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Changes in digestive enzymes through developmental and molt stages in the spiny lobster, *Panulirus argus*

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ABSTRACT

Changes in major digestive enzymes through developmental and molt stages were studied for the spiny lobster *Panulirus argus*. There were significant positive relationships between specific activity of trypsin and amylase enzymes and lobster size, whereas esterase and lipase specific activities decreased as lobsters aged. No relationship was found between amylase/trypsin ratio and lobster size. Positive trends were found, however, for trypsin/lipase and amylase/lipase ratios. Results suggest that changes in enzyme activity respond to the lobsters' physiological needs for particular dietary components although multivariate analysis suggested that enzyme activities could be not totally independent of diet. On the other hand, the pattern of changes of major enzyme activities through molt cycle was similar for most enzymes studied. Following molt, trypsin, chymotrypsin, amylase, and lipase activities gradually increased to maximal levels at late intermolt (C_4) and premolt (D). There were no variations in the electrophoretic pattern of digestive enzymes through developmental and molt stages and thus, it is demonstrated that regulation is exerted quantitatively rather than qualitatively. Further studies on the effect of other intrinsic and extrinsic factors on digestive enzyme activities are needed to fully understand digestive abilities and regulation mechanisms in spiny lobsters.

1. Introduction

Spiny lobsters are ecologically key predators in benthic communities of tropical and temperate seas (Lipcius and Eggleston, 2000) and support important fisheries all around the world. Additionally, tropical species (e.g. *Panulirus argus*, *Panulirus ornatus*) continue to attract great interest for aquaculture, especially for the growout of post-pueruli collected from the wild. Cage growout of spiny lobsters *P. ornatus* in Vietnam accounted for around US$ 40 million in 2004 (Thuy, 2004) and US$ 100 million in 2006 (Jones and Williams, 2007).

Knowledge of digestive capacities of spiny lobsters through their complex life cycle is limited to a few studies although it is relevant from ecological and aquaculture viewpoints. After hatching, pelagic larvae (phyllosoma) drift in ocean waters for several months feeding on plankton. Information on the natural diet of phyllosoma is scarce but phyllosoma from different palinurids have been successfully reared in laboratory on a diet of *Artemia* and other seafood. Final-stage phyllosoma larvae molt into the colorless lobster-like pueruli and migrate into shallow waters such as mangrove areas for settling to the bottom. There is evidence (Nishida et al., 1990; Wolfe and Felgenhauer, 1991; Lemmens and Knott, 1994; Nishida et al., 1995; Abronhosa and Kittaka, 1997) that pueruli do not feed but rather exhibit secondary lecithotrophy. Shortly after settlement, *P. argus* pueruli molt to become post-pueruli and start to feed on a wide variety of invertebrates such as crustaceans (copepods), holothurians, foraminiferans, and sponges (Lalana and Ortiz, 1991). The natural diet of juvenile and adult *P. argus* comprises mainly gastropods but also bivalves, chitons, anomurans, brachyurans, and sea urchins, depending on availability in the wild (Colinas-Sanchez and Briones-Foorzan, 1990; Herrera et al., 1991; Cox et al., 1997). Ontogenetic variations in digestive enzymes activities in spiny lobsters have been presented before only for *Jasus edwardsii* (Johnston, 2003). However, many other studies have shown that digestive enzymes in Crustacea vary according to the developmental stage. Still, there is little information on the forces that drive these variations and the level at which regulation occur.

Additional information on the physiological meaning of enzyme variations and regulation mechanisms can be obtained from the study of the molt cycle. The molt cycle drives extensive behavioral, integumentary, physiological, and biochemical changes in crustaceans.

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Besides its role in digestion, the digestive gland or hepatopancreas actively participates in the molt cycle, being the major site for storage glycogen, fats, and calcium during premolt and thus, in the mobilization of these reserves when needed in subsequent molt stages. Enzyme variations during the molt cycle of crustaceans have been studied for some species (Bauchan and Mengeot, 1965; Van Wormhoudt, 1974; Fernández et al., 1997; Fernández-Gimenez et al., 2001, 2002) and results are to some extent contradictory. We are aware of no previous reports for spiny lobsters.

Enzymes present in the digestive tract of spiny lobsters have been studied in *Panulirus japonicus* (Galgani and Nagayama, 1987; Iida et al., 1991), *J. edwardsii* (Johnston, 2003), *Panulirus interruptus* (Celis-Gerrero et al., 2004; Navarrete del Toro et al., 2006), and recently in *P. argus* (Perera et al., 2008). With few exceptions, such studies have covered mainly biochemical aspects of enzymes.

This study was undertaken to examine developmental and molt stage variations in the main digestive enzymes of *P. argus*. Results are correlated with feeding behavior and reveal the capacity of the lobster digestive system to meet the physiological requirement of each developmental or molt stage. Also, results suggest the level at which the regulation of enzyme activity is exerted.

2. Materials and methods

2.1. Staging of lobsters

The distal half of a single pleopod was excised from each lobster upon capture in the wild and used within minutes of removal, to avoid misleading epidermal retraction, for staging according to Lyle and MacDonald (1983).

Differentiation among stage C subdivisions is difficult based only on histology because of variations in onset, rate, and degree of formation of the different cuticle layers, as has been shown in the spiny lobster *Panulirus marginatus* (Lyle and MacDonald, 1983). After molt, clawed and spiny lobsters harden progressively through the different stages (Aiken, 1980; Quackenbush and Herrnkind, 1983) and although shell hardness is unreliable as a unique indicator of molt stage, it was conveniently used herein to subdivide arbitrarily stage C as in Aiken (1980). In our study, all C substages were considered as "intermolt" and the term "molt" was used in synonymy with ecdysis. Due to limited availability of premolt lobsters, data from premolt were pooled. In summary, for this study lobsters were classified into premolt (D), molt (E), postmolt (AB), and intermolt stages (C_2, C_3, C_4).

2.2. Sample collection and preparation of extracts

Lobsters were collected in the Golf of Batabanó, Cuba. Post-larvae (post-pueruli) were collected by sandwich floating collectors, and juveniles and adults were collected by diving. Small juveniles could not be collected due to difficulties in locating them. Only late intermolt (C_4) lobsters were used to study developmental trends in enzyme activities whereas lobsters at all other stages were analyzed for variations during the molt cycle. Animals were anesthetized by placing them on ice for 10 min and then dissected to collect the hepatopancreas. Samples were immediately frozen in liquid nitrogen, lyophylized, and then stored at −20 °C until used. Before analysis, the powders were homogenized in cold distilled H_2O and centrifuged at 4 °C at 8000 *g* for 15 min. Supernatants were immediately used for enzyme assays and electrophoresis.

2.3. Assays for enzyme activity

All chemicals used in this study were reagent grade and were obtained from Sigma except DMSO which was purchased from Merck. The concentration of crude extracts for each assay was adjusted to obtain linearity of enzyme activities with respect to both protein concentration and time. Assays were always run in duplicate and enzyme activities were expressed as change in absorbance per minute per milligram of protein (ΔAbs min^{-1} mg $protein^{-1}$) or per gram of dry tissue (ΔAbs min^{-1} g dry $tissue^{-1}$). The protein content of extracts was measured according to Bradford (1976) using BSA as standard.

2.3.1. Trypsin and chymotrypsin-like activities

Trypsin-like activity was measured using 1.25 mM BApNA in 0.2 M Tris–HCl, 20 mM $CaCl_2$, pH 8.4. Chymotrypsin-like activity was measured with 0.1 mM SApNA in the same buffer. Substrate stock solutions of BApNA (125 mM) and SApNA (10 mM) were prepared in DMSO and brought to working concentration by diluting with buffer prior to assaying. In a 96-well microplate, 10 μL of enzyme extract were mixed with 200 μL of respective substrate, and liberation of *p*-nitroaniline was kinetically followed at 405 nm in a microplate reader Multiscan EX (Thermolab Systems).

2.3.2. Non-specific esterase activity

Esterase activity in extracts was assessed by the hydrolysis of 0.3 mM *p*-nitrophenyl butyrate (*p*-NPB) according to Gilham and Lehner (2005) with slight modifications. Stock solutions (100 mM) were prepared for the *p*-nitrophenyl esters in CH_2Cl_2 and diluted immediately prior to assaying with 20 mM Tris–HCl, 150 mM NaCl, pH 8.0. For assays, 20 μL of enzyme extract were mixed with 200 μL substrate solution in 96-well microplate and the liberation of *p*-nitrophenol was measured kinetically at 405 nm in a microplate reader.

2.3.3. Lipase activity

Lipase activity was measured using ß-naphthyl caprylate in DMSO as the substrate. The assay mixture contained: 100 μL of 100 mM sodium taurocholate, 900 μL of 50 mM Tris–HCl pH 7.5, 10 μL enzyme extract, and 10 μL of substrate stock solution (100 mM). The reaction mixture was incubated for 30 min at 37 °C for the reaction to proceed and then 10 μL of 100 mM Fast Blue BB in DMSO was added. The reaction was blocked with 100 μL TCA 12%. Finally, 1.35 mL of 1:1 (v:v) ethyl acetate/ethanol solution was added and absorbance was recorded at 510 nm.

2.3.4. Amylase activity

Amylase activity was determined according to the Somogy-Nelson method using soluble starch (2% w:v) as substrate, as described in Robyt and Whelan (1968). Briefly, 20 μL of enzyme extract and 125 μL of buffer pH 5 were incubated with 125 μL of substrate for 30 min. Activities were measured by calculating the reducing sugars released at 600 nm.

2.4. Zymograms of digestive enzymes

Substrate-SDS-PAGE (5% stacking gel, 13% separating gel) was used to examine the composition of proteases in digestive tract (García-Carreño et al., 1993) using casein as the substrate. Samples were neither boiled nor treated with mercaptoethanol before loading into the gel and they were run in a vertical electrophoresis device (Hoeffer SE260, 8×10×0.75 cm). Bands were revealed as described before (Perera et al., 2008).

Substrate-SDS-PAGE for amylase was performed on 5% stacking gel and 12% resolving gel in the same conditions as for proteases. Gels were immersed in a starch solution (1%) at pH 6 for 60 min and then stained with iodine/KI solution (10%) (Perera et al., 2008).

Zymograms for esterases were performed under native conditions (5% stacking gel and 8% resolving gel) as described by Perera et al. (2008). High degree of polymorphism for esterases harms us to analyse variation through developmental and molt stages.

2.5. Statistical analysis

Regressions describing the relationship between the enzymes activities and the body size (carapace length, CL) were obtained by the

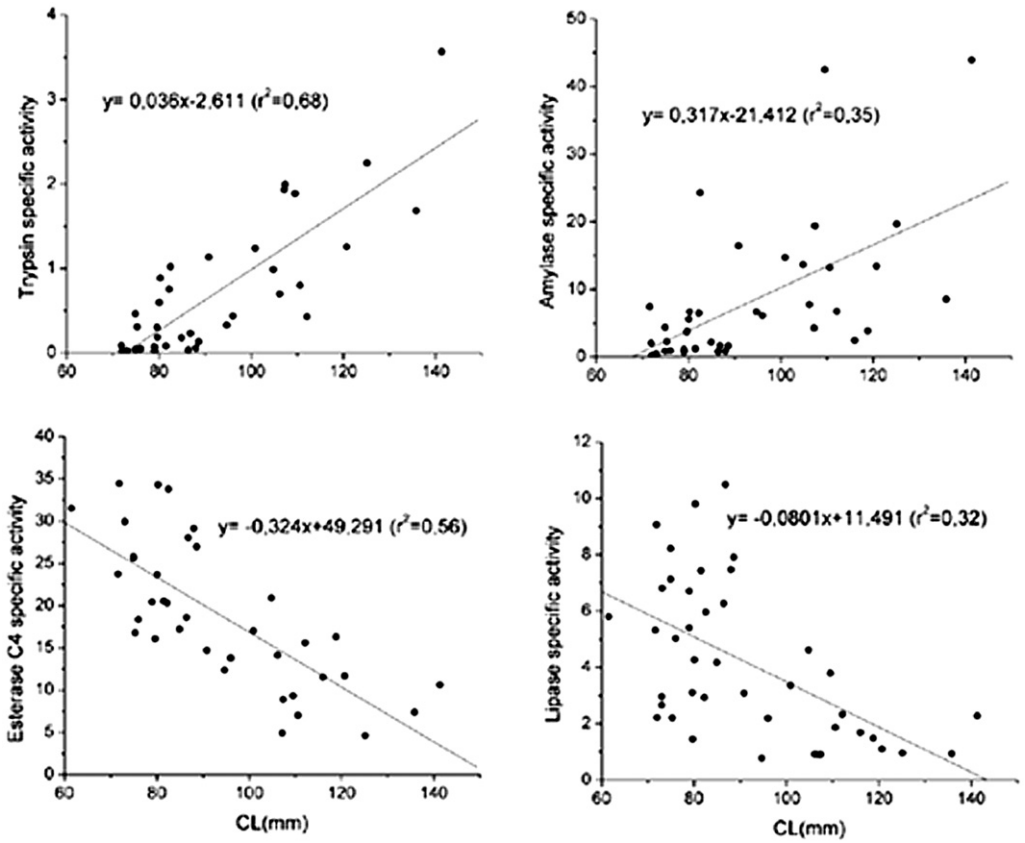

Fig. 1. Specific activity (ΔAbs min^{-1} mg protein^{-1}) of trypsin, amylase, esterases and lipase in the digestive gland of intermolt *P. argus* from late juvenile to adult. Substrates were BApNA for trypsin, starch for amylase, *p*-nitrophenyl butyrate for esterases and ß-Naphthyl caprilate for lipase. Data points are mean of duplicate measurements for an individual animal.

least-squares method. The significance of regression slope *b* was tested by analysis of variance. R^2 values were calculated as a measure of relative goodness of fit of regression curves. Data points were means of duplicate assays for each lobster.

Similarities in digestive capacities among developmental stages were explored by multivariate analysis, taking into account all enzymes studied (multiple dimensions). A hierarchical and agglomerative method was selected and the number of clusters was not pre-established, but randomly generated by the software. Single linkage was selected as amalgamation rule and Euclidean distances were used for computing distances between objects in the multi-dimensional space.

One-way ANOVAs were performed to test for differences among different molt stages. Data were previously checked for normality and homogeneity of variance by Kolmogorov–Smirnov and Levine's tests, respectively, and $\log_{10}$ transformation of data was necessary to achieve requirement for analysis. SNK tests were used to determine post-hoc differences among means. The software Statistica 6.0 (Statsoft, Inc.) was used for all tests, performed with α=0.05.

The ratio of amylase to protease activity was estimated from the amylase: trypsin ratio as in Lovett and Felder (1990) for shrimp and Johnston (2003) for spiny lobsters. Trypsin accounts for almost 60% of protease activity in *P. argus* (Perera et al., 2008). Additionally, trypsin/lipase and amylase/lipase ratios were calculated.

3. Results

3.1. Postlarvae (post-pueruli) to first juvenile stages: trends in digestive enzyme activity

There were no trends in the relationship between specific enzyme activities (trypsin, amylase, esterase and lipase) and lobster size for animals from 6 to 20 mm CL, i.e. from first post-pueruli to first juvenile stages. Mean specific activities as ΔAbs min^{-1} mg protein^{-1} were as follows: trypsin (6.5±2.36), chymotrypsin (3.1±1.24), amylase (12.2±7.02), esterase C_4 (14.7±12.1), and lipase (1.2±0.63).

3.2. Juvenile to adult: trends in digestive enzyme activity

There was a significant positive relationship between specific activity and lobster size for trypsin and amylase enzymes in juveniles and adults. (Fig. 1, Table 1). Trypsin and amylase activities expressed as units per gram of dry tissue followed the same trends (not shown). Chymotrypsin activity also increase with age (F=15.2, $p \leq 0.001$) but r^2 was extremely low (0.29) thus considered as non-relevant. Esterase and lipase activities exhibited negative linear relationships with

Table 1
Summary of statistics for the calculated regressions

Regressions	*b*	*a*	*N*	*r*	*F*
Enzyme activities on size					
Trypsin on CL	0.036	−2.6109	38	0.82	76.19***
Amylase on CL	0.3168	−21.412	41	0.59	20.98***
Esterase C_4 on CL	−0.3244	49.291	37	−0.75	44.47***
Lipase on CL	−0.0801	11.491	42	−0.56	18.40***
Enzymes ratios on size					
A/L on CL	0.2231	−16.668	41	0.74	48.12***
T/L on CL	0.0286	−2.2011	38	0.78	55.46***
A/T on CL	−0.1217	25.652	38	0.36	ns

Enzymes activities as specific activities (ΔAbs min^{-1} mg protein^{-1}). CL: cephalothorax length. A/L: amylase to lipase ratio. T/L: trypsin to lipase ratio. A/T: amylase to trypsin ratio. Slope (*b*) and intercept (*a*) are from the regression model $Y=a+bx$. *N*: number of observations, *r*: correlation coefficient. *F*: variance ratio of regression and residual mean squares. The values for *F* correspond to a probability of $p \leq 0.001$ (***). ns: not significant.

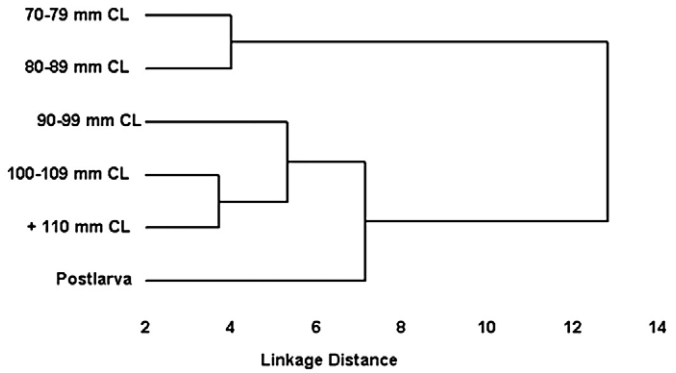

Fig. 2. Result of the cluster multivariate analysis of all digestive enzyme activities analysed at different developmental stages.

lobster size (Fig. 1, Table 1). Esterase and lipase activities expressed as units per gram of dry tissue followed the same trends (not shown).

Multivariate analysis revealed two main clusters, the one for lobsters of less than 90 mm CL and the one for bigger animals. Postlarva appear more related to old than to young lobsters (Fig. 2).

3.3. Enzyme ratios

There was no significant ontogenetic trend in amylase/trypsin ratio between juvenile and adult lobsters. Positive trends were found for trypsin/lipase and amylase/lipase ratios (Table 1).

3.4. Molt cycle variation in digestive enzyme activities

The pattern of changes in enzyme activity through the molt cycle was similar for all enzymes studied except esterases. After molt, trypsin, chymotrypsin, amylase, and lipase activities gradually increased to maximal levels at late intermolt (C_4) and premolt (D) (Fig. 3). Lipase and amylase activities significantly dropped near or at molt while proteases dropped after molt. Most enzyme activities remained relatively low until early intermolt (C_2) (Fig. 3). Esterase activity did not significantly vary through the different molt stages except for higher values at late intermolt (C_4) (Fig. 3).

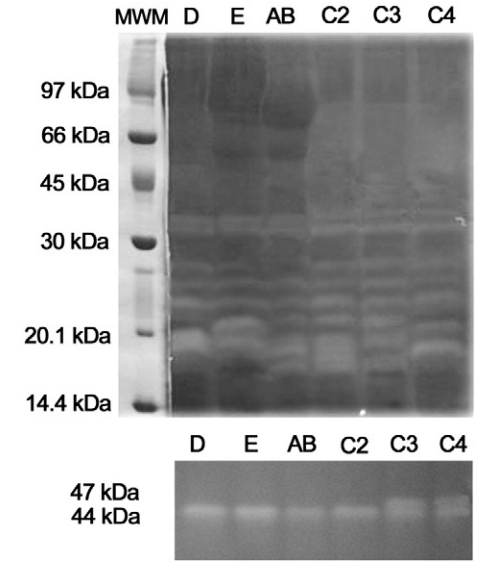

Fig. 4. Isoenzyme pattern of proteases (top panel) and amylases (button panel) at different molt stages. Variations correspond to polymorphism reported by Perera et al. (2008) for trypsin (proteases of lower MW in the top panel) and amylase, and not to molt stages. For example: many individuals in C_3 and C_4 present only the 44 kDa isoform of amylase whereas individuals from D to C_2 can express also the 47 kDa enzyme (not shown).

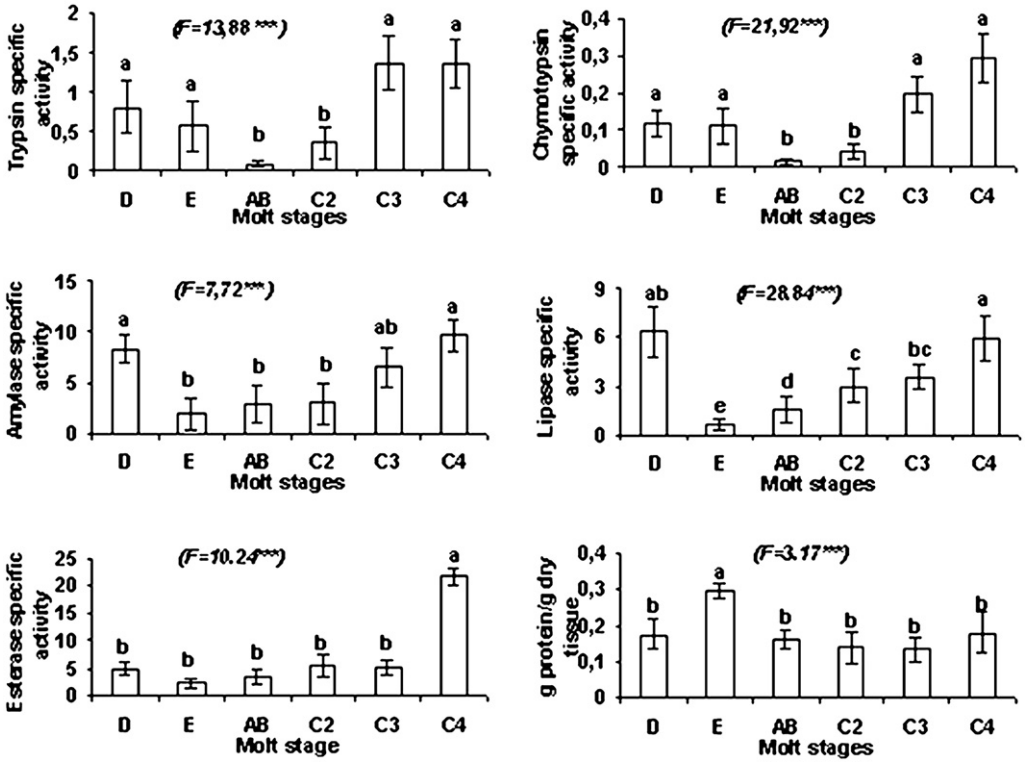

Fig. 3. Enzyme activities and total soluble protein variations in the hepatopancreas throughout the molt cycle of *P. argus*. *F* values for one-way ANOVAs are shown and *** means statistical differences at $p \leq 0.001$. Different letters above the bars in each figure represent stages that differ by SNK tests at $p \leq 0.05$. Number of individual analyzed for each molt stage was: 5 individuals in D, 3 individuals at E, 5 individuals in AB, 5 individuals in C2, 6 individuals in C3, and 14 individuals in C4.

Variations described above refer to specific activity of enzymes. No significant variation was found in the protein content of the digestive gland among molt stages, except for higher values at molt (Fig. 3). Activities of the different enzymes through the molt cycle are similar than above if expressed as activity units per g of dry tissue (not shown).

3.5. Changes in the isoenzyme pattern of digestive enzymes

There were no variations on the electrophoretic pattern of proteases and amylase through the molt cycle (Fig. 4), except those correspond to the polymorphism previously reported for digestive enzymes in *P. argus* (Perera et al., 2008). Also, postlarva and adults present the same electrophoretic pattern of digestive enzymes (not shown).

4. Discussion

No variations were detected in protein content of hepatopancreas among developmental and molt stages of *P. argus*, except for a significant increase at molt. Thus, variations in specific enzyme activity were assumed to be unaffected by protein content of extracts and reflect actual variation in digestive capacities.

4.1. Variations among developmental stages

After an extended period of feeding on plankton in ocean waters, spiny lobster *P. argus* larvae metamorphose into pueruli, which then migrate into shallow waters to settle. The short-lived pueruli stage do not feed and their digestive system experiences a series of changes adapted for benthic feeding behavior as described for *J. edwardsii* (Nishida et al., 1990), *P. argus* (Wolfe and Felgenhauer, 1991), and *P. cygnus* (Lemmens and Knott, 1994; Abronhosa and Kittaka, 1997). This phase does not feed and although the presence of digestive enzymes has been demonstrated (Johnston, 2003), their meaning is still poorly understood. Our work focuses on feeding stages.

There were no trends in specific enzyme activities (trypsin, amylase, esterase and lipase) in lobsters from 6 to 20 mm CL (from post-pueruli to first juvenile stages). The most frequent items in natural diet of *P. argus* post-pueruli are copepods, holothurians, foraminiferans, and sponges (Lalana and Ortiz, 1991). All *P. argus* post-pueruli stages feed on similar items but brachyurans do not appear in stomachs until post-pueruli of 8 mm CL and increase their importance in diet of larger post-pueruli (11–13 mm CL) (Lalana and Ortiz, 1991). Since this progressive change in diet does not correspond to any trend in enzyme activities, it is proposed that activities of enzymes present since early post-pueruli stages are enough for an efficient digestion of a varied diet. Changes observed in diet composition could reflect just a steady adaptation to preying on larger items. Specific activities for amylase, esterase, and lipase in *P. argus* post-pueruli are similar to those found in adults, which supports the statement above. However, values for proteases in post-pueruli are above the ones for juvenile and adults. Since prey items of post-pueruli are rich in highly digestible proteins, this result could indicate that enzyme activities respond to the physiological requirements of this stage rather than to a compensatory mechanism in the presence of a deficient diet. The requirement for dietary protein has been determined to be very high (45%) in post-pueruli *P. argus* (Fraga, 1996), whereas the estimates are at or below 35% in juveniles (Perera et al., 2005).

Variations in digestive enzyme activities could then reflect changing physiological requirements as lobsters grow. Metabolism of juvenile *P. argus* relies on protein for energy and, when feeding on major natural prey (e.g. gastropods, bivalves, and crustaceans), lipids can somewhat spare proteins from oxidation (Díaz-Iglesias et al., 2002). Higher activity of enzymes involved in lipid digestion in young lobsters could ensure a higher proportion of ingested proteins to be channeled toward growth in correspondence with the higher growth rate of juveniles. The same idea can be drawn from Trypsin/Lipase ratio.

Amylolytic activity was measured here at the optimal pH, which is acidic and thus distant from the physiological pH in the digestive gland. However, high amylolytic activity is present in the gastric juice of *P. argus* (Perera et al., 2008) where acidic pH occurs (5.9±0.2, mean±standard deviation, unpublished results) as in other spiny lobsters (Johnston, 2003; Navarrete del Toro et al., 2006). Thus, amylolytic activity measured in the hepatopancreas at acidic pH is likely a good indicator of that occurring in the gastric chamber. The positive trends observed for amylase suggest that the efficiency of carbohydrate digestion increases as lobsters age. Since there is no evidence of a significant role of carbohydrates in energy metabolism of *P. argus* (Díaz-Iglesias et al., 2002; Perera et al., 2005), whether this behavior of amylase activity could energetically pay off for the drop in lipolytic activity in older lobsters remains to be studied, but it is unlikely. No trend in Amylase/Trypsin ratio was observed in this work for *P. argus* and in Johnston (2003) for *J. edwardsii*. An alternative hypothesis is related to the bigger and stronger exoskeleton in larger lobsters and a possible increase in glycogen needs for chitin synthesis after molt as animals grow.

Interestingly, small *J. edwardsii* exhibited higher amylase activity than large lobsters (Johnston, 2003) which is the opposite of our finding for *P. argus*. Feeding habits of *J. edwardsii* are somewhat different from those of *P. argus* (Edmunds, 1995). It is not known to what extent these differences can be explained by a different diet or by a distinct specie-specific pattern of expression of enzymes. Our results indicate that changes in enzyme activities are developmental clued but since our lobsters were not grown in captivity on a single diet but sampled from the wild, further studies are required to determine if activities can be affected by changes in the composition of diet. Despite significant trends, variations in enzyme activities can not be explained to a great extent by the increase in size even for trypsin where lobster size only accounts for 68% of the variation. Our results indicate that factors other than size could influence digestive enzyme activities in lobsters. Multivariate analysis suggested that digestive enzyme activities appear to be not totally independent of diet. Two main clusters were obtained and a size of 90 mm CL, correspond to a size where some prey items appear for the first time in stomachs (e.g. *Strombus gigas*) and other big gastropods become more frequent preys (Herrera et al., 1991).

Cross-reactivity can generate a false association between lipase and esterase activities but the substrates employed in this study discriminate the patterns of lipase and esterase activities through the molt cycle (see below), suggesting that observed connection between activities correspond to the physiological role of these enzymes rather than to an artifact of methodology. On the other hand, although several proteolytic enzymes exhibit esterase activity, trends for proteases and esterases were reverse. Finally, the method used for esterases does not discriminate digestive esterases from intracellular esterases, but the former were assumed more abundant than the latter.

To our knowledge, there is only one previous study on ontogenetic variations of digestive enzymes in spiny lobsters. Johnston (2003) found a positive relationship between total activity (units per digestive gland) of all enzymes and size in the spiny lobster *J. edwardsii*, coinciding with an increase in size of digestive gland. However, she observed no ontogenetic trend in specific activities except for negative correlations for amylase and laminarinase. In contrast, we report here that trypsin and amylase specific activities tend to increase with lobster size whereas esterase and lipase specific activities fall as lobsters age.

4.2. Molt cycle variations

Variations in the activity of digestive enzymes in *P. argus* resemble the foraging and feeding patterns observed in previous studies.

Feeding rate in *P. argus* gradually increases during intermolt to high levels at late C_4 and early D. Then, it gradually decreases to minimal rates at D_3-B_1. Finally, food consumption rises again at late postmolt (B_2) and early intermolt (C_1) (Lipcius and Herrnkind, 1982).

During late stage C, few glycogen granules are evident in the hepatopancreas of *P. argus* but their number increases during stage D both in the hepatopancreas and epidermis. This glycogen disappears some days after molt, indicating glycogen is a necessary precursor for chitin formation (Travis, 1955). In this scenario, the observed increase in amylase activity at late intermolt and during premolt might enhances carbohydrate assimilation and formation of glycogen reserves.

The lipid content of the digestive gland is positively correlated with growth in spiny lobsters (Johnston et al., 2003). Lipids are continuously stored in the hepatopancreas of *P. argus* during intermolt and early premolt (Travis, 1955) and in correspondence high lipolytic activity occurs in these stages. Also, during late premolt some glycogen is thought to come from the conversion of fat stored in the hepatopancreas (Travis, 1955).

Protease activity increases gradually following molt to maximal activity at late intermolt in correspondence to the carnivorous behavior of lobsters. Protease activity remains high during premolt and thus allowing the animal an efficient use of dietary protein during the feeding phase of premolt. Results in other crustaceans indicate that active synthesis of digestive enzymes remains during early premolt. Chymotrypsin mRNA (Van Wormhoudt et al., 1995) and trypsin mRNA (Klein et al., 1996) increase during premolt in the shrimp *L. vannamei*. Also, the high protease activity in premolt lobsters could be associated to the storage of enzymes produced. Both active synthesis and storage in premolt could promote the high activities observed.

Esterase enzymes are involved in different functions besides digestion and perhaps that is why there is not a tight connection between enzyme activity and feeding behavior.

In general, the activities of the main digestive enzyme in *P. argus* were highest at late intermolt and premolt. Our results totally or partially agree with those observed in *Palaemon serratus* (Van Wormhoudt, 1974) and *Penaeus notialis* (Fernández et al., 1997) but differ from other studies. Bauchan and Mengeot (1965) reported for *Carcinus maenas* high protease activity in postmolt and intermolt and low activity during premolt. In *Pleoticus muelleri*, the activities of trypsin and chymotrypsin were highest in postmolt (Fernández-Gimenez et al., 2001) whereas in *Artemesia longinaris* they were highest during intermolt and dropped at premolt (Fernández-Gimenez et al., 2002). Many factors can contribute to these contradictory results in different crustacean species, such as criteria for staging, duration of each stage, and species-specific patterns of expression and synthesis of digestive enzymes. Klein et al. (1996) studied trypsin expression through the molt cycle of *L. vannamei* and found highest mRNA levels at premolt and lowest levels near molt. Trypsin expression gradually increased, especially during the intermolt. These results compare better to those obtained in this work.

There were no variations on the electrophoretic pattern of digestive enzymes through the molt stages, except those correspond to the polymorphism previously reported for digestive enzymes of *P. argus* (Perera et al., 2008). Also, postlarva and adults present the same electrophoretic pattern of digestive enzymes (not shown). Individual enzymes in some fishes develop independently during ontogenesis with variations related to species, temperature and feeding habits (Kolkovski, 2001, Rathore et al., 2005). Our results shown that the lobsters *P. argus* express all proteases and amylase enzymes (Fig. 4) at first benthic feeding and all molt stages. Several studies have reported the influence of different environmental factors on trypsin mRNA levels, but to our knowledge, never the absence of isoforms. Sanchez-Paz et al. (2003) and Sainz et al. (2004) have concluded that trypsin activity in *L. vannamei* is regulated quantitatively at the transcription level, but not qualitatively. The lobster *P. argus* appears to regulate trypsin (and others digestive enzymes) activity in a similar fashion. Additionally, the high degree of polymorphism for trypsin enzymes in *P. argus* (Perera et al., 2008) provides several points for this regulation to occur. Yet, electrophoresis has been the method of choice in most studies and thus only isoforms with different electrophoretic mobilities could be detected. Five trypsin-like enzymes were found in gels by Perera et al. (2008) but current studies in our laboratory have shown that after gel filtration and anion-exchange chromatography, up to seven trypsin-like enzymes occur in *P. argus* digestive gland (unpublished results). Sainz et al. (2004) showed that the three isotrypsins of *P. vannamei* have different kinetic properties. The high amount of trypsin isoforms makes spiny lobsters good models for studying regulation mechanisms in decapod crustaceans.

Our results suggest that changes in the activities of digestive enzymes of *P. argus* across developmental and molt stages respond to the lobsters' physiological needs for particular dietary components and thus, are thought to be closely regulated by internal factors. It has been reported that endocrine cells in the midgut of the cockroach are stimulated to synthesize and secrete crustacean cardioactive peptide (CCAP) by nutrients, and CCAP then up-regulates the activity of digestive enzymes like α-amylase and proteases (Sakai et al., 2006). However, Chung et al. (2006) fund that the expression patterns of CCAP mRNA in crustaceans thoracic ganglia throughout the moult cycle shown few changes. Yet, other internal signal like cholecystokinine-like, secretin-like, gastrin-like substances and ecdysteroids could be related with digestive enzyme regulation during development and the molt cycle. Also here it is demonstrated that this regulation is exerted quantitatively rather than qualitatively. Further studies are needed on the effects of other intrinsic and extrinsic factors on digestive enzyme to fully understand digestive abilities of spiny lobsters and regulation mechanisms.

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