Trypsin isozymes in the lobster *Panulirus argus* (Latreille, 1804): from molecules to physiology

E. Perera^{1,4*†}, L. Rodríguez-Viera¹, R. Perdomo-Morales², V. Montero-Alejo², F.J. Moyano³, G. Martínez-Rodríguez⁴, J.M. Mancera⁵

¹ Center for Marine Research, University of Havana, Havana, Cuba

² Biochemistry Department, Center for Pharmaceuticals Research and Development, Havana, Cuba

³ Department of Applied Biology, University of Almeria, Almeria, Spain

⁴ Instituto de Ciencias Marinas de Andalucía (ICMAN), CSIC, Cádiz, Spain

⁵ Department of Biology, Faculty of Marine and Environmental Science, University of Cadiz, Spain

* Corresponding author, [†] Current address: Instituto de Ciencias Marinas de Andalucía (ICMAN), CSIC, Apartado Oficial, 11510-Puerto Real, Cádiz, Spain. Tel. +34 956 832612 ext. 334, E-mail address: erick.perera@icman.csic.es; erickpbcim@yahoo.es (E. Perera).

Running title: Trypsins in lobster: from molecules to physiology

Abstract

Trypsin enzymes have been studied in a wide variety of animal taxa due to their central role in protein digestion as well as in other important physiological and biotechnological processes. Crustacean trypsins exhibit a high number of isoforms. However, while differences in properties of isoenzymes are known to play important roles in regulating different physiological processes, there is little information on this aspect for decapod trypsins. The aim of this review is to integrate recent findings at the molecular level on trypsins enzymes of the spiny lobster *Panulirus argus*, into higher levels of organization (biochemical, organism) and to interpret those findings in relation to the feeding ecology of these crustaceans. Trypsin in lobster is a polymorphic enzyme, showing isoforms that differ in their biochemical features and catalytic efficiencies. Molecular studies suggest that polymorphism in lobster trypsins may be non-neutral. Trypsin isoenzymes are differentially regulated by dietary proteins, and it seems that some isoenzymes have undergone adaptive evolution coupled with a divergence in expression rate to increase fitness. This review highlights important but poorly studied issues in crustaceans in general, such as the relation among trypsin polymorphism, phenotypic (digestive) flexibility, digestion efficiency, and feeding ecology.

Keywords: digestion biochemistry; ecological physiology; *Panulirus argus*; protein digestion; trypsin polymorphism; spiny lobster

1. Introduction

Trypsin and trypsin-like enzymes have been the subjects of a huge amount of studies due to their central role in protein digestion as well as in other important physiological (e.g. blood coagulation, fibrinolysis, development, fertilization, apoptosis and immunity, [Page and Di Cera 2008](#)), and biotechnological processes ([Anheller et al. 1989](#); [Nyberg et al. 2006](#); [Shahidi and Kamil 2001](#); [Bougatef 2013](#)). Historically, the most studied trypsin is the bovine trypsin since it was purified by crystallization at the beginnings of the 1930s ([Northrup and Kunitz 1931](#)), and then characterized ([Northrup et al. 1948](#)) and sequenced ([Walsh et al. 1964](#); [Walsh and Wilcox 1970](#)). The three-dimensional structure of the bovine trypsin was resolved in the 1970s by X-ray crystallography ([Huber et al. 1974](#); [Sweet et al. 1974](#); [Kossiakoff et al. 1977](#)), and further research during the last decades has expanded the knowledge of this enzyme in terms of catalysis, structure-function relationship, evolution, and regulatory mechanisms ([Perona and Craik 1995](#); [Hedstrom 1996](#); [Thimister et al. 1996](#); [Page and Di Cera 2008](#)). In addition, trypsins and trypsin-like serine peptidases have been studied in a wide variety of other animal taxa ([Borovsky 2003](#); [Srinivasan et al. 2006](#); [Rascón et al. 2011](#)), including several crustaceans ([Muhlia-Almazán et al. 2008](#); [Carrillo et al. 2008](#); [Sainz and Córdova-Murueta 2009](#)). Trypsins are the main digestive endopeptidases in most crustaceans, although in some species other proteinases are predominantly expressed. Caridean shrimps such as *Crangon crangon* and *Crangon allmani* showed extremely variable levels of tryptic activity, with some shrimp showing very low or none tryptic activities, while cysteine proteinases such as cathepsin L-like enzymes dominates in their midgut gland ([Teschke and Saborowski 2005](#)).

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) grouped in 1992 the serine peptidases of crustaceans under the term brachyurins (EC 3.4.21.32) ([Tsu and Craik 1998](#); [Barret et al. 1998](#)) that include three different types: Ia, Ib and II. Type Ia brachyurins exhibit wide specificity, including trypsin, chymotrypsin and elastase activity, and they are known as crustacean collagenolytic serine-proteases. Type Ib brachyurins share the specificity of type Ia, but with a drastic reduction of the trypsin activity, being known as crustacean chymotrypsins. Type II brachyurins are enzymes with strict trypsin specificity, they are referred as crustacean trypsins and, as all other trypsins, they belong to the clan PA, family S1 (MEROPS database: [URL: http://www.merops.co.uk](http://www.merops.co.uk)). Trypsins have a catalytic triad composed by the residues His57, Asp102 and Ser195 (chymotrypsin nomenclature) and cleave peptide bounds at the carboxylic side of the residues Arg and Lys in P1 position of proteinaseous substrates ([Stryer 1988](#)).

The remarkable distinct features of crustacean collagenolytic serine proteases have prompted several studies, and the structural bases of their wide specificity are now relatively well known (e.g. large groove, multiple binding sites in S1 pocket for different amino acid side chains) (Perona et al. 1997; Tsu et al. 1997). Conversely, up until now, the X-ray structure of crustacean trypsins has been revealed just in the crayfish *Astacus leptodactylus* (Fodor et al. 2005), although Hehemann et al. (2008) and Perera et al. (2010b) modeled the three-dimensional structure of trypsins from the crab *Cancer pagurus* and the spiny lobster *Panulirus argus*, respectively. Nevertheless, the available information on the structure of the different crustacean trypsins (Fodor et al. 2005; Hehemann et al. 2008; Perera et al. 2010b) point to these enzymes sharing the structural elements required for trypsin specificity with their vertebrate counterparts (Perona and Craik 1995; Hedstrom 1996).

Crustaceans (and invertebrates in general) present several trypsin isoenzymes (Muhlía-Almazán et al. 2008), as also occurs in fish (Rungruangsak-Torrissen 2012). Conversely, the number of trypsin isoenzymes in mammals is reduced: only two trypsins (one cationic and one anionic form) have been reported in humans (Figarella et al. 1975), bovines (Puigserver and Desnuelle 1971), pigs (Voytek and Gjessing 1971) and dogs (Ohlsson and Tegner 1973). The lower number of isoforms seems to be correlated to an increase in the complexity within the family of serine-peptidases, with several genes acquiring additional domains other than the trypsin domain (Patthy 1999).

Genes coding for trypsin enzymes in crustaceans are arranged into multi-gene families (Klein et al. 1998), leading to several isoforms (Brockerhoff et al. 1970; Galgani and Nagayama 1987; Kim et al. 1992; Klein et al. 1996; Sainz et al. 2004, 2005; Perera et al. 2008a). According to the combination of isoforms in individuals, different trypsin phenotypes have been described in the penaeid shrimp *Litopenaeus vannamei* (Sainz et al. 2004, 2005) and in the spiny lobster *P. argus* (Perera et al. 2010a). However, to our knowledge, there is no information about the digestive role of trypsin isoforms in decapods, although there is evidence of digestive advantage of certain phenotypes in other crustaceans (*Daphnia*, Schwarzenberger et al. 2010). However, the physiological impacts of trypsin polymorphism have been found on fish size (Torrissen 1987), growth rate (Torrissen 1991; Torrissen et al. 1993), protein utilization (Torrissen et al. 1994) and feed efficiency (Torrissen and Shearer 1992; Rungruangsak-Torrissen et al. 1998), as reviewed in Rungruangsak-Torrissen and Male (2000) and Rungruangsak-Torrissen (2012). Examples of the physiological impact of enzyme polymorphism exist for other digestive enzymes such as amylase (*Drosophila*, Kirpichnikov and Muske 1980; *Crassostrea gigas*, Prudence et al. 2006; chickens, Hughes et al. 1994).

Two fundamental questions in physiology and evolutionary biology are how genetic differences produce different physiological performances and how fitness is achieved. According to Nelson and Hedgecock (1980), heterozygosity in decapod crustaceans is triggered by a broad food spectrum, thus coining the term ‘adaptive polymorphism’. The extremely high heterogeneity of digestive serine-endopeptidases found in the opportunistic predator caridean shrimp *Crangon crangon* is thought to be an adaptation to the variability of food (Saborowski et al. 2012), although the underlying mechanisms are still not completely understood. During the last years some reviews have been published on crustacean trypsins (Muhlía-Almazán et al. 2008; Carrillo et al. 2008; Sainz and Córdova-Murueta 2009), integrating essential information on their molecular and biochemical properties. However, due to the lack of experimental evidence, some important issues such as the adaptive value of polymorphisms and the regulatory mechanisms behind protein digestion have been poorly assessed in crustaceans. In this review, we use the spiny lobster *P. argus* as a model organism for a deeper advance in the understanding of trypsin polymorphism in crustaceans, by integrating updated molecular, biochemical and ecological information. The present review provides insights into the biochemical basis of the ecological success of *P. argus* as a top predator in benthic communities of tropical seas (Marx and Herrnkind 1985; Colinas-Sanchez and Briones-Foorzan 1990; Lalana and Ortiz 1991; Herrera et al. 1991; Cox et al. 1997).

2. First approaches: studies on crude extracts

The first attempt to characterize trypsin-like enzymes in *Panulirus argus* was performed by a combination of biochemical assays and casein zymograms performed on crude extracts of the digestive gland and gastric juice of this species (Perera et al. 2008a). The optimal pH for trypsin activity was found to be around 7.0, while the activity remained high over a broad alkaline pH range. The optimal temperature was 60°C, and the optimal ionic strength was between 100-200 mM NaCl. Enzymes responsible for tryptic activity were stable up to 55°C for at least 1 h, although activities were notably affected at 60°C, and were resistant to moderate acid conditions (i.e. pH 5-6). Similar results have been obtained in other spiny lobster species (*Panulirus interruptus*, Celis-Guerrero et al. 2004). Lobster trypsins were found to be relatively resistant to autolysis (Perera et al. 2008a, 2010b), as those of shrimp (Sainz and Córdova-Murueta 2009) and crabs (Hehemann et al. 2008).

Trypsin activity dominates over all other proteinase activities in the digestive gland and the gastric juice of *P. argus* (Perera et al. 2008a), which is in agreement with previous reports in

other spiny lobster species (*Panulirus japonicus*, [Galgani and Nagayama 1987](#); *Jasus edwardsii*, [Johnston 2003](#); *Panulirus interruptus*, [Celis-Guerrero et al. 2004](#)). Also, as described in other crustaceans ([Sainz et al. 2004](#); [Klein et al. 1996](#)) and lobster species ([Celis-Guerrero et al. 2004](#)), high trypsin activity in *P. argus* occurs associated with several isoforms of the enzyme ([Perera et al. 2008a](#)). Among the five isoforms found in casein zymograms, the three faster bands were the most active ([Perera et al. 2008a](#)). In a further study, three different trypsin phenotypes were defined according to the presence of these three main isoenzymes in individual lobsters ([Fig. 1](#)) ([Perera et al. 2010a](#)). Specimens with the three main isoenzymes were named phenotype A, individuals lacking the isoenzyme of higher electrophoretic mobility were named phenotype B, while lobsters lacking the isoenzyme of middle electrophoretic mobility were named phenotype C ([Fig. 1](#)). The trypsin isoform of minor electrophoretic mobility was present in all lobsters analyzed so far in our laboratory. The studied lobster specimens exhibited the following phenotype frequency: A (41.2%) > B (35.3%) > C (23.5%). Using the same method (substrate-SDS-PAGE), similar trypsin phenotypes have been described in the shrimp *Litopenaeus vannamei* ([Sainz et al. 2004, 2005](#); [Sainz and Córdova-Murueta 2009](#)). The separation of trypsin isoenzymes from Atlantic salmon by a more resolutive technique, isoelectric focusing on agarose-IEF gel, allowed the identification of a higher number of phenotypes. Three common isoenzymes (TRP-1*100, TRP-2*100, and TRP-3) were also identified, but with two variants in the TRP-1 system (TRP-1*75, TRP-1*91) and one variant in the TRP-2 system (TRP-2*92) ([Torrissen 1987](#); [Rungruangsak-Torrissen and Male 2000](#); [Rungruangsak-Torrissen 2012](#)). The application of isoelectric focusing to lobster or shrimp enzymes would identify more isoenzymes and resultant phenotypes.

Trypsin phenotype is not affected by dietary treatment, molt cycle, or time, either in shrimp ([Sainz et al. 2005](#)) or in the lobster ([Perera et al. 2008b](#)), indicating that the regulation of trypsin activity occurs quantitatively by changing the concentration of the different isoforms. It has been postulated that the three main isotrypsins described in shrimp depend on two loci: one homozygous locus yields one isoenzyme and one heterozygous locus yields the other two isoenzymes ([Sainz et al. 2005](#)).

3. Biochemical features of digestive trypsins

Biochemical characterization of *Panulirus argus* trypsins was accomplished after purification of the five isoenzymes including the three main isoforms ([Perera et al. 2012a](#)). The molecular masses of lobster trypsins range from 35 to 36 kDa, being similar to those reported for other crustaceans. All isolated enzymes have restricted trypsin activity (type II brachyurins, [Barret et](#)

al. 1998), and show the distinctive trypsin preference for Arg over Lys in the P1 position. As anticipated by studies on crude extracts of the digestive gland and gastric juice of *P. argus*, optimal pH for most isoforms ranges from 7 to 8, although one trypsin exhibits maximum activity at pH 6-6.5, and this same isoenzyme has double the activity of the other isoforms at pH 5 (Perera et al. 2012a). Additionally, all lobster trypsins require calcium coordination for maximal activity, and most of them need this ion for stability in acidic media (Perera et al. 2012a). Interestingly, one of the isoenzymes appears to be fairly resistant to relatively low pH values, irrespective of the presence of calcium. In general, kinetic constants obtained for trypsins in *P. argus* compare well with those reported for other trypsins. While *P. argus* trypsins are less efficient than those present in fish (e.g. *Engraulis japonicus*, Ahsan and Watabe 2001; *Oncorhynchus keta*, Toyota et al. 2007; *Oreochromis niloticus*, Unajak et al. 2012) and cold-adapted crustaceans (e.g. *Thenus orientalis*, Johnston et al. 1995; *Euphausia pacifica*, Wu et al. 2008), they are more efficient than the bovine trypsin (Rascón et al. 2011). All *P. argus* trypsins have similar K_m values (0.08-0.19 mmol L⁻¹) and most of them present similar turnover numbers (k_{cat} : 0.57-1.15 s⁻¹), leading to similar catalytic efficiencies (k_{cat}/K_m : 4.82-6.04 s⁻¹ mmol L⁻¹). However, the trypsin isoenzyme of middle electrophoretic mobility has a low k_{cat} (0.16 s⁻¹), resulting in the lowest catalytic efficiency (2.02 s⁻¹ mmol L⁻¹) among isoenzymes (Perera et al. 2012a).

However, what does all this information could mean? While *P. argus* trypsins share most of the general features of the clan PA, family S1A, including optimal conditions for activity, calcium requirement, effect of inhibitors, and kinetic constants, they slightly differ in some important functional features including catalytic efficiency (Perera et al. 2012a), which likely lead to diverse digestive performances in the living organism. It has been suggested previously in the penaeid shrimp *Litopenaeus vannamei* that differences in catalytic properties among trypsin isoforms could provide digestive advantages to individuals carrying certain phenotypes (Sainz et al. 2004; Sainz and Córdova-Murueta 2009). Recently, we have provided experimental evidence of advantages in the digestion related to the presence of some isoforms in *P. argus*. Efficiency in protein digestion is greater in individuals having all three major isoenzymes, while it varies in individuals with other phenotypes depending on the protein substrate (Perera et al. 2010a, 2012a).

Variations in protein digestion related to biochemical properties of the different isoforms were confirmed after purification of *P. argus* isotrypsins, since the isoenzyme of the lowest catalytic efficiency is mainly present in less efficient (according to *in vitro* digestion studies) individuals (Perera et al. 2012a), although the impact of these variations on growth performance still needs

to be tested. There are few but consistent examples of advantages related to the presence of some digestive isoenzymes in other organisms, such as amylase in *Drosophila* (Kirpichnikov and Muske 1980), *Crassostrea gigas* (Prudence et al. 2006) and chickens (Hughes et al. 1994), or trypsins in *Salmo salar* (Rungruangsak-Torrissen and Male 2000; Rungruangsak-Torrissen 2012) and *Daphnia* (Schwarzenberger et al. 2010), which in most cases are beneficial rewards for growth to individuals with certain genotypes. The best studied case is Atlantic salmon, in which increased growth rate in fish possessing the trypsin isoform TRP-2*92 (Torrissen 1987, 1991) is due to better food conversion and protein efficiency (Rungruangsak-Torrissen et al. 1998). While initial attempt to explain these differences in fish by protein *in vivo* digestibility studies failed probably due to poor sensitivity of the chromic oxide method (Torrissen and Shearer 1992), a further study using a more sensitive *in vitro* method demonstrated differences in digestion ability among isoenzymes (Bassompierre et al. 1998). However, there are indications that major effect of the variant TRP-2*92 is postabsorptive promoted by higher and faster postprandial free amino acid in plasma which stimulate higher secretion of plasma insulin leading to stimulation of protein synthesis in white muscle (Rungruangsak-Torrissen 2012).

It is known that gene duplication followed by sequence divergence provided a mechanism for the evolution of isoenzymes and eventually new functional properties (Taylor and Raes 2004). Although the structural features of *P. argus* isotrypsins responsible for their functional variation remain largely unknown, recent molecular studies on *P. argus* trypsins have provided insights on the enzyme regions that may account for this variation (Perera et al. 2012a; Perera et al. 2010b).

4. Molecular characterization of digestive trypsins

Several transcripts of 870-875 bp of total length and 798-802 bp of open reading frame for anionic trypsins were identified in the digestive gland of *Panulirus argus* (*PaTry1a*, *PaTry1b*, *PaTry2*, *PaTry3* and *PaTry4* with GenBank accession numbers GU338026, GU338027, GU338028, GU338029 and GU338030 respectively) (Perera et al. 2010b). According to their nucleotide variation, these transcripts were suggested to belong to three different gene families (Perera et al. 2010b), as in the shrimp *Litopenaeus vannamei* (Klein et al. 1996), although definitive genomic studies are needed in *P. argus*. The predicted proteins for these transcripts have the signal and activation peptides well conserved among crustacean trypsins, with the exception of the parasite copepod *Lepeophtheirus salmonis* (Johnson et al. 2002), indicating that there are few differences in the secretion and activation mechanisms of these enzymes. The residues of the catalytic triad His74, Asp125 and Ser218 (equivalent to His57, Asp102 and Ser

195 in chymotrypsin nomenclature) are conserved across all studied *P. argus* trypsins, as well as residues conferring trypsin specificity (Perera et al. 2010b), which indicates these are active enzymes.

Three-dimensional (3D) homology models were obtained for all *P. argus* deduced trypsins. The model data for *PaTry1a*, *PaTry1b*, *PaTry2*, *PaTry3* and *PaTry4* are available in the PMDB database under the accession numbers PM0076235, PM0076234, PM0076233, PM0076232 and PM0076231, respectively. All *P. argus* trypsins (e.g. *PaTry3*, Fig. 2a) showed the conserved core structure of the chymotrypsin fold, consisting of two six-stranded β -barrel domains packed against each other, with the catalytic residues located at the junction of the two barrels (Perera et al. 2010b). These 3D models also anticipate all other structural features of trypsins (i.e. disulfide bonds, superficial loops, calcium-binding site, the configuration of the active centre and the enzyme pocket), with no major difference among lobster isoenzymes (Perera et al. 2010b). Considering the importance of the 3D structure in the functionality of enzymes, it is not surprising the presence of a well conserved architecture in lobster trypsins. However, when lobster trypsins were compared to crayfish trypsin (Fodor et al. 2005) structural differences were noticed, especially in the surface loops. Variation in these regions, which are not core elements of the fold, but important for inhibitors/substrate binding (Fodor et al. 2005), may be a way for evolutionary adaptation (Perona and Craik 1997). In insects, surface loops notably vary among isoenzymes even within the same species, and this is suggested to be related to the presence of proteinase inhibitors in the diet (Díaz-Mendoza et al. 2005). Both the high number of gene copies and the high degree of polymorphism found in crustacean trypsins (Klein et al. 1998) point to a huge source of variation for adaptive evolution to occur in species differing in feeding habits (e.g. crayfish and spiny lobster). On the other hand, despite the low intra-specific variation in overall 3D structure of *P. argus* trypsins, amino acid substitutions occur among isoforms (Perera et al. 2010b), these being presumably the structural basis of the observed intra-specific differences in catalytic efficiencies. Insights into the possible effect of these point variations among isoforms arise from an evolutionary assessment of lobster trypsin sequences (see next section).

Unfortunately, transcripts and isoenzyme in lobster have been not matched due to high sequence similarity (Perera et al. 2010b) and difficulties for isolating high amount of the isoenzymes (Perera et al. 2012a). To circumvent these drawbacks we made an attempt to identify, at least indirectly, the isoenzymes in *P. argus*. We selected individuals lacking some particular isoenzyme in casein zymograms and used them for RT-qPCR analysis using specific primers for the most abundant transcripts: *PaTry1a*, *PaTry2*, and *PaTry3*. We found that all lobsters highly

express *PaTry3*. This transcript should correspond to the isoenzyme of minor electrophoretic mobility since this form of the enzyme is always present in zymograms. On the other hand, we observed that lobsters that lack the isoenzyme of higher electrophoretic mobility in zymograms exhibit very low or no expression of *PaTry1a*, and the same occurred with *PaTry2* in lobsters without the isoenzyme of middle electrophoretic mobility (Fig. 3).

5. Approach to the evolution of trypsins in lobster

The assessment of the ratio of non-synonymous to synonymous substitution (K_a/K_s) (Hurst 2002) in lobster trypsins has provided some insights into the evolution of these isoenzymes (Perera et al. 2012b). Nucleotide synonymous substitutions do not change amino acids in a protein and generally have neutral evolutionary effects, while non-synonymous mutations result in amino acid substitutions and occur more often under selective pressure (Hurst 2002). Calculated K_a/K_s suggested that isoenzymes in lobsters are under purified or neutral selection (Perera et al. 2012b). It is well known that enzymes are subjected to functional constraints, thus the result was somewhat expected. However, because averaging K_a/K_s for all sites does not consider the type of selective pressure applied to individual amino acids, maximum likelihood approach was later used for detecting positively evolving sites in a background of purifying/neutral selection. According to this strategy, several positively evolving sites (25 codons) were found in *P. argus* trypsins (Perera et al. 2012b). It is interesting to remark that not all trypsin isoenzymes appear to have been under positive selection at these sites, since 72% of the positive selected sites imply unique substitutions in one particular transcript, *PaTry3* (Perera et al. 2012b). Unequal patterns of molecular evolution for different trypsin isoenzymes have been reported before in other invertebrates (e.g. *Drosophila*, Wang et al. 1999).

Positive selection may be an important force driving adaptive evolution (Wu et al. 2009), but trypsin isoenzyme sequences from other spiny lobster species are required to truly determine whether some trypsins are evolving under selective pressure at certain sites to undergo adaptive evolution. It is noteworthy that 55% of all positively evolving sites in *P. argus PaTry3* are within (or in close vicinity to) functionally relevant motifs, like surface loops as well as the calcium binding site (Perera et al. 2012b), supporting the hypothesis that trypsin polymorphism in *P. argus* may be non-neutral and, thus, have an adaptive value in relation to feeding. These regions are known to be the most susceptible to variation through trypsin evolution (Rypniewski et al. 1994).

The role of interaction with environmental factors in lobster trypsins evolution is also supported by analysis of the 3D structures (Perera et al. 2010b) and mapping of the positively selected residues to these structures in relation with proteinase inhibitors (Fig. 2b). Canonical inhibitors are known to bind the enzymes in a substrate-like fashion and thus, are suited for the study of possible interactions with proteinaceous substrates. A cluster of aromatic residues (mostly Phe) in Loop37 of the crayfish *Astacus leptodactylus* trypsin forms the binding surface for positions P4'-P5' of the C-terminal of the trypsin inhibitor from desert locust *Schistocerca gregaria* (SGTI), and might play a role in the orientation of the inhibitor (Fodor et al. 2005). This hydrophobic loop of the crayfish trypsin plays a similar role during the interaction with bovine pancreatic trypsin inhibitor (BPTI) (Molnár et al. 2013). In the lobster, these hydrophobic residues are conserved (Fig. 2b), but there are two additional and positive selected Phe residues in the distal part of loop 37 of *PaTry3* isoform. Given the role of hydrophobic residues in this loop, these two positive selected residues may be involved in more extended interactions with dietary substrates (Fig. 2b). The two positive selected sites found in trypsin-like serine proteases in the malaria mosquito *Anopheles gambiae* are also located at the surface, in regions that contact proteinaceous substrates (Wu et al. 2009). A positive selected residue in *PaTry3* calcium binding loop may favor a stronger calcium binding (Perera et al. 2012b). Besides positive selected residues in lobster trypsins probably contacting the substrate (Fig. 2b) or the calcium ion, others lie in important regions for activity, like those in loops 1, 2, and 3 (Perera et al. 2012b). Since these other substitutions are in the neighborhood of primary or secondary specificity determinants in these loops, an effect on the function of the enzyme cannot be ruled out. Some residues in these surface loops of trypsins may ensure the optimal positioning of key residues or stabilize the geometry of the enzyme (Rypniewski et al. 1994). Adaptive evolution in lobster trypsin may have occurred on both substrate binding and activity. Trypsins from different insects differ in the strength with which their subsites bind the substrate or the transition state (Marana et al. 2002).

Trypsin diversity is particularly high in insects, decapod crustaceans, and fishes. The phylogenetic relationship of a selected group of trypsin isoenzymes from these three groups is shown in Fig. 4. In general, most trypsin isoforms trend to group in accordance with the phylogenetic relationships of species, although crustacean trypsins clustered with vertebrate trypsin and not with those of the other Arthropod group analyzed. It is accepted that members of multigene families do not usually evolve independently, so greater sequence similarity is commonly found within a species than between species (between paralogues than between orthologues) (Liao 1999). However, as in Lepidoptera (Lopes et al. 2004), mosquito (Wu et al. 2009), and *P. argus* (Perera et al. 2012b), some degree of independent evolution for particular

trypsin isoenzymes may occur in other crustaceans, since the topology of phylogenetic tree of related species (e.g. penaeid shrimp) reflects relationships among some trypsin variants rather than among species (Perera et al. 2010b) (Fig. 4). Thus, some trypsins isoform in crustaceans may have escaped from homogenization of DNA sequences (i.e. concerted evolution, Liao 1999), as demonstrated to occur in *Drosophila* (Wang et al. 1999), to undergo adaptive diversification.

Trypsin diversification is less understood in terms of functionality in crustaceans respect to insects and fishes. Major diversification occurs in insects, with the highest amino acid sequence identity shared by two putative trypsins in some species being just 61.3% (Chen et al. 2013). The occurrence of multiple isoforms is thought to provide an adaptive advantage for insects feeding on plants containing inhibitors, although the effects of small variations in amino acid sequences on enzyme activity and sensitivity to inhibitors are still unclear (Srinivasan et al. 2006; Spit et al. 2014). Functional properties of enzymes cannot be predicted based only on sequences, and experimental data are needed to afford true evidence of functional diversification (Srinivasan et al. 2006). However, conventional purification methods have limited resolution in dealing with protease isoforms, expression in heterologous systems faces problems such as toxicity of the expressed protease and misfolding of the recombinant polypeptide, and minor changes in activity may not be detectable with synthetic substrates (Srinivasan et al. 2006). In few insect groups such as Lepidoptera (Lopes et al. 2004) and mosquito (Soares et al. 2011) transcripts have been matched with the enzyme, but major evidences for functional diversification were found for lepidopteran trypsins (Lopes et al. 2004). Comparing inhibitor-sensitive and insensitive lepidopteran trypsins, several residues have been proposed as critical for trypsin resistance to plant inhibitors, resulting in increased inhibitor hydrolysis (preference for lysine at the P1 position), and decreased binding to inhibitor hydrophilic reactive sites (substrate binding subsites more hydrophobic than trypsins from insects in other orders) (Lopes et al. 2004). The topology of general feeder lepidopteran trypsins in Fig. 4 indicates sequence divergence between sensitive and insensitive trypsins (Lopes et al. 2004). Specialist feeders Lepidoptera such as *Manduca sexta* exhibit less enzyme diversification than general feeders (Srinivasan et al. 2006) (Fig. 4). In *Drosophila melanogaster*, trypsin isoforms are known exhibit different pattern of evolution: two genes are evolving in concert (gamma and delta trypsin genes), a third gene appears to be evolving independently (epsilon trypsin gene), and the remaining two genes show an intermediate pattern (Wang et al. 1999) (Fig. 4). In fishes, trypsins are separated in two major clusters (Psochiou et al. 2007). The first one gathered trypsinogens of fish species adapted to extreme cold environments, and the second cluster grouped all other fish trypsinogens (Psochiou et al. 2007) (Fig. 4).

The trypsin isoform of lobster with minor electrophoretic mobility in Fig. 1 is present in all *P. argus* individuals (Perera et al. 2008a, 2010a), is the most reactive toward native protein substrates (Perera et al. 2012a), and its transcript was indirectly identified as *PaTry3*, for which some positive selected sites were found (Perera et al. 2012b). Forces driving diversification (e.g. diet features, environmental temperature) of crustacean trypsins and structural changes involved need further evaluation.

6. Positive correlation between apparent adaptive evolution and expression rate

Several factors are known to be involved in the regulation of transcription in eukaryotes (Brivanlou and Darnell 2002) and many genome regions that interact with these factors have been also identified (Nègre et al. 2001), usually in (Cis) 5' regions (Wray et al. 2003). Unfortunately, there is no information on the genomic sequence of trypsin isoenzymes of lobster and, therefore, neither on genomic regulatory elements. In the copepod *Lepeophtheirus salmonis*, several candidate promoter sequences have been identified in association with trypsin genes (Kvamme et al. 2005), but in general the information on this issue is scarce in crustaceans in comparison to insects (Xiong and Jacobs-Lorena 1995; Mazumdar-Leighton and Broadway 2001). Trypsin isoenzymes in *Panulirus argus* greatly differ in expression rates both in fasting individuals and during feeding (Perera et al. 2010b, 2012b), thus they constitute a suitable model for studying the regulation of gene expression in crustaceans.

The transcript for which positive selected sites were detected is by far the most expressed in the digestive gland of *P. argus* (Perera et al. 2012b). More interestingly, it is the only transcript for which dietary up-regulation could be demonstrated, while all other trypsin isoforms appear to be expressed in a constitutive fashion (Perera et al. 2012b). This result also supports the suggestion that isoenzyme variation in lobster arose as a result of adaptive evolution to increase fitness (Perera et al. 2012b). Although the transcriptional regulation of crustacean trypsins by dietary proteins was reported before (Le Moullac et al. 1996; Muhlia-Almazán et al. 2003), this is the first demonstration of a differenced response in expression of trypsin isoforms to dietary proteins for a crustacean species. However, this is not the first time that differences in response to diet occur among isoforms of digestive enzymes in invertebrates. In the oyster *Crassostrea gigas*, one amylase gene is regulated at the mRNA level when food varied quantitatively, whereas the other gene present is either not regulated under these conditions or it is expressed at low mRNA levels (Huvet et al. 2003, 2008). While positive correlation between apparent adaptive evolution and expression rate is likely to occur in *P. argus* isotrypsins, more studies are

needed to confirm this hypothesis. It is known that high expression levels usually correlate with low rates of protein evolution (Drummond et al. 2005; Larracuenta et al. 2008). However, some works have provided evidence of concomitant adaptive evolution in highly expressed genes of the *Drosophila* genome (Holloway et al. 2007), and trypsin-like genes in mosquito with increased expression after meal (Wu et al. 2009).

7. Trypsins isoenzymes and physiological needs

As key enzymes in protein digestion, gene expression and, more important, activity of trypsins in lobster should be related with the amino acid needs for energy and growth. Variations of trypsin activity in spiny lobsters are developmentally clued (*Jasus edwardsii*, Johnston 2003; *Panulirus argus*, Perera et al. 2008b) and could reflect changing physiological requirements as lobsters grow, but the meaning of these variations is not clear because few studies are still available. Johnston (2003) found a positive relationship between total trypsin activity and size in the spiny lobster *J. edwardsii*, coinciding with an increase in size of the digestive gland, but not a similar trend with specific activity. In contrast, trypsin specific activities tend to increase with age in *P. argus*, although the increase in size only accounted for 68% of the variation (Perera et al. 2008b). Further studies are necessary in order to understand how variations of trypsin activity in lobsters of different size correlate with their physiological needs, and particularly the role of trypsin isoforms.

In thoroughly studied fish such as Atlantic salmon, the trypsin isoenzyme composition have been shown to affect absorption and transport of free amino acids (FAA), including the ratio of essential to non-essential FAA (Torrissen et al. 1994; Rungruangsak-Torrissen and Male 2000; Rungruangsak-Torrissen 2012). Higher protein digestion and FAA absorption in fish carrying certain trypsin phenotypes produce a higher rate of protein synthesis in white muscle in association with higher secretion of plasma insulin (Rungruangsak-Torrissen et al. 1999; Rungruangsak-Torrissen and Male 2000; Rungruangsak-Torrissen 2012). These studies in fish may provide guidance for future studies in crustaceans, although differences are expected to occur (e.g. endocrine factors). Also, studies in salmon fish have indicated that digestive efficiency and growth are not always related with trypsin specific activity, but with the activity ratio of trypsin to chymotrypsin (T/C ratio) (Rungruangsak-Torrissen 2012).

Since the molt cycle drives extensive physiological changes in crustaceans, it constitutes a good model for assessing the physiological meaning of variations in digestive enzymes (Perera et al. 2008b). Whereas changes in digestive enzymes during the molt cycle were studied in different

crustaceans (Van Wormhoudt 1974; Klein et al. 1996; Fernández et al. 1997; Fernández-Gimenez et al. 2001, 2002), there is just one study in spiny lobsters (Perera et al. 2008b). Variations in the activity of trypsins and other digestive enzymes in *P. argus* (Perera et al. 2008b) resemble the foraging and feeding patterns observed in previous studies (Lipcius and Herrnkind 1982), showing a gradual increment during intermolt to highest levels at late intermolt and early premolt (when requirements of proteins are high for energy and growth), and then a gradual decrease to minimal activity at late premolt and postmolt (when lobsters do not eat and use the energy stored as lipids and glycogen). In a recent study on the blue swimming crab, *Portunus pelagicus*, variations in T/C ratio were found to match with expected growth rate through the molt cycle (Rungruangsak-Torrissen 2012), similar to the observations on fish mentioned above. However, the validation of T/C ratio as feed efficiency indicator in crustacea needs further studies. No discernible pattern of T/C ratio during the mol cycle can be deduced from data of trypsins and chymotrypsin activity in the digestive gland of the lobster *P. argus* (Perera et al. 2008b), despite variation of both trypsin and chymotrypsin activity are rather similar in the lobster (Perera et al. 2008b) and the blue swimming crab (Rungruangsak-Torrissen 2012). In addition, a substantial share of proteolytic activity in some crustaceans such as carideans is caused by cysteine proteinases, instead of trypsin or chymotrypsin enzymes (Teschke and Saborowski 2005). Enzyme activity variations should be under genetic and hormonal control, but also appearing to be closely regulated by feeding.

8. Trypsin regulation and feeding

From early studies it is known that new digestive enzyme produced in the digestive gland of crustaceans are subsequently discharged into the gland lumen and then accumulated (i.e. extracellular storage) in its active form in the cardiac stomach (Vogt et al. 1989). Studies in lobster (Perera et al. 2008a; Simon 2009; Perera et al. 2012b) and other crustaceans have regularly reported high trypsin activity in the gastric fluid of unfed animals. However, despite crustacean zymogen granules differ from that of vertebrates (Vogt et al. 1989), both the deduced cDNA sequence (i.e. presence of trypsinogen activation peptide) of several crustacean trypsins (Muhlia-Almazán et al. 2008) including *P. argus* (Perera et al. 2010), and immunohistology studies (Sainz et al. 2004) support that trypsins are also stored as inactive zymogen in crustaceans to be secreted after feeding. Significant amount of trypsins enzymes are secreted by lobster after feeding with maximal activity in gastric fluid attained four hours after ingestion (Simon 2009; Perera et al. 2012b). Likewise, in continuous digester insects (e.g. Lepidoptera and Diptera larvae) feeding stimulates secretion, but enzymes are released continuously at a basal rate in unfed animals (Woodring et al. 2009). That is why trypsin and other digestive

enzymes are always present in the gut lumen of continuous digesters insects, while in batch digesters such as mosquitoes trypsin is not present in the unfed insect (Lehane et al. 1995). In mosquito, trypsin is not stored in the epithelial cells as an inactive enzyme, but is synthesized, activated and released into the gut lumen after the blood meal (Borovsky 2003). In humans and other higher vertebrates, trypsins are synthesized, often stored for a long time, and then secreted as inactive zymogens, which are normally activated only upon entry into the duodenum (van Acker et al. 2006). In mammalian pancreatic cells, the pancreatic secretory trypsin inhibitor (PSTI) prevents the premature activation of trypsin and other digestive enzymes (Hirota et al. 2006). In several insect species protease inhibitors that structurally resemble the mammalian PSTI have been predicted *in silico*, and a PSTI-like protein from the African migratory locust, *Locusta migratoria*, was cloned, sequenced, and demonstrated to be involved in controlling proteolytic activity (van Hoef et al. 2011). These results in insects point to the existence of an ancient protective mechanism in metazoan digestive systems (van Hoef et al. 2011). Protease inhibitors have been described in the digestive gland of some crustaceans (de Albuquerque-Cavalcanti et al. 2002) but their role is still unknown.

While regulatory mechanisms behind protein digestion in crustaceans remain fairly unknown, a recent study has provided evidence of dietary regulation of trypsin enzymes in *P. argus* (Perera et al. 2012b). Experimental evidence leads us to propose that trypsin enzymes in *P. argus* are regulated at the transcription and secretion level by the quality of dietary proteins, through two distinct signaling pathways. Intact proteins rather than protein hydrolysates stimulate trypsin secretion in the lobster (Fig. 5), while the signal for differential isotrypsin transcription seems to be the appearance of small peptides or free amino acids (i.e. protein digestion end-products) in the digestive gland (Fig. 5). These facts support a model of stepwise regulation of trypsin enzymes in lobsters during digestion (Perera et al. 2012b). While just the removal of gastric fluid is known to stimulate secretion in some crustaceans (Vogt et al. 1989), a regulatory mechanisms for trypsin secretion by dietary proteins should have evolved early during evolution, as trypsin secretion is also more stimulated by intact proteins in very distant groups like insects (Blakemore et al. 1995; Lehane et al. 1995; Graf et al. 1998; Noriega and Wells 1999) [although contradictory results have been obtained in some species (Woodring et al. 2009)], fishes (Cahu et al. 2004; Koven et al. 2002) and rats (Green and Miyasaka 1983; Owyang 1994). Such a general mechanism for regulating trypsin secretion may be related to the fact that intact proteins in the digestive tract always mean recent food ingestion.

Mechanisms controlling trypsin secretion are relatively well known in vertebrates. Feedback inhibition of pancreatic enzyme secretion is mediated by trypsin and other proteases in the

intestine in rat, chickens, pigs and humans by means of the trypsin-sensitive cholecystokinin-releasing peptide. Intact dietary protein in the intestine competes for the trypsin that would otherwise inactivate the factor (Owyang 1994). The resulting increase of cholecystokinin-releasing peptide releases cholecystokinin (CCK) and stimulates pancreatic enzyme secretion (Owyang 1994). Results in rat indicate that the effect of trypsin inhibitor SBTI, which often stimulate secretion, acts by a mechanism not involved in masking (i.e. inhibiting) trypsin activity but acting as a protein, since a non-peptidic trypsin inhibitor did not induce secretion (Hira et al. 1997). Although the action mechanism is not so well understood as in mammals, humoral CCK is also recognized as major regulator of trypsin secretion in fishes (Kofuji et al. 2007; Einarsson et al. 1997; Buddington and Krogdahl 2004; Jönsson et al. 2006; Zambonino-Infante and Cahu 2007), regulated by the trypsin-sensitive cholecystokinin-releasing peptide (Zambonino-Infante and Cahu 2007; Tillner et al. 2013).

However, amino acids and peptides are more effective than intact proteins in stimulating pancreatic exocrine secretion in dogs (Meyer and Kelly 1976) and humans (Thimister et al. 1996). Actually, digestion of protein is required to stimulate plasma cholecystokinin (CCK) release, gallbladder emptying, and pancreatic enzyme secretion in humans, while undigested protein is only a weak stimulus to pancreas (Thimister et al. 1996). It is though that under unstimulated conditions, the feedback regulation between pancreatic enzyme secretion and protease activity in the lumen of the small intestine is operative also in humans, but that in contrast with rats, protease-dependent feedback regulation of pancreatic exocrine secretion in humans is probably not mediated by humoral CCK. In humans and dogs the major pathways of CCK action appear not to be endocrine but neural, involving the sensory receptors at afferent nerves and long vagovagal or short enteropancreatic reflexes (Konturek et al. 2003), although there are difference in the contribution of the different pathways between low and high loads of amino acids (and fatty acids) (Singer 1987). Other hypothesis is that CCK-mediated feedback control of pancreaticobiliary secretion in humans is principally regulated by bile acid sequestration and not by protease activity. There are strong evidences for the presence of CCK-like peptides in crustaceans (van Wormhoudt et al. 1989; Favrel et al. 1991; Resch-Sedlmeier and Sedlmeier 1999; Ravallec-Plé and van Wormhoudt 2003; Ma et al. 2009) but their role on digestive enzyme secretion has been not clarified (Staljanssens et al. 2011).

Studies in crustaceans have indicated that regulation of trypsin activity occurs also at the transcription level (Klein et al. 1996). Up-regulation of trypsin expression in *P. argus* by protein digestion end-products (Perera et al. 2012b) appears to be related with the preparation for next feeding (Fig. 5), and matches with the periodic feeding habits of spiny lobsters. Lack of stimuli

(low concentration of digestion end-products) for synthesis of new trypsin enzymes (Perera et al. 2012b) may be related with low amount/activity during fasting periods such as late premolt and postmolt (Perera et al. 2008b). This transcriptional induction of some trypsin isoform in the lobster resemble that occurs in mosquito, where partially digested proteins and peptides released during the earlier part of the digestive cycle, affect the transcription of the late trypsin (see below). Differential expression of trypsin isoforms have been also observed in other insects after feeding different plants (Srinivasan et al. 2006; Chen et al. 2013) although the exact nature of the signaling mechanism is not well understood (Srinivasan et al. 2006). In mammals, transcriptional differences among trypsin isoenzymes have been also reported. Three of the four rat trypsinogen mRNA studied by Hara et al. (2000) increased in response to casein level in diet, while only one reacted to CCK administration.

There are also reports on regulation of trypsin and other digestive enzymes at the translation level or at both transcription and translation. In the female mosquito trypsin expression is biphasic: an early phase that is translationally regulated, and a late phase that is transcriptionally regulated. Transcription of the early trypsin gene occurs after mosquito female emergence under the control of juvenile hormone, but the transcript remains untranslated before blood feeding (Noriega and Wells 1999). Early trypsin appeared in the mosquito midgut after the blood meal (Noriega and Wells 1999) and its synthesis appears regulated by soluble proteins of variable molecular weights (Borovsky 2003) and the size of the free amino acid pool in the midgut (Noriega et al. 1999) by the Target of Rapamycin (TOR) signaling pathway (Brandon et al. 2008). Unlike early trypsin, the transcription of the late trypsin gene is regulated by uncharacterized proteolysis products (Noriega and Wells 1999), probably small peptides (Borovsky 2003). The termination of trypsin biosynthesis, at the translation level, is controlled by the trypsin modulating oostatic factor (TMOF) secreted by the mosquito ovary several hours after feeding (Borovsky 2003). In other hematophagous insects such as *Stomoxys calcitrans*, increased synthesis of trypsin following the blood meal appears to be regulated also at the translational level (Moffatt et al. 1995). Amino acids also stimulate the synthesis of digestive enzymes at the translational level in rats (Bragado et al. 1998; Sans and Williams, 2002). A study demonstrated that branched-chain amino acids (leucine, isoleucine, and valine), and most notable leucine, stimulate translation initiation in mouse pancreas independent of CCK, although amino acids by themselves are not sufficient for increasing protein synthesis (Sans et al. 2006). Amino acids “prime” the mouse pancreatic translational machinery for protein translation to occur, but a hormonal and/or cholinergic stimulus would be required for a complete stimulation of protein synthesis (Sans et al. 2006).

The high degree of polymorphism (Perera et al. 2008a), the marked differences in expression rates (Perera et al. 2010b), and the differences in activity among isoforms (Perera et al. 2012a), provide several points at which regulation of digestive trypsin may occur in *P. argus*. Our result on the signals inducing transcription and secretion of trypsin isoforms in lobster provide the first evidence of these mechanisms in crustaceans, but they are far to be well understood as in insects or higher vertebrates. The regulation of trypsin and other digestive enzymes in crustaceans by feed is an area that deserves further studies. Besides contributing to understand the evolution of these mechanisms, it has practical implications on the optimization of aquafeeds. There have been contradictory results on the effect of protein hydrolysates on both fish and crustacean growth. High inclusion of protein hydrolysate in diets depressed growth in fishes (Zambonino-Infante and Cahu 2007) and crustaceans (Córdova-Murueta and García-Carreño 2002), while moderate levels have positive effects in fish (Zambonino-Infante and Cahu 2007; Savoie et al. 2011), crustaceans (Córdova-Murueta and García-Carreño 2002), and other invertebrate taxa such as cephalopods (Aguila et al. 2007). Squid meal, which is rich in peptides and small molecular weight proteins (Córdova-Murueta and García-Carreño 2002; Perera et al. 2010a) also promote better growth rates in crustaceans at low supplementation levels (Córdova-Murueta and García-Carreño 2002). Conversely, high levels of krill hydrolysates still enhance growth in shrimps (Córdova-Murueta and García-Carreño 2002) and spiny lobsters (Smith et al. 2005; Barclay et al. 2006). These feed components may exert different effect on the expression, synthesis and secretion of digestive enzymes in the different groups, although more general effects on overall metabolism cannot be ruled out.

9. Trypsin and feeding ecology

9.1. Features of natural diet

Little is known on the natural feeding of *Panulirus argus* larvae (phyllosoma) although it is accepted that, in general, spiny lobster are carnivorous throughout their entire larval cycle (Kittaka 1997). Pelagic phyllosoma from different palinurids have been successfully reared in laboratory on a diet of *Artemia* and other seafood (Kittaka 1997). A recent assessment of DNA material in the digestive tract of larvae provided indication of salps, ctenophores, and medusae as main zooplankton dietary items in some species (Chow et al. 2011), with preference for some prey items (e.g. chaetognaths) (Saunders et al. 2012). It is known that pelagic zooplankton generally has higher protein and lipid and lower carbohydrate contents than phytoplankton (Le Vay et al. 2001).

Shortly after settlement, early *Panulirus argus* juveniles feed on a wide variety of small and relatively soft invertebrates, mostly mollusks and crustaceans (Marx and Herrnkind 1985; Lalana and Ortiz 1991), and gradually change into bigger size preys (Lalana and Ortiz 1991). The diet of older juveniles remains similar, but there is an increase in prey size and armor (Herrera et al. 1991). The proportions of prey types in the diet of *P. argus* juveniles vary according to their abundance in the environment (Colinas-Sanchez and Briones-Foorzan 1990; Herrera et al. 1991; Cox et al. 1997). Chemical composition of the natural diet in juvenile and adult spiny lobsters is thought to have a high protein, low lipid and moderate carbohydrate contents (Williams 2007).

As outlined above, spiny lobsters eat a diverse assemblage of zooplankton species during larval stages and a wide variety of benthic and infaunal invertebrates during juvenile and adult stages. Although the natural diet of spiny lobsters is hence very diverse, it presents two common features: i) high protein content, and ii) wide variation in protein quality. Less variation in quality is expected for dietary carbohydrates and lipids. Glycogen is likely to be the main dietary carbohydrate in lobster diet (Hickman and Illingworth 1980), and triacylglycerols and phospholipids are the main lipids in prey items, probably with a balanced content of all fatty acid families (Zlatanos et al. 2009). Therefore, it is feasible that the ecological success of lobsters relies to a great extent in their capacity to efficiently digest a wide variety of dietary proteins, a process that largely relies on trypsin enzymes (Perera et al. 2008a, 2010a).

9.2. Trypsins and natural diet

Enzyme activities in decapod larvae reflect adaptations to the trophic levels utilized according to a general pattern. Herbivorous larvae adapt to low energy food by high daily ingestion rates, high enzyme activities, rapid food turnover, and low assimilation efficiency, while carnivorous larvae (which rely upon high-energy digestible preys) exhibit low levels of enzyme activity and longer gut retention times to maximize assimilation efficiency (Le Vay et al. 2001). Trypsins are the major proteases in the digestive system of most decapod crustaceans, and a relationship between trypsin activity and trophic level has been established according to this model in larvae from different decapods (Le Vay et al. 2001), including spiny lobsters (Jones et al. 1997). Carnivorous lobster larvae show comparatively low trypsin activity at first feeding respect to herbivorous penaeid and brachyuran larval stages (Jones et al. 1997). Trypsin activity, however, increased during spiny lobster larval development reflecting enlarges in their digestive capacity (Johnston et al. 2004), a trend that continues in adult stages (Johnston 2003; Perera et al. 2008b).

Whereas adult stages of phytoplankton feeder decapods seem to fit to the abovementioned model (Johnston and Freeman 2005), there is another pattern well supported by studies in adult crustaceans. Carnivorous species exhibit a wide range and high activity of proteolytic enzymes, whereas herbivores and omnivores possess highly active carbohydrases. In crabs, key enzymes present in the gut are generally consistent with this other pattern (Johnston and Freeman 2005), and high trypsin activity has been regularly reported in spiny lobster juveniles and adults (Johnston 2003; Celis-Guerrero et al. 2004; Perera et al. 2008a, 2010a, 2012b; Simon 2009). However, there are some contradictory observations in which omnivores, or even herbivorous species, have higher protease activity than predator crustaceans (Johnston and Freeman 2005). Similar difficulties for correlating proteases and diet have been evidenced also in fish (German et al. 2010), probably because all animals need protein, so selection should not be strong for low protein-processing capability, and with the fact that herbivores have compensatorily higher biochemical capacity (Karasov et al. 2011). In contrast, positive correlation of other digestive enzymes (e.g. amylase) and diet are usual in very different groups, such as insects (Inomata and Nakashima 2008), crustaceans (Pavasovic et al. 2007), and humans (Perry et al. 2007).

9.3. Trypsins and digestive flexibility

Feeding rate and digestive efficiency are two parameters of nutritional and ecological importance (Karasov et al. 2011). Feeding rate in spiny lobsters is quite limited compare with other crustaceans due to small foregut capacity, long foregut clearing time and slow appetite revival (Simon and Jeff 2008), thus digestive efficiency of a single ration (at night) is expected to be high. This partially relies on efficient mechanisms for mechanical (mouthparts, gastric mill) and chemical digestion (enzymes). Trypsin accounts for around 60% of proteolysis of dietary proteins in *Panulirus argus* (Perera et al. 2008a). Considerations of evolutionary economic design suggest that enzymatic capacities should be only slightly in excess of their corresponding loads (Karasov et al. 2011), but excess of trypsin enzymes would lie behind the capacity of spiny lobster for switching to more abundant prey items in the environment without impacting protein digestion efficiency. High amounts of trypsin enzymes in the gastric chamber of fasting spiny lobsters, in addition to the ones secreted after feeding (Simon 2009; Perera et al. 2012b), may indicate the need for excess enzymes in anticipation to feeding on preys with high protein content, but of unexpected quality.

While excess of trypsin expression and secretion may provide a general mechanism to cope sudden changes in dietary protein quality (e.g. when switching to most abundant prey items in

the environment), differences in specificity or cleavage ability among isoforms in *P. argus* (Perera et al. 2012a) may provide additional flexibility. Moreover, some degree of physiological flexibility may also occur by adapting the production of trypsins to a particular dietary composition. Although proteins in the natural diet of *P. argus* and other spiny lobsters are supposed to be highly digestible and a great adaptation capacity may be not necessary, some results suggest that this capacity is present. In the spiny lobster *Jasus edwardsii*, the trypsin activity tended to be lower in specimens fed with fresh mussel than formulated feeds (Simon and Jeffs 2011), indicating perhaps an adaptation in response to lower digestibility of the formulated feeds. The protein source (animal vs. vegetal) in formulated feeds also differentially affects the expression and secretion of trypsins in *P. argus* (Perera et al. 2005; Perera et al. 2012b). This flexibility is poorly understood in terms of ecological significance. From studies in larvae of other crustaceans it is well known that greater enzymatic flexibility occurs in herbivorous species (Jones et al. 1997). Among invertebrates, flexibility in digestive enzymes has been studied in detail in some insects such as Lepidoptera. Lepidopteran insects employ different strategies to deal with plant inhibitors (PI) such as the expression of inhibitor-insensitive proteases, over-expression of constitutive proteases to outnumber inhibitors present in the digestive tract, induction of proteases capable of degrading the PI, or switching to enzymes with altered substrate specificity such as the up-regulation of chymotrypsin-like activity (Jongsma and Bolter, 1997; Spit et al. 2014).

Gene copy number variations and single-nucleotide polymorphisms are sources of phenotypic variation in digestive biochemistry (Karasov et al. 2011). While these processes are known to occur in *P. argus* trypsins (Perera et al. 2010b, 2012a), we are just starting to understand how these could provide an adaptive value and some degree of digestive flexibility. It is feasible than high basal and postprandial trypsin activity (Perera et al. 2008a; Perera et al. 2010a), difference in activity, cleavage ability and other functional properties among isoforms (Perera et al. 2012a), as well as difference in expression rate and responsiveness to dietary proteins among isoforms (Perera et al. 2012b), may provide the digestive flexibility required by lobsters to meet their high protein physiological needs with a varied diet.

10. Open issues

Trypsin enzymes are not responsible of all protein digestion in crustaceans since other endo- and exo-proteinases are deeply involved (Muhlia-Almazán et al. 2008; Sainz and Córdova-Murueta 2009). However, trypsin account for most of the protein hydrolysis in *P. argus* (Perera et al. 2008a) and it is the most studied *P. argus* enzyme to date, thus constituting a good model

for assessing the digestive adaptation of this crustacean to its feeding ecology. Unfortunately, molecular or even biochemical information about trypsins in spiny lobsters, and other crustaceans, is available just for few species. Although this review is focused on studies using one spiny lobsters species as a model organism, it provides baseline information and highlights important but poorly studied issues in crustaceans in general. Information in other spiny lobsters and crustaceans on trypsin isoenzymes richness, molecular and biochemical difference among isoforms, molecular evolution, and the response of these enzymes to feeding, would lead to a generalized model of trypsin evolution and regulation in crustaceans in relation to their feeding ecology. Finally, although the aquaculture of spiny lobsters is currently a growing activity (Phillips and Matsuda 2011), lobsters are still fed exclusively on fresh fishery by-catches due to the absence of suitable artificial feeds. Major difficulties with the artificial feeding in spiny lobsters have been associated with deficiencies in protein digestion and utilization (Williams 2007). These issues should be further addressed via the capacity of spiny lobsters to deal with less digestible/soluble proteins and very different feed formats (e.g. dry, particulate) respect to their natural diet.

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Fig.1 Schematic representation of the main three trypsin isoenzyme patterns or phenotypes in the spiny lobster *Panulirus argus*, according to the presence of major isoforms in individual lobsters, as revealed in casein zymograms (Perera et al. 2008a, 2010a). Trypsin identity was corroborated by in-gel assays with specific proteinase inhibitors (Perera et al. 2008a). Lobsters with the three main trypsin isoenzymes are named phenotype A. Individuals lacking the trypsin isoenzyme of higher electrophoretic mobility are named phenotype B, while lobsters lacking the trypsin isoenzyme of middle electrophoretic mobility are named phenotype C. Above these three main isotrypsins, other serine (even trypsin-like) proteinases occur in gels, but notably less active and without a discernible pattern through individual lobsters (Perera et al. 2008a)

Fig. 2 Three-dimensional models of the most abundant *Panulirus argus* trypsin isoform, *PaTry3* (PMDB database: accession number PM0076232), showing (a) the two β -barrels (yellow) making up the fold, connecting α -helices (red) and surface loops (green), the catalytic residues His74, Asp125 and Ser218 (His57, Asp102, Ser 195, chymotrypsin numbering), primary specificity residues [Asp212 (189, chymotrypsin numbering) residue near the base of the substrate-binding pocket to stabilize the positive charge of P1 Arg or Lys side chains, and the small residues Gly239 and Gly249 (216 and 226, chymotrypsin numbering) on one wall of the pocket to allow the access of bulky residues to the S1 site], and the calcium-binding site, and (b) predicted hydrophobic binding sites in loop 37 of lobster trypsin (*PaTry3*) to a proteinase inhibitor (SGTI accession no. 1KJ0, in green) using the docking approach when ranking models based on cluster size by the program ClusPro 2 (<http://cluspro.bu.edu>) (Comeau et al. 2004a,b; Kozakov et al. 2006, 2013), and the mapping of positively selected sites (Phe 52 and Phe 54) in loop 37 of *PaTry3*, showing they may play a role in more extended interactions with hydrophobic elements of larger inhibitors and presumably, of proteinaceous substrates in this variant of the enzyme. Site numbers start at the first residue of lobster *PaTry3* trypsinogen (Perera et al. 2010a). Loops 37 and 60 are used according to the nomenclature in crayfish trypsin (Fodor et al. 2005). Loops 1 and 2 are according to vertebrate nomenclature (Perona and Craik 1995; Hedstrom 1996). Figures were generated with Open-Source PyMOL™ Molecular Graphics System, Version 1.6.x (Schrödinger, DeLano Scientific LLC, San Carlos, California, USA)

Fig. 3 Substrate-SDS-PAGE showing caseinolytic activity bands in digestive gland of individuals *P. argus* with low expression of trypsin transcripts *PaTry1a* (GU338026.1) and *PaTry2* (GU338028.1) as revealed by RT-qPCR assays. The central lane corresponds to an individual expressing all major transcripts, *PaTry1a*, *PaTry2*, and *PaTry3* (GU338029.1). Black boxes indicate absence or low amount/activity of particular isoenzymes. Arrows on the left

indicate transcripts possibly matching with isoenzymes. *PaTry3* is always highly expressed and putative corresponding isoenzyme is always present in activity gels. LMW: low molecular weight markers.

Fig. 4 Structural and functional diversification is better understood in Lepidoptera and fishes than in decapod crustaceans. NJ distance analysis tree showing trypsin isoform structural diversification in selected groups of insects, crustaceans, and fishes. Non-redundant trypsin sequences were aligned using the Clustal algorithm. The best-fit model of evolution (JTT+G, gamma shape parameter = 2.69) was selected and tree constructed with MEGA5 (Tamura et al. 2007). Topology robustness was tested with 1000 bootstrap replicates. Only bootstrap values higher than 50% are shown. Species and accession numbers are shown in the tree.

Fig. 5 Proposed model for food-mediated regulation of trypsin enzymes during the feeding cycle in the spiny lobster *Panulirus argus*. Bold dotted lines represent constitutive processes and bold continuous lines represent inducible events. High expression and secretion of trypsins occur in fasted lobsters, suggesting an advantage of excess of enzyme in anticipation to feeding. During feeding, soluble intact proteins stimulate enzyme secretion to attain maximal activity in the gastric chamber four hour after ingestion. Through the progression of digestion, trypsin phenotype (composition of isoenzymes) significantly affects the efficiency of protein hydrolysis. Later, protein digestion end-products induce the expression of the most abundant isoforms (*PaTry3*) in the digestive gland, together with the constitutive expression of the other isoforms, most likely as preparation for the next feeding. Superscripts represent research papers where the indicated processes were studied in *P. argus*: ¹Perera et al. 2008a; ²Perera et al. 2008b; ³Perera et al. 2010a; ⁴Perera et al. 2010b; ⁵Perera et al. 2012a; ⁶Perera et al. 2012b

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Trypsin phenotypes

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