "Programa de Movilidad Académica" and a Doctoral scholarship from CONICET (Argentina).
408

409 References

410 Alexandre, D., Ozório, R. A., Derner, R. B., Fracalossi, D. M., Oliveira, G. B., Samuels, R. I., Terra W.

411 R., and Silva, C. P. 2014. Spatial distribution of digestive proteinases in the midgut of the Pacific white

412 shrimp (*Litopenaeus vannamei*) indicates the existence of endo-ectoperitrophic circulation in
413 Crustacea. Comp. Biochem. Physiol. 172: 90-95.

414 Aragón-Axomulco, H., Chiappa-Carrara, X., Soto, L., Cuzon, G., Arena, L., Maldonado, C., and Gaxiola,

415 G. 2012. Seasonal variability in trypsin and α -amylase activities caused by the molting cycle and feeding

416	habits of juvenile pink shrimp Farfantepenaeus duorarum (Burkenroad, 1939). J. Crustac. Biol. 32: 89-
417	99.
418	Asaro, A., del Valle, J.C., and López Mañanes, A.A. 2011. Amylase, maltase and sucrase activities in
419	hepatopancreas of the euryhaline crab Neohelice granulata (Decapoda:Brachyura:Varunidae): partial
420	characterization and response to low environmental salinity. Sci. Mar. 75: 517-524.
421	Baldisserotto, B., Martos-Sitcha, J.A., Menezes, C.C., Toni, C., Prati, R.L., Garcia, L.D.O., Salbego, J.,
422	Mancera, J.M., and Martínez-Rodríguez, G. 2014. The effects of ammonia and water hardness on the
423	hormonal, osmoregulatory and metabolic responses of the freshwater silver catfish Rhamdia quelen.
424	Aquat. Toxicol. 152: 341-352.
425	Bezerra, C.A., Macedo, L.L.P., Amorim, T.M.L., Santos, V.O., Fragoso, R.D.R., Lucena, W.A.,
426	Meneguim, A.M., Valencia-Jimenez, A., Engler, G., Silva, M.C.M., Albuquerque, E.V.S., and Grossi-de-
427	Sa, M.F. 2014. Molecular cloning and characterization of an α-amylase cDNA highly expressed in major
428	feeding stages of the coffee berry borer, Hypothenemus hampei. Gene, 553: 7-16.
429	Biesiot, P., and Capuzzo, J.M. 1990. Changes in the digestive enzyme activities during early development
430	of the American lobster Homarus umericanus Milne Edwards. J. Exp. Mar. Biol. Ecol. 136: 107-122.
431	Bortolus, A., and Iribarne, O. 1999. Effects of the SW Atlantic burrowing crab Chasmagnathus granulata
432	on a Spartina salt marsh. Mar. Ecol. Prog. Ser. 178: 79-88.
433	Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgran quantities of
434	protein-dye binding. Anal. Biochem. 72: 248-254.
435	Charmantier, G., and Anger, K. 2011. Ontogeny of osmoregulatory patterns in the South American
436	shrimp Macrobrachium amazonicum: Loss of hypo-regulation in a land-locked population indicates
437	phylogenetic separation from estuarine ancestors. J. Exp. Mar. Biol. Ecol. 396: 89-98.
438	Date, K., Satoh, A., Iidam, K., and Ogawa, H. 2015. Pancreatic α-amylase controls glucose assimilation
439	by duodenal retrieval through N-Glycan-specific binding, endocytosis, and degradation. J. Biol. Chem.
440	290 : 17439-17450.
441	del Valle, J.C., Busch, C., and López Mañanes, A.A. 2006. Phenotypic plasticity in response to low

- 442 quality diet in the South American omnivorous rodent Akodon azarae (Rodentia: Sigmodontinae). Comp.
- 443 Biochem. Physiol. A 145: 397-405.

- del Valle, J.C., López Mañanes, A.A., and Busch, C. 2004. Phenotypic flexibility of digestive
 morphology and physiology of the South American omnivorous rodent *Akodon azarae*(Rodentia:Sigmodontinae). Comp. Biochem. Physiol. A 139: 503-512.
- Drach, P., and Tchernigovtzeff C. 1967. Sur le methode de determination des stades d'intermude et son
 application generale aux crustaces. Vie Milieu, 18: 595–607.
- 449 Ezquerra, J., García-Carreño, F.L., and Haard, N.F. 1997. Effects of feed diets on digestive proteases
- 450 from the hepatopancreas of white shrimp (*Penaeus vannamei*). J. Food Biochem. 21(2): 401-419.
- 451 Fanjul, E., Grela, M.A., Canepuccia, A., and Iribarne, O. 2008. The Southwest Atlantic intertidal
- 452 burrowing crab *Neohelice granulata* modifies nutrient loads of phreatic waters entering coastal area.
- 453 Estuar. Coast Mar. Sci. 79: 300-306.
- 454 Hehemann, J.H., Redecke, L., Murugaiyan, J., Von Bergen, M., Betzel, C., and Saborowski, R. 2008.
- 455 Autoproteolytic stability of a trypsin from the marine crab *Cancer pagurus*. Biochem. Biophys. Res.
 456 Commun. **370**(4): 566-571.
- 457 Iribarne, O., Martinetto, P., Schwindt, E., Botto, F., Bortolus, A., and Borboroglu, P.G. 2003. Evidences
 458 of habitat displacement between two common soft-bottom SW Atlantic intertidal crabs. J. Exp. Mar. Bio.
- 459 Ecol. **296**(2): 167-182.
- 460 Jiang, K.J., Zhang, F.Y., Zhang, D., Tao, Q.C., Zhang, Y., Pi, Y., Qiao, Z., and Ma, L.B. 2011.
- 461 Identification of a trypsin gene from *Scylla paramamosain* and its expression profiling during larval
 462 development. African J. Agric. Res. 6: 6613-6621.
- 463 Johnson, S., Ewart, K., Osborne, J., Delage, D., Ross, N., and Murray, H. 2002. Molecular cloning of
- 464 trypsin cDNAs and trypsin gene expression in the salmon louse *Lepeophtheirus salmonis* (Copepoda:
- 465 Caligidae). Parasitol. Res. **88**(9): 789-796.
- 466 Karasov, W.H., and Douglas, A.E. 2013. Comparative digestive physiology. Compr. Physiol. 271-283.
- 467 Karasov, W.H., Martínez del Riom C., Caviedes-Vidal, E. 2011. Ecological physiology of diet and
- 468 digestive systems. Annu. Rev. Physiol. 73: 69–93.
- Kelly, S.A., Panhuis, T.M., and Stoehr, A.M. 2012. Phenotypic plasticity: molecular mechanisms and
 adaptive significance. Compr. Physiol. 2: 1417–1439.
- 471 Kumar S., Stecher G., and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version
- 472 7.0 for bigger datasets. Mol. Biol.Evol. 33: 1870-1874.

Page 17 of 39

- 473 Kumari, A., Singh, K., and Kayastha, A.M. 2012. α-Amylase: general properties, mechanism and 474 biotechnological applications-A Review. Curr. Biotechnol. 1(1): 98-107. 475 Kuriki, T., and Imanaka, T. 1999. The concept of -amylase family: Structural similarity and common 476 catalytic mechanism. J. Biosci. Bioeng. 87: 557-65. 477 Lancia, J.P., Bas, C., and Spivak, E. 2013. Food manipulation and selection in the omnivorous grapsoid 478 crab Neohelice granulata (Decapoda: Varunidae). Sci. Mar. 78(4): 529-536. 479 Le Moullac, G., Klein, B., Sellos, D., and Van Wormhoudt, A. 1997. Adaptation of trypsin, chymotrypsin 480 and α -amylase to case level and protein source in *Penaeus vannamei* (Crustacea Decapoda). J. Exp. 481 Mar. Biol. Ecol. 208: 107-125. 482 Lemos, D., Ezquerra, J.M., and Garcia-Carreño, F.L. 2000. Protein digestion in penaeid shrimp: digestive 483 proteinases, proteinase inhibitors and feed digestibility. Aquaculture, 186(1): 89-105. 484 Li, E., Chen, L., Zeng, C., Yu, N., Xiong, Z., Chen, X., and Qin, J.G. 2008. Comparison of digestive and 485 antioxidant enzymes activities, haemolymph oxyhemocyanin contents and hepatopancreas histology of 486 white shrimp, Litopenaeus vannamei, at various salinities. Aquaculture, 274: 80-86. 487 Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$ Ct method. Methods, **25**: 402–408. 488 López Mañanes, A.A., Magnoni, L.J., and Goldemberg, A.L. 2000. Branchial carbonic anhydrase (CA) of 489 490 gills of Chasmagnathus granulata (Crustacea Decapoda). Comp. Biochem. Physiol. B 127: 85-95. 491 Luppi, T., Bas, C., Méndez Casariego, A., Albano, M., Lancia, J., Kittlein, M., Rosenthal, A., Farías, N.,
 - 492 Spivak, E., and Iribarne, O. 2013. The influence of habitat, season and tidal regime in the activity of the

493 intertidal crab *Neohelice* (=Chasmagnathus) granulata. Helgol. Mar. Res. 67: 1-5.

- 494 McNamara, J.C., and Faria, S.C. 2012. Evolution of osmoregulatory patterns and gill ion transport
- 495 mechanisms in the decapod Crustacea: a review. J. Comp. Physiol. B 182: 997–1014.
- 496 Méndez, E., López Mañanes, A.A., and Pinoni, S.A. 2011. Lipase activity in the hepatopancreas of the
- 497 euryhaline crab *Neohelice granulata*: short and long term postfeeding response. Biocell, **35**: A142–A164.
- 498 Méndez, E., López Mañanes, A.A., and Pinoni, S.A. 2012. Proteolytic activity in hepatopancreas of the 499 euryhaline crab *Neohelice granulata*: response to hyper-regulation and feeding. Biocell, **36**: A32–A61.
- 500 Michiels, M.S. 2015. Fisiología bioquímica digestiva de crustáceos decápodos: estudio integrativo sobre
- 501 la actividad de enzimas digestivas en cangrejos eurihalinos. Tesis doctoral, Universidad Nacional de Mar
- 502 del Plata. Mar del Plata, Argentina

- 503 Michiels, M.S., del Valle, J.C., and López Mañanes, A.A. 2015. Biochemical characteristics and 504 modulation by external and internal factors of aminopeptidase-N activity in the hepatopancreas of a 505 euryhaline burrowing crab. J. Comp. Physiol. B **185**: 501-510.
- 506 Michiels, M.S., del Valle, J.C., and López Mañanes, A.A. 2013. Effect of environmental salinity and
- dopamine injections on key digestive enzymes in hepatopancreas of the euryhaline crab *Cyrtograpsus angulatus* (Decapoda: Brachyura: Varunidae). Sci. Mar. 77:129–136.
- 509 Miller, G.L. 1959. Use of dinitrosalicylic acid regent for determination of reducing sugar. Anal. Chem.
 510 31: 426-428.
- 511 Muhlia-Almazán, A., and Garcia-Carreño, F.L. 2003. Digestion physiology and proteolytic enzymes of
- 512 crustacean species of the Mexican Pacific Ocean. Contributions to the study of east Pacific crustaceans, 2:
 513 77-91.
- 514 Muhlia-Almazán, A., Sánchez Paz, A., and García Carreño, F.L. 2008. Invertebrate trypsins: a review. J.
- 515 Comp. Physiol. B **178**: 655–672.
- 516 Nikapitiya, C., Kim, W.S., Park, K., and Kwak, I.S. 2014. Identification of potential markers and
- 517 sensitive tissues for low or high salinity stress in an intertidal mud crab (*Macrophthalmus japonicus*).
- 518 Fish Shellfish Immunol. **41**(2): 407-416.
- Peng, T., Wang, D., Yu, Y., Liu, C., and Zhu, B. 2015. Identification and expression of an ecdysteroidresponsive amylase from red crayfish *Procambarus clarkii*. Fisheries Sci. 81: 345-352.
- 521 Perera, E., Moyano, F.J., Díaz, M., Perdomo-Morales, R., Montero-Alejo, V., Alonso, E., Carrillo, O.,
- 522 and Galich, G.S. 2008a. Polymorphism and partial characterization of digestive enzymes in the spiny
- 523 lobster *Panulirus argus*. Comp. Biochem. Physiol. B 150: 247–254.
- 524 Perera, E., Moyano, F.J., Díaz, M., Perdomo-Morales, R., Montero-Alejo, V., Rodriguez-Viera, L.,
- 525 Alonso, E., Carrillo, O., and Galich, G.S. 2008b. Changes in digestive enzymes through developmental
- 526 and molt stages in the spiny lobster, *Panulirus argus*. Comp. Biochem. Physiol. B 151: 250–256.
- 527 Perera, E., Moyano, F.J., Rodriguez-Viera, L., Cervantes, A., Martínez-Rodríguez, G., and Mancera, J.M.
- 528 2010a. In vitro digestion of protein sources by crude enzyme extracts of the spiny lobster Panulirus argus
- 529 (Latreille, 1804) hepatopancreas with different trypsin isoenzyme patterns. Aquaculture, **310**(1): 178-185.
- 530 Perera, E., Pons, T., Hernandez, D., Moyano, F.J., Martínez-Rodríguez, G., and Mancera, J.M. 2010b.
- 531 New members of the brachyurins family in lobster include a trypsin-like enzyme with amino acid
- substitutions in the substrate-binding pocket. FEBS J. 277(17): 3489-501.

533 Perera, E., Rodríguez-Viera, L., Perdomo-Morales, R., Montero-Alejo, V., Moyano, F.J., Martínez-

- 534 Rodríguez, G., and Mancera, J.M. 2015. Trypsin isozymes in the lobster Panulirus argus (Latreille,
- 535 1804): from molecules to physiology. J. Comp. Physiol. B 185(1):17-35.
- 536 Perera, E., Rodríguez-Viera, L., Rodríguez-Casariego, J., Fraga, I., Carrillo, O., Martínez-Rodríguez, G.,
- 537 and Mancera, J.M. 2012. Dietary protein quality differentially regulates trypsin enzymes at the secretion
- 538 and transcription level in *Panulirus argus* by distinct signaling pathways. J. Exp. Biol. **215**(5): 853-862.
- 539 Pfenning, D.W., Wund, M.A., Snell-Rood, E.C., Cruickshank, T., Schlichting, C.D., and Moczek, A.P.
- 540 2010. Phenotypic plasticity's impacts on diversification and speciation. Trends Ecol. Evol. 25: 459–467.
- 541 Piersma, T., and Drent, J. 2003. Phenotypic flexibility and the evolution of organismal design. Trends
 542 Ecol. Evol. 18: 228-233.
- 543 Pinoni, S.A., Goldemberg, A.L., and López Mañanes, A.A. 2005. Alkaline phosphatase activities in
 544 muscle of the euryhaline crab *Chasmagnathus granulatus*: response to environmental salinity. J. Exp.
 545 Mar. Biol. Ecol. 326: 217–226.
- 546 Pinoni, S.A., and López Mañanes, A.A. 2008. Partial characterization and response under hyperregulating
 547 conditions of Na⁺/K⁺-ATPase and levamisole-sensitive alkaline phosphatase activities in chela muscle of
 548 the euryhaline crab *Cyrtograpsus angulatus*. Sci. Mar. **72**: 15–24.
- 549 Pinoni, S.A., and López Mañanes, A.A. 2009. Na⁺ATPase activities in chela muscle of the euryhaline
 550 crab *Neohelice granulata*: differential response to environmental salinity. J. Exp. Mar. Biol. Ecol. 372:
 551 91–97.
- 552 Pinoni, S.A., Méndez, E., and López Mañanes, A.A. 2015. Digestive flexibility in a euryhaline crab from
- 553 a SW Atlantic coastal lagoon: alkaline phosphatase activity sensitive to salinity in the hepatopáncreas. J.
- 554 Mar. Biol. Assoc. U.K. **95**: 1133-1140.
- 555 Pinoni, S.A., Michiels, M.S., and López Mañanes, A.A. 2013. Phenotypic flexibility in response to
- 556 environmental salinity in the euryhaline crab *Neohelice granulata* from the mudflat and the saltmarsh of a
- 557 SW coastal lagoon. Mar. Biol. 160: 2647-2661.
- 558 Rodríguez-Viera, L., Perera, E., Casuso, A., Perdomo-Morales, R., Gutierrez, O., Scull, I., Carrillo, O.,
- 559 Martos-Sitcha, J.A., García-Galano, T., and Mancera, J.M. 2014. A holistic view of dietary carbohydrate
- 560 utilization in lobster: digestion, postprandial nutrient flux, and metabolism. PLoS ONE 9(9): e108875
- 561 http://dx.doi.org/10.1371/journal.pone.0108875.

- 562 Rodríguez-Viera, L., Perera, E., Martos-Sitcha, J.A., Perdomo-Morales, R., Casuso, A., Montero-Alejo,
- 563 V., et al., 2016. Molecular, biochemical, and dietary regulation features of α-Amylase in a carnivorous
- 564 crustacean, the spiny lobster *Panulirus argus*. PLoSONE 11(7): e0158919
- 565 http://dx.doi.org/10.1371/journal.pone.0158919.
- 566 Romano, N., and Zeng, C. 2012. Osmoregulation in decapod crustaceans: implications to aquaculture
- 567 productivity, methods for potential improvement and interactions with elevated ammonia exposure.
- 568 Aquaculture, **334**: 12–23.
- 569 Rudenskaya, G.N., Kislitsin, Y.A., and Rebrikov, D.V. 2004. Collagenolytic serine protease PC and
- 570 trypsin PC from king crab Paralithodes camtschaticus: cDNA cloning and primary structure of the
- 571 enzymes. BMC Struct. Biol. 4(1): 2-10.
- Sellos DY, and Van Wormhoudt A. 2002. Structure of the of alpha-amylase genes in crustaceans and
 molluscs: evolution of the exon/intron organization. Biol. Brat. 57: 191-196.
- 574 Shi, X., Ren, Q., Zhao, X., and Wang, J. 2009. Expression of four trypsin-like serine proteases from the
- 575 Chinese shrimp, Fenneropenaeus chinensis, as regulated by pathogenic infection. Comp. Biochem.
- 576 Physiol. B 153: 54–60.
- 577 Singh, K., and Kayastha, A.M. 2014. α-Amylase from wheat (Triticum aestivum) seeds: Its purification,
- 578 biochemical attributes and active site studies. Food Chem. 162: 1–9.
- 579 Spivak, E. 1997. Cangrejos estuariales del Atlántico sudoccidental (25°-41°S) (Crustacea: Decapoda:
- 580 Brachyura). Invest. Mar Valparaíso, 25: 105-12.
- 581 Spivak, E. 2010. The crab Neohelice (=Chasmagnathus) granulata: an emergent animal model from
- 582 emergent countries. Helgol. Mar. Res. 64: 149-154.
- 583 Spivak, E., Anger, K., Luppi, T., Bas, C., and Ismael, D. 1994. Distribution and habitat preferences of
- two grapsid crab species in Mar Chiquita lagoon (Pcia. Bs As. Argentina). Helgol. Meeres. 48: 59-78.
- 585 Tiwari, S.P., Srivastava, R., Singh, C.S., Shukla, K., Singh, R.K., Singh, P., Singh, R., Singh, N.L., and
- 586 Sharma, R. 2015. Amylases: an overview with special reference to alpha amylase. J. Global Biosc. 4:
- 587 1886-1901.
- 588 Van Wormhoudt, A., and Sellos, D.1996. Cloning and sequencing analysis of three amylase cDNAs in
- 589 the shrimp *Penaeus vannamei* (Crustacea decapoda): evolutionary aspects. J. Mol. Evol. **42**: 543-51.
- 590 Wang W, Wu X, Liu Z, Zheng H, Cheng Y. 2014. Insights into hepatopancreatic functions for nutrition
- 591 metabolism and ovarian development in the crab Portunus trituberculatus: gene discovery in the

- 593 http://dx.doi.org/10.1371/journal.pone.0084921.
- 594 Zeng, H., Ye, H., Li, S., Wang, G., and Huang, J. 2010. Hepatopancreas cell cultures from mud crab,
- 595 Scylla paramamosain. In Vitro Cell Dev. Biol. Anim. 46: 431-437.

597 **Table 1.** Nucleotide sequence of degenerate primers designed for molecular identification of α -amylase,

598 tryps n and β -actin partial cDNA sequences, as well as specific primers used for 3'- Rapid Amplification

of cDNA Ends (RACE).

600

Degenerate primers	Nucleotide sequence	Size amplified	
α -amy Fw ₁	5'-ATBGTSCAYYTBTTYGARTGG-3'	1.250 hn	
α -amy Rw ₁	5'-TVACVNBCTTBCCMGTGCA-3'	1,250 op	
α -amy Fw ₂	5'-GGCCDTGGTGGGARMGDTAC-3'	1 155 hr	
α -amy Rw ₂	5'-CCRGARATSACRTCRCAGTA-3'	1,155 bp	
α -amy Fw ₃	5'-GCYDSCAARCACATGTGGCC-3'	550 hn	
α-amy Rw ₃	5'-TTNCGGAADNBVACCATGTT-3'	550 bp	
tryp Fw ₁	5'-CCAARATCATCCARCACGARG-3'	294 hp	
tryp Rv ₁	5'-AGTCACCCTGGCANGMGTC-3'	284 Up	
tryp Fw ₂	5'-TTCTGCGGHGCBTCCATCTACA-3'	122 hn	
tryp Rv ₂	5'-CYTCGTGYTGGATGATYTTGG-3'	152 Up	
β -act Fw ₁	5'-ACCACAGCYGARMGKGAAAT-3'	202 hn	
β -act Rv ₁	5'-TCCKGTCWGCRATGCCAGGGT-3'	293 Op	
Primers for 3'RACE	Nucleotide sequence	Position	Direction
Ng α-Amyelong3 Fw1	5'-TCCGGGGGACATTGAGAACTAC-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

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'-TCCGGGGGACATTGAGAACTAC-3'	600 nM	136 bn	
Ng qPCR α-Amy Rv	5'-CGTACCCGATCAGATTGTTCA-3'	000 1111	150 op	
Ng qPCR Tryp 1 Fw	5'-CCCTTCTCCAACTGTCCTCC-3'	400 nM	145 bp	
Ng qPCR Tryp 1Rv	5'-TGAAGGATAGAGGGAGTGCT-3'	400 1111	145 op	
Ng qPCR Tryp 2 Fw	5'-GCGAAGTTTACGGAGAAGAAG-3'	400 nM	176 bp	
Ng qPCR Tryp 2 Rv	5'-GGCGTACACGCCGGGGCTGTC-3'	400 1111	170 op	
Ng qPCR β -act Fw	5'-ATTCCAGCCTTCCTTCTTGG-3'	600 nM	105 bp	
Ng qPCR β-act Rv	5'-TTTGCAAACAGGTCCTTCCT-3'	000 11101	105 Up	

606

- 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.

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

612 613

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. trituberculatus l	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

Table 5. Osmolality (mOsmol kg⁻¹) in external environment and *N. granulata* hemolymph of individuals 620

622

10 p	su	35 _I	osu	37 psu				
Environment	Hemolymph	Environment	Hemolymph	Environment	Hemolymph			
255 ± 15	$646 \pm 30*$	813 ± 41	869 ± 33	974 ± 33	899 ± 19*			

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

624

⁶²¹ acclimated to different environmental salinity (10, 35 and 37 psu).

627 Legends

628

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

633

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

640

Fig. 3. Nucleotide and deduced amino acid sequence of *N. granulata* trypsin 1 cDNA. The deduced amino acid sequence is displayed in bold capital letters above nucleotide sequence. Stop codon (taa) and consensus polyadenylation signal (aataaa) are represented in bold, italic and underlined. 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 shaded in black. The conservative motif of catalyzing-site is boxed. The cysteine residues are marked with a circle.

647

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

654

656 Fig. 5. Alignment of protein sequences of crab trypsins using Clustal Omega. The conserved amino acids 657 that participate in the catalytic triad (His,Asp,Ser) are shaded in grey. The amino acids that participate in 658 the substrate-binding pocket (Asp, Ser, Gly) are marked in bold with an open arrow on the column. The 659 conserved motif for catalyzing-site is boxed. The cysteine residues are marked with a triangle on the 660 column. Amino acid differences between isoforms of N. granulata are indicated in black boxes. Sylla 661 paramamosain (ADB55592), Eriocheir sinensis 1 (ABQ02519.1), E. sinensis 2 (ABQ02520.1), E. 662 sinensis 3 (ABQ02521.1), Portunus trituberculatus 1 (ABQ02537.1), P. trituberculatus 2 663 (ABQ02538.1), P. trituberculatus 3 (ABQ02539.1), Charybdis japonica 1 (ABQ02512.1), C. japonica 2 664 (ABQ02513.1), C. japonica 3 (ABQ02514.1), Portunus pelagicus (ABM65758.1), Paralithodes 665 *camtschaticus* (AAL67442.1). The symbols indicate: identity (*), conserved substitutions (:), and semi-666 conserved substitutions (.).

667

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.

671

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.

677

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.

- **Fig. 9.** Gene expression (a) and activity of α-amylase (b) in hepatopancreas of *N. granulata* acclimated to different environmental salinities (10, 35 and 37 psu). Different letters indicate statistically significant 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 \pm
- 692 SE for 5–7 individuals.

		Ρ	W	W	Е	R	Y	Q	Ρ	v	s	Y	N	L	v	т	R	s	G	18
5´	-gg	сса	tgg	rtgo	ggag	gogt	tac	cag	lago	gt	gtco	tac	aac	ctg	gto	faco	ccgo	tca	ggg	56
D	Е	s	А	F	х	D	м	v	s	R	С	х	х	v	х	v	R	I	Y	38
gac	gag	tca	gcc	tto	ccar	ngad	cato	gtg	ago	cage	gtgo	anc	anc	gtg	gno	gto	cago	gatt	tac	116
А	D	I	v	м	N	н	м	т	G	W	Q	P	s	х	н	G	G	D	W	58
gct	gac	ata	gtg	fato	gaad	ccat	cato	faco	ggo	ctg	gcaa	lace	tct	ngg	cac	add	lddo	gac	tgg	176
W	F	L	v	R	С	х	A	Q	s	L	т	х	Α	v	Ρ	Y	s	Α	Y	78
tgg	Ittc	ctc	gtt	cga	atgo	cong	ggct	cag	ragt	ccti	taco	ncc	gcc	gta	ccc	tac	etco	gec	tat	236
D	F	N	D	G	N	С	N	s	G	s	G	D	I	E	N	Y	G	D	A	98
gac	ttc	aac	gat	ggg	gaat	tgo	caac	tcg	làda	gtc	cada	gac	att	gag	aac	tac	eggt	gac	gcg	296
N	Q	v	R	N	С	ĸ	L	s	G	L	N	D	L	N	Q	G	т	D	Y	118
aac	cag	gtc	cgc	aac	ctgo	caaq	gcto	ago	ggo	ccti	taac	gac	ctc	aac	cag	ldde	aca	igac	tac	356
v	R	G	м	I	R	D	Y	м	N	N	L	Ι	G	Y	G	v	А	G	F	138
gtg	lcdd	ggc	atg	fatt	cga	agao	ctac	atg	aac	caat	tctç	fato	ggg	tac	ggo	gto	gct	ggg	ittc	416
R	v	D	А	s	ĸ	н	м	W	Р	G	D	м	Q	А	I	F	s	s	L	158
cga	gtt	gac	gcc	tco	caaa	acat	cato	ıtgg	rcct	cgg	cgat	atg	cag	gcc	ato	tto	ago	age	ctc	476
N	D	L	s	т	N	Y	F	Ρ	А	G	s	R	Ρ	F	v	Y	Q	Е	v	178
aac	gac	ctg	tcc	aco	gaat	tac	ette	cac	gco	cgga	atca	icgo	CCC	ttc	gtt	tac	ccaç	Igaa	gtg	536
I	D	L	G	G	Е	Α	I	т	s	D	E	Y	v	G	Ι	G	R	v	т	198
att	gat	ctc	ggc	gga	agag	ggco	cato	acc	ago	cgat	tgag	gtac	gtg	ggc	ato	gga	icgt	gtg	aca	596
Е	F	ĸ	Y	G	ĸ	F	L	G	Е	Α	F	R	G	s	N	Q	L	к	W	218
gag	Ittc	aag	tat	ggo	aaq	gtto	ccto	lddo	gag	ggci	ttto	agg	ggc	agc	aac	caa	acto	aag	ıtgg	656
г	v	N	F	G	Е	G	W	G	м	м	D	R	G	N	Α	L	v	F	v	238
ctg	gtc	aac	ttc	ggt	gag	gggo	ctgg	lddo	ato	gat	ggao	cgo	ggc	aac	gct	ct	ggto	ttc	gtt	716
D	N	н	D	N	Q	R	G	н	G	А	G	G	D	м	Ι	L	т	F	R	258
gat	aac	cac	gac	aac	ccaq	goga	aggo	cac	ggt	gc	cggo	ggt	gat	atg	ato	cto	jaco	ttc	cgt	776
D	P	ĸ	L	Y	ĸ	м	Α	N	Α	F	м	L	А	W	Р	Y	G	Y	т	278
gac	cct	aaa	ctg	rtac	caaq	gato	adco	aac	gco	ctt	cato	rcto	gcc	tgg	ccc	tat	ggt	tac	acc	836
R	v	м	s	s	Y	Y	W	D	Q	N	W	v	G	G	s	D	Q	N	D	298
cgc	gtc	atg	tcc	tco	tad	ctad	ctgg	gac	caa	aaa	ctgg	gtg	ggt	ggc	tct	gao	ccaa	laat	gac	896
W	I	G	₽	P	н	D	D	R	Y	N	т	L	s	P	т	F	I	Α	D	318
tgg	atc	ggc	ccc	cct	ccad	cgad	cgat	aga	tac	caa	caco	ctt	agc	ccc	aco	tto	ato	gcg	gac	956
G	s	С	G	N	D	W	м	С	Е	н	R	W	R	Q	Ι	Y	I	м	v	338
ggc	agc	tgc	ggc	aac	gad	tg	gato	ıtgt	gag	gca	ccgt	tgg	agg	cag	ato	tac	ato	atg	gtg	1016
Е	F	R	N	v	А	н	G	т	D	м	N	D	W	W	D	N	D	N	N	358
gag	Ittc	aga	aat	gtt	cgct	ccad	cggo	cacg	gao	cat	gaac	gac	tgg	tgg	gao	aac	gac	aac	aac	1076
Q	I	А	F	С	R	G	G	R	G	F	I	Α	Ι	N	N	E	G	Y	v	378
cag	ata	gcc	ttc	tgo	cago	gggt	:ggc	cga	iggo	ctt	cato	gcc	atc	aac	aac	gag	idda	tat	gtc	1136
L	A	Q	N	F	Q	т	С	L	s	Е	G	т	Y	С	D	v	I	s	G	398
cto	gcg	cag	aac	tto	cag	gaco	ctgo	ttg	rtco	ga	gggo	aca	tac	tgc	gao	gto	ato	tcc	ggc	1196
s	L	E	G	; 0	; ;	\	1	G	; P	κ :	s v	г т	v	G	5	: 1	0	; т	A	418
tcc	ctg	gag	ggc	ggg	ggcg	gtgo	cacq	iddo	aaq	gagt	tgtg	Jaco	gtg	ggc	ago	gad	egga	acc	gcc	1256
Y	I	Е	I	Α	т	s	E	D	D	G	v	L	Α	I	н	Α	N	s	ĸ	438
tac	atc	gag	atc	gco	caco	ctco	ggag	gac	gad	cgg	cgto	getg	gcc	atc	cac	geo	caac	tcc	aag	1316
L	*																			439
ctt	<u>taa</u>	aca	tca	aco	cago	ccad	caco	gaa	itca	ata	caga	igca	gag	gag	agg	gtca	aaa	cac	taa	1376
gaa	aaa	саа	aga	laaa	ataa	acct	cgg	Jaag	aaa	agti	tarc	aag	aaa	att	aca	laca	acc	tac	cat	1436
aac	aac	aac	aac	aac	caad	caad	caac	agg	rcca	aaaq	ggaa	iaat	aag	gaa	aac	aaa	acaa	laaa	aaa	1496
gaa	aaa	gaa	aag	gata	aca	agag	gact	ctt	cct	cat	caaa	aca	aag	gag	aga	laaa	itca	cac	ctg	1556
tca	gat	taa	tca	aaa	aggt	gad	gtt	tga	igga	atta	aaaq	jaaa	aga	aaa	aaa	agt	gga	tat	ata	1616
cat	ata	aaa	caa	laaa	ataa	aaaa	a –	Зí												1637

Figure 1

215x279mm (300 x 300 DPI)

N.g.	ranulata		0
M.j.	aponicus	MLRVVPLVVLLAAASLAQWDPNSSNG-QAIVHLFEWKWPDIAAECENFLGPRGFAG	55
$L \cdot V $	annamei	MLRVAPLVVLLAAAAQAQWDPNSSNG-QVIVHLFEWKWSDIAAECENFLGPRGFAG	55
P.a.	rgus	MLRMVVKVALAVTLLAASVLAQWDPHVTNG-QAIVHLFEWRWSDIASECENFLGPRGYGG	59
D.pt	ulex	MKT-IVLLLVAAFVADQATGQWNANYASGRTTMVHLFEWKWDDIAAECERFLGPKGYAG	58
		Υ	
N.g.	ranulata	PWWERYQPVSYNLVTRSGDESAFXDMVSRCXXVXVRIYADI	41
M. J.	aponicus	VQVSPPNEYVEVYQGEVKRPWWERYQPVSYKLVTRSGDENAFKDMVTRCNNVGVKIYVDI	115
L.V.	annameı	VQVSPPNEYVEVYQGDVKRPWWERYQPVSYKLVTRSGDENAFKDMVTRCNNVGVRIYVDA	115
P.a.	rgus	VQVSPPNENVVVYQDEKSRPWWERYQPVSYKIATRSGDEASFKDMVNRCNNAGVRIYVDA	119
D.pt	uiex	VQVSPPNEHIVIMQSTVQRPWWERIQPVSYKLVTRSGDENAFKSMVDRCNAVGVRIYVDC	118
Nor	ranulata	VMMHMTGWOPSXHGGDWWFLVRCXAOSLTXAVPYSAYDFND-GNCNSGSGDTENYGDANO	100
M. 1	anonicus	VMNHMSGGWPOGTGGSGGSSEDSGAO-SYPGVPYSAEDEND-GNCHTGSGNTENYGDANO	173
L.V	apnamei	VINHMSGGWPMGTGASGGSSFDSGAE-SYPGVPYSAFDFND-GNCHTGSGNTENYGDANO	173
P.a.	raus	VINHMSGWWPTGTAATGGSTFDAGAE-SYPAVPYSAFDFND-ANCHTASGTTEDYGDAEO	177
D.0	ulex	VFNOMTPGSGTGIGGSSVN-GGSMSYPAVPYGPNDFTPRSSCPSNSGDIENYGNA00	174
		::*:**** : ** **:*:*:*	
		▼	
N.g:	ranulata	VRNCKLSGLNDLNQGTDYVRGMIRDYMNNLIGYGVAGFRVDASKHMWPGDMQAIFSSLND	160
M.j.	aponicus	VRNCKLVGLNDLNQGTDYVRGKIRDYLNTLIGYGVAGFRIDASKHMWPGDMKAIFDSLNN	233
$L \cdot V $	annamei	VRNCKLVGLNDLNQGTDYVRGKIREFMNKLISYGVAGFRIDASKHMWPGDMKAIFDSLDN	233
P.a:	rgus	VRNCKLVGLNDLNQGTDYVRGKIRDYLNTLTSWGVAGFRVDATKHMWPGDLKAIFDSLDD	237
D.pi	ulex	VRNCKLSGMPDLYQGSEYVRGKILEFLNKLTSYGVAGFRWDASKHMWPGDLKVLSDRLDN	234
		······································	
N		I CT-NYEDA CODEZYOEVIDI CCEA ITODEVUCI CDI TEEVYCKEI CEA EDCONOL KNI	210
M.g.	Lanuiata	LST-NTFFAGSRFFVTQEVTDLGGEATISDETVGIGRVTEFRIGRFLGEAFRGSNQERWE	202
19. Je	aponicus	LNT-DEFKAGARDET FORVIDLGGEAT SGGETVGNGRVIEFRIGKILGEAFRGNNGLGIL	292
P =	raug	LSP-HYFDAGSRPFTFOFVIDLGGEPTTSSOVVCNGRVTEFKYCKSLGESFNGNNPLKWF	296
D 70	uler	LPTAKGEPAGSRPYTYMEVIDOGGEPITANEVFYTGRVTEFKYGRKLSDVFHKKTOLKYL	294
210	a con	* . * **:**:: **** *** *: .*. *****:**: * *.	
N.g.	ranulata	VNFGEGWGMMDRGNALVFVDNHDNQRGHGAGGDMILTFRDPKLYKMANAFMLAWPYGYTR	279
M.j.	aponicus	${\tt KNFGEDWGMMDRANALVFIDNHDNQRGHGAGGDMILTFRVSKWYKMANAFMLGWPYGYTR}$	352
$L.v_{c}$	annamei	${\tt NNFGEGWGMIDRHDALVFIDNHDNQRGHGAGGDMILTFRVSKWYKMATAYMLAWPYGYTR}$	352
P.a:	rgus	STFGESWGMVDRGNAVVFIDNHDNQRTGGDMILTFRSSKWYKMANAYMLAWPYGFPR	353
D.pt	ulex	VNWGVGWGLMPDGNALVFIDNHDNQRGHGGGGDL-LTFRESKLYKMAVSYMLAWPYGDTR	353
		* **:: :*:**:************************	
			220
N.g.	ranulata	VMSS11WDQNWVGGSDQNDWIGFFHDDRINILSF1FIADGSCGNDWMCEHRWRQ111MVE	410
12. Je	aponicus	VMS511WDQWWENGQDKNDWIGFFQDD5FN115F5FNADGICGAGWICERKWKQIINMVE	412
D. 00	annanter	VMS511WDQWWENGQDRADWIGFFHDG5FWIISF5INADG5CGAGWICERRWRQIIWMVE	412
г.а. П. п.	ular	VMSST1WDQWWEAGHDRADWIGPPHDGS1WIVGPIFAFDDSCGAGWICEHRWRQI1MMVQ VMSSYYWDONIANGODONDWIGPPHDSNENTLSPTINADDSCGAGWICEHRWRQI1MMVQ	413
2.0	4267	****	110
		· · · · · · · · · · · · · · · · · · ·	
N.g.	ranulata	FRNVAHGTDMNDWWDNDNNQIAFCRGGRGFIAINNEGYVLAQNFQTCLSEGTYCDVISGS	399
M. j.	aponicus	FRNVAHGTDMNDWWDNGSNQIAFCRGDKGFLAINNDGWDLKETLQTCLPAGTYCDVISGS	472
$L \cdot v_i$	annamei	FRNVAHGTDMNDWWDNGSNQIAFCRGNKGFLAINNDGWDLKETLQTCLPAGTYCDVISGS	472
P.a.	rgus	FRNVAHGTDMNDWWDNGSNQISFCRGDRGFIAINNDPWDMKESRQTCLSSGTYCDVISGS	473
D.pt	ulex	${\tt FRNIVAGTTMNDWWDNGNNQIAFCRGGKGFIAINNEGSNMSQTLQTCLSAGTYCDIISGN}$	473
		:. ** **** .***:**** :**:****: : :. **** ****:***.	
N ~~	rann1a+-	LEGGACTCKSVTVGSDGTAVIELATSEDDGVLATHANSKI, 439	
M. 1	aponicus	KEGGSCTGKSVTVGGDGKAYIEITTMEDDGVLAIHANSKL 512	
	annamei	KDGGSCTGKSVTVGGDGKAYIETTTMEDDGVLATHANSKL 512	
P. A	raus	KIOGSCTGKTVTVNADGFAYIEILNSEEDGVLAIHRDSKL 513	
D.0	ulex	LVNGOCTGKSVTVGSDGKALISIGNAEDDGVLAIHVESKL 513	
[01		* ****:*** .** * *.* . *:****** :***	

Figure 2

209x297mm (300 x 300 DPI)



	_	\sim		_		_			_					\sim	_	
	F	C	G	A	s	I	Y	s	Е	N	W	Α	v	C	A	15
5	-tto	etge	dda	cgco	ctco	cato	ctac	cago	cgaq	yaac	ctgg	Idco	cgt	gtgt	gcc	45
G	н	C	v	Q	G	E	D	F	Е	N	P	D	Y	г	Q	31
ggt	cad	etge	gto	ccaç	ldds	igaç	ggad	etto	cgao	yaac	ccct	gao	cta	cctt	cag	93
v	v	Α	G	E	Q	D	г	D	v	v	Е	G	N	E	Q	47
gtt	gtt	gct	ggo	gag	caa	gat	ttg	gac	gtt	gtt	gag	ggt	aat	gag	cag	141
Е	v	v	L	s	ĸ	I	I	Q	н	Е	D	Y	N	G	F	63
gag	gtt	gtt	ctg	itco	aag	ato	atc	cag	JCad	gaa	igat	tac	caad	cggg	ttc	189
т	I	s	N	D	I	s	L	L	Q	L	s	s	Ρ	L	т	79
acc	ato	agc	aac	gac	att	tcc	ctt	cto	caa	ictg	itcc	tcc	cct	ctg	acc	237
F	N	т	N	v	G	s	I	G	L	Q	т	v	ĸ	Е	Y	95
ttc	aac	cacc	aac	gtt	gga	tct	atc	ggt	cto	ICag	act	gta	aac	ggag	tac	285
I	G	D	0	v	v	s	G	W	G	т	L	т	Е	D	G	111
atc	gqa	igac	tgo	gtt	gto	tcc	adc	tqq	laad	aca	ctc	acq	Iqaa	agat	ddc	333
s	T	P	s	Ī	Ľ	0	Ŷ	v	D	v	P	т	v	s	D	127
age	act	ccc	tct	atc	ctt	cac	Itat	gto	gat	gtc	ccc	act	gto	agt	gac	381
Ā	Е	C	R	D	A	Y	G	Q	้ท	D	I	D	D	s	м	143
gct	gaa	itqt	cqc	gac	get	tac	gga	icaa	aad	gac	atc	gat	gad	ctcc	atg	429
Ī	(C)	A	G	Ľ	P	Е	G	G	v	D	А	(C)	Q	G	D	159
atc	tac	act	aac	cta	cee	aac	iada	idda	ato	raac	acc	tac	cad	aat	gac	477
S	Ğ	G	P	L	А	6	G	G	Ľ	L	т	Ğ	I	v	s	175
tct	aat	aaa	cct	cto	aco	rtat	aac	aac	ot	cta:	race	aac	ato	ata	tcc	525
W	é	Ŷ	G	ര്	ัล	Ŕ	P	Ğ	Y	P	G	v	Y	Ť	Е	191
taa	aac	tat	aac	tat	acc	cac	cct	ada	tac	acc	aac	ato	tac	act	dad	573
v	Δ 2	Y	- ७७ च	- с. 9 с Т	D	w	v	E.	Δ	N	Δ	s	*		949	204
at a		- +	++-	-		+ 00	at c	-	act		act	+ ~ =	+ = =	att	ac 2	621
+++	tat	tat	ata	t acy	gau	at o	tac	.yaa .taa	get	adu	.get	+ ==	and	acc	gea	660
			CCC art	, Lgg		y Ly	24	, LgC	cau	jaca		Ldo	aCo	icgg	cac	600
aca	ogo	jude	agt	LCT	cat	CE-										083

215x279mm (300 x 300 DPI)

	к	I	I	Q	н	Е	Q	Y	N	G	F	т	L	s	N	15
5'- c	caaa	atc	ato	cag	cac	gaa	саа	ata	caac	add	ittc	aco	ccto	cago	caac	47
DI	S	L	L	Q	L	s	Q	P	L	т	F	N	т	F	v	32
gatat	ttcc	ctt	cto	caa	ctg	rtct	cac	geet	tetg	racc	ttc	aac	caco	tto	gtt	98
GS	I	D	г	Q	s	Е	к	E	Y.	s	G	Е	୍ତ	т	v	49
ggatct	atc	gat	ctg	caga	agto	gaai	aag	gag	tac	agt	gga	gaa	tgc	act	gtg	149
IG	W	G	T	т	Е	E	G	G	Е	т	P	S	v	<u>ь</u>	Q	66
ategge	:tgg	ggca	act.	acco	jago	gago	ada	ggc	gaa		CCCI	CCT.	gtc	CUU	cag	200
E V	D	ц п. п. п. п.	P	A	v	T		A	E	G	R	E	v	¥	6	051
E E E	igati W	ELCI T	000	ger	JUC	acto T	A	gci N	gag	w	geo	Jaa N	guu	C	yga V	100
	v at a	н ~~~~	ара.		nt au	- - +	U.	A aat	aaa	v at a:		n			at a	302
yaayaa	i <u>na</u>		gac c		a c ga	acc c	G	P	ууа 1 т.	y ca r	้ด	geg G	gga c	yya T.	T.	117
gacgee	tac	caa	aat	ract	tete	aate	rga	cct	cta.	- a.c.o.	e	unc.	aac	ctc	cta	353
T G	I	v	8	W	e	Y	G	õ	A	R	P	D	s	P	G	134
accord	atc	ata:	tcc	taa	gqti	- tato	adc	tqt	dcc.	cqc	- cct	gac	age	ccc	aac	404
vŸ	A	E	v	A	Ŷ	F	т	Ď	ัพ	v	E	A	Ň	v	S	151
gtgtac	gcc	gag	gtg	gaat	tact	ttca	acg	gac	tgg	gtc	gaag	gcc	aat	gtc	tcg	455
*																
<u>taa</u> atg	jcca	ttt	tcc	tct	ctg	gct	ggt	gtg	cag	tca	gaca	ac <u>a</u>	ata	<u>aa</u> c	aag	506
tcacag	Jaga	act	ttg	ctt	tca	gtc	ttt	cta	gtt	gta	atg	gtg	ttt	cga	cat	557
gtatag	gtcc	aga	ccg	gtg	ccaa	aaa	tca	aat	tat	acgi	agti	tat	aaa	tat	tga	608
gtacgt	taa	gac	gac	ggg	gtaa	atco	gtg	tgt	ttt	aag	ggaa	agc	gcc.	tgg	ggt	659
tgattt	ttt	aac	gaa	tati	ttc	tta	aat	сса	cga	aati	atgi	ttt	ttc	tcg	ctc	710
aacatg	gtcg	aggi	ata	ccat	tgaa	aati	atc	agg	tag	tga	agti	ttt	ttt	ttt	cta	761
gaattt	aaa	gaat	tgg	cgt	ccci	ttti	aag	aca	ttt	acti	aaco	cac	taa	agc	tct	812
ggcgtt	aaa	act	ctc	ttc	caat	taa	gta	gcc	act	gati	ata	taa	ctt	ttc	gta	863
tacato	caaa	ttc	tct	gaca	acaa	aagi	act	cac	gca	aaca	acgo	cac	gaa	caa	tac	914
aacgac	act	caca	acc	aaca	acta	aca	aaa	cac	aca	cac	aca	cac	aca	cac	aca	965
cacaco	ggt	atca	agt	atca	agt	cag	gtg	agg	tgt	ggt	gtg	gca	ggt	cac	ctt	1016
cctctt	aat	taa	caa	ctca	acci	tga	act	gtc	tca	taa	cat	cac	caa	atc.	aat	1067
<u>aaa</u> gto	aat	acci	tgt	tcta	att	tag	agg	cgc	cca	aaa	caca	att	tct	aag	cgt	1118
tgccga	igto	cga	ttt	ccaa	aaca	acg	atc	ata	aac	aaa	cace	gtg	tct	att	aac	1169
gtaaa-	- 3															1174

Figure 4

215x279mm (300 x 300 DPI)

•	T
N.granulata1	FCG
N.granulata2	
E.sinensisl	DISFGFQFHFCG
E.sinensis2	DISFSSPFHFCG
E.sinensis3	DISFGFQFHFCG
S.paramamosain	MKTLVLCLLVAGALAAPSRKPTFRRGLNRIVGGEDTVHGEFQYQLSLQDTSYSNPWHFCG
P.trituberculatus1	DISFGFAFHFCG
P.trituberculatus2	DVSFGFAFHFCG
P.trituberculatus3	DISFGFAFHFCG
C.japonical	DTSYGYKFHFCG
C.japonica2	DVSYGYKFHFCG
C. Japonicas R pologique	MODILIU CLIVECTI ANDERVETERRCINETICORDELICORDELICORDELICORDESCENTRO
P.camtschaticus	MKIDVICLLVIGALAAFSAKFIFKAGLAKIVGGEDILMGEIQIQLSIQDISIIEFWHFCG MKYLVFCLLLGAAFAAPSRKPTFRRGLNKIVGGTEVTPGEIFYQLSFQDTSFGGEFHFCG
	V V
N.granulatal	ASIYSENWAVCAGHCVQGEDFENPDYLQVVAGEQDLDVVEGNEQEVVLSKIIQHE-DYNG
N.granulata2	
E.SINCASISI B.SINCASISI	ASTINENWGVCAGHCVQGEDFNNPDTLQVVAGEQDLDVDEGNEQATVLSKTTQHE-DING
E.SINCHSIS2	ASTINKNWGVCAGHCVQGDDFNNPDTLQVVAGEQELTVDEGNEQATVLSKTIQHE-DING
5.DINCHDIDS	ASTINENWOVCACHCVQCEDINNPDIEQVVACEQDEDVDECNEQALVESKIIQHE-DING
D tritubarculaturi	STRUMENTACHCOCEDNNNDDVIONIACEUNDDVDECNEOTVILEVIIONE DVNC
P trituberculatur?	AST TREMENT CACHCYOCEDMINIED I DOVY COMPARING VIESNI VIESNI CHE DING
P trituberculatur?	AST VSENNOVCECHNOCENNNDDDVI ODIACENNDDVJECNEOVUT CVTTOVE-EVNO
C.isponical	ASTYSENWUCAGHWUCGENMYSODVLOUVAGDHTLYUNDGYEOXUTLSKTTONETDYNS
C. japonica2	ASTYSENWU/CAGHWVOGENMYSODVLOVVAGDHTLYVNDGYEOXVTLSKTTOHF-FVNC
C iaponica3	AST VNENWAT CAGHCVOGDDEDNPNVLOVVAGEHNEDVNEGNEOTVVLSKTTOPE-DVNG
P.nelagicus	GTLYNDHWGITACHCLOY-DVANPGTVOAVAGEYYLKVNDGSEOFRKIDETTIND-NEDS
P.camtschaticus	ASIYKDTWAICAGHCVOGEDFDSPASLOIVAGDHTLYSAEGNEOKIAVSKITOHE-DYNG
	V :** * ::
N.granulata1	FTUSNIISLLQLSSPLTFNTNVGSICLQTVK-EYIGDCVVSGWGTUTEDGSTPSILQVVD
N.granulata2	FTWSNUTSILQLS@PLTFNTWVGSTULQS#K-EYBGBCWVGWGTWDEGGUTPSWLQWVD
E.SINENSISI B. sissesis 2	FTISNDISLLQLSSPLTFNSFVGPVGLQSVK-EYIGDCVVSGWGTTSEGGSTPSVLQYVD
E.Sinensis2	FIISNDISLEVESFEIFNSFVGFVGEVGEVEFEIMGDCVVSGWGTISEGGSSFSVEVIVD
C.Sinensiss	FIISNBISELUESFEIFNSFVGSVGEUSVA-EIIGDCVVSGWGIISEGGSIFSVEUIVD
D tritubereuletuel	STEWETRI I VI SODI FENNEVODI NI JENONDACOCIVISWOALSEGGSAATVLQAVA
P.trituberculatus1	FTISNDISLLKESQPETENNFVGPTALPEAGHAASGDCIVSGWGALSEGGSSPSVLQXVS
P trituberculatue?	FTISNETSILKI SODI FENNEVGETAL PEAGNAASGDCTVSGWGTISEGGSTSVIOKVS
C isponical	FTIGNUSLIGI SESLEFINDVALDI OSEK_EFI GEOV/SCHOPTEEGISEDNI VV/D
C japonica?	FTLCNDVSLLOUSESLTEDNDVKATDLOSEK-EFLCECVVSCHCTTTEECDSDDNLVVVD
C isponice?	FTISNDISLIVESODI SENDVUDAT DI DAOGNA SCRUTUSCHOTTISEGOSTDSVI OVUT
P nelagique	GLI INDVALTNEPEAMIYDEVUNDIGI OFEKNI VGVDCTVTGVGALSEGGNAASVI OXVN
P.camtschaticus	FSISNDISLLQFASPLTFNSFVGPIALPAQGQVASGDCTCTGWGTTTEGGYSSDALLKVT
	Î
N.granulata1	WPT-VSDAECRDAYGONDIDDSMICAGIRDGGVDACOGDSGGELBCGGLLTGIVSW
N.granulata2	FW-VIDAECKEWYGD-DWEDSMICAGWSMGGVDACQGDSGGELMCGGLLTGIVSW
E.SINENSISI	VPT-V5DADCRAATGENDIDDSMICAGIPEGGVDACQGDSGGHMVCGGLLT
E.sinensis2	VPT-VSDADCRAAYGESDIDDSMICAGLPEGGVDACQGDSGGFTVCGGLLT
E.sinensis3	VPT-VSDADCRAAYGENDIDDSMICAGLPEGGVDACQGDSGG9MVCGGLLT
s paramamosain	VPT-VSDEECKISYYGIEDSMICAGYPEGGKDACQGDSGGHMVCKGYLTGIVSW
F.trituberculatus1	VPI-VSDAECRDAYGQNDIDDSMICAGVPEGGKDSCQGDSGGHLACSDTGSPYLA
P.trituberculatus2	VPI-VSDEECRAAYGDSEVEDSMICAGVPEGGK D SCQGDSGGFLACSDTGSTYLA
r.trituperculatus3	VPI-VIDAECCKHIGENQIDDSMICVGIPEGGKDACQGDSGGHLACSDTGSPYLA
c.japonical C.japonical	LETV-TUTECRMHIGENQIDDSMICAGLEEGGKDACQGDSGGHLACSDTGSTYLA
c.japonicaz C.jemenice2	DET VEDRECKHIGENGEDEREKEGENDECKERGEREKEREKERE
c.japonicas D.m.l.m.	VFI-VSDDECRDAIQSDIEDSRICAGVPEGGRDSCOGDSCGEBGACSDIGSTYLA
r.peiagicus D. semtesbetisu:	VFI-VSDEECRISIS-GIEDSMICAGIFEGGKDACQGDSGGHNVCKGYDIGIVSW
r,cantSchatzeuS	:* 1:* * ::******* ** *:***************
N.granulata1	
N.granulata2	GYGCARPBSPGVYMEVAYFTDWVEANNS 151
E.sinensis1	180
E.sinensis2	100
E.sinensis3	180
S.paramamosain	GYGCARPNYPGVYTEVAYFVDWIIANAV 259
P.trituberculatus1	185
P.trituberculatus2	185
P.trituberculatus3	185
C.japonical	185
C.japonica2	185
C.japonica3	185
P.pelagicus	GYGCARPNYPGVYTEVAYFVDWITANTV 259
P. camtachaticus	GYGCARPNYPGVYCEVAYYVDWULANSS 266

Figure 5

209x296mm (300 x 300 DPI)

		т	A	E	R	Е	I	v	R	D	I	ĸ	Е	K	L	С	Y	v	А	18
5'- ccacagctgagagggaaatcgtgcgcgatatcaaggagaagctttgctatgtcgct														56						
L	D	F	Е	s	Е	м	N	v	А	А	А	s	s	s	L	Е	к	s	Y	38
cttgacttcgaaagtgagatgaacgtggctgctgctcctcctccttgagaagtcctac													116							
Е	L	Ρ	D	G	Q	v	I	т	I	G	N	Е	R	F	R	А	Ρ	Е	S	58
gagetgeecgatggteaggteateaceateggeaaegagegetteegtgeaeeagaatet													176							
L	F	Q	Ρ	s	F	L	G	М	Е	s	v	G	I	н	Е	т	v	Y	N	78
ctattccagccttccttcttgggtatggaatctgttggcattcacgagaccgtctacaac													236							
s	I	М	R	С	D	I	D	I	R	к	D	L	F	А	N	N	v	М	s	98
tccataatgagatgcgacattgacatcaggaaggacctgtttgcaaacaacgtaatgtct													296							
G	G	т	т	М	Y	Ρ	G	I	А	*										108
ggcggcaccaccatgtaccctggcatcgca <mark><i>tga</i>ccggatctgcggaaaaattg-3`</mark>											349									

Figure 6

215x279mm (300 x 300 DPI)









0.2

Figure 8 48x65mm (300 x 300 DPI)

Can. J. Zool. Downloaded from www.nrcresearchpress.com by UNIVERSITY OF CONNECTICUT on 09/01/17 For personal use only. This Just-IN manuscript is the accepted manuscript prior to copy editing and page composition. It may differ from the final official version of record.









Figure 10 156x284mm (300 x 300 DPI)