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Ontogenetic changes in digestive enzymatic capacities of the spider crab, *Maja brachydactyla* (Decapoda: Majidae)

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ABSTRACT

Ontogenetic changes in digestive capabilities were analyzed in larvae and first juveniles of the spider crab *Maja brachydactyla*. Activities of five proteinases (total proteases, trypsin, chymotrypsin, pepsin-like and aminopeptidase), three carbohydrases (amylase, maltase and chitinase), an esterase and an alkaline phosphatase were studied to evaluate digestive enzyme profiles of the species. Both quantitative (spectrophotometry and fluorometry) and qualitative (SDS-PAGE) approaches were used. All assayed enzymes were active from hatching (zoea I-ZI) throughout larval development and in first juveniles. Significant variations during ontogeny were found only in total activities likely as a consequence of digestive system development. Specific activity varied little over ontogeny, being significant only for chitinase. Total proteases, trypsin and pepsin-like activities showed a similar pattern of increase as larval ontogeny advanced, decreasing significantly in juveniles. Chymotrypsin continued to increase, showing maximum activity after metamorphosis. Proteinase zymograms confirmed strong proteolytic activity in first zoeas, with increasing bands over the course of ontogeny, decreasing after metamorphosis. A group of bands with high molecular mass was specific to larval stages. Amylase and maltase showed a parallel pattern of continuous increase of total activity as development advanced. Gel-SDS-PAGE showed unchanged patterns of amylase activity in first zoeas of different ages and the most complex set of bands during larval ontogeny in second zoea. Esterase total activity increased significantly as ZI's aged likely reflecting introduction of a lipid-enriched diet. The importance of lipid accumulation at the beginning of ontogeny was also confirmed by the protease/esterase and amylase/esterase activity ratios, which decreased from hatch to late ZI and might be explained as an adaptation, ensuring the next molt. The results suggest that larvae of *M. brachydactyla* are capable of digesting a variety of dietary substrates as soon as they hatch.

1. Introduction

Fulfilment of nutritional requirements is essential during larval development of all marine organisms, including crustaceans. Hence, digestion becomes a key process in metabolism, since it determines availability of the nutrients needed for biological functions (McConaugha, 1985). During development, important ontogenetic changes take place in the structure and physiology of the digestive system of decapod crustaceans with adaptation to different food types (Factor, 1981). Zoeal stages of brachyuran crabs are adapted to planktonic life, having complex morphology in their external mastication organs but a relatively simple cardiac stomach where simple longitudinal cuticle folds in the inner surface helps in mechanical maceration of food. With development, mandible structure becomes less complex, but in contrast, additional longitudinal cuticle ridges, ossicles and teeth appear as robust skeletal elements in the cardiac chamber (gastric mill). These structural changes are accompanied by an increase in the hepatopancreas (midgut gland) volume, which results in an increase of enzymatic secretion and efficiency of extracellular digestion (Anger, 2001). Thus, crustacean larval digestive physiology is closely related to feeding strategies and trophic position of the individuals (Jones et al., 1997; Le Vay et al., 2001). This results in direct relationships between the presence/absence, activity ratios and concentration of the main digestive enzymes and the relative importance of major nutrients in the diet (Johnston, 2003).

In this context, the analysis of digestive enzyme activities has proven to be an effective approach for understanding digestive physiology and determining the nutritional characteristics of natural food in a great variety of decapod crustaceans like shrimps and prawns (Kamarudin et al., 1994; Lemos et al., 2002; Lovett and Felder, 1990), lobsters (Biesiot and Capuzzo, 1990; Johnston et al., 2004; Kumlu and Jones, 1997; Perera et al., 2008b) and crabs (Harms et al., 1991, 1994; Hirche and Anger, 1987; Rotllant et al., 2008; Saborowski et al., 2006).

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Major proteases (mainly trypsin-like protease), carbohydrases and esterases have been found in all the crustacean species studied so far (Jones et al., 1997). The general trend shows higher protease activity in those species and developmental stages which feed on phytoplankton, decreasing in the omnivorous and carnivorous feeders (Le Vay et al., 2001). Besides trypsin and amylase, some studies have demonstrated the presence of a great variety of other proteases and carbohydrases, such as chymotrypsin, cystein proteinases, aminopeptidase, chitinase and alkaline phosphatase (Frank et al., 1975; Johnston, 2003; Lovett and Felder, 1990; Tesche and Saborowski, 2005), which indicates the complexity of digestive processes in this group of species.

The spider crab *Maja brachydactyla* has a high economic and ecological significance, supporting fisheries along the NE Atlantic coasts (Spain, Portugal, France, Ireland and UK). The high fishing pressure tolerated by populations of this crab (Freire et al., 2002), together with its growth and reproductive characteristics (González-Gurriarán et al., 1995) defines the species as potentially interesting for aquaculture (Andrés et al., 2007). *M. brachydactyla* has a short larval development characterized by three stages: two zoeas (ZI and ZII) and one megalopa (M) (reviewed by Guerao et al., 2008). Studies on its larval ecology and distribution are scarce (for references, see Martin and Planque, 2006) and no work has been published concerning the natural diet of planktonic stages. However, laboratory spawning and larval rearing of this species have been successful with a wide variety of diets (Andrés et al., 2007; Urcera et al., 1993). Recently, Rotllant et al. (2008) reported digestive activities of trypsin, amylase and esterase in larval and first juvenile stages of this species, using the larvae of *M. brachydactyla* as a model for comparison between two different techniques of enzymatic analysis, spectrophotometry and fluorometry. The findings agreed with results obtained for trypsin and amylase activities in another spider crab species (*Hyas araneus*, Hirche and Anger, 1987). These studies suggest that in the wild, larvae of *M. brachydactyla* might be able to ingest and digest live prey as soon as they hatch, showing an opportunistic feeding behaviour in which prey capture occurs by encounter probability without any selection of food items. Larval stages of *M. brachydactyla* are thus considered omnivores.

At this point, our knowledge of *M. brachydactyla* requires deeper insight into its digestive biochemistry since adequate larval nutrition is essential for the future of profitable aquaculture of this species. Effectiveness of feeds administered depends on our knowledge of how larvae use various components of their diet (Carrillo-Farnés et al., 2007). The aim of this study is to provide insight into the larval digestive physiology of cultured *M. brachydactyla* by examining the profiles of the main digestive enzymes and identifying their quantitative and qualitative changes over the course of development.

2. Materials and methods

2.1. Broodstock maintenance and sampling

Adult specimens of *M. brachydactyla* were captured at the Atlantic North-West coast of Spain and brought to the IRTA (Sant Carles de la Ràpita, Tarragona, Spain) by surface transport in high humidity containers at temperatures of about 8 °C. At IRTA, broodstock was kept in 2000-L tanks connected to a recirculation unit, with constant salinity of 36‰ and temperature of 18 °C. Adults were fed fresh mussels (*Mytilus* sp., five times per week) and frozen crab (*Liocarcinus depurator*, twice per week).

Upon arrival, three adults were randomly chosen for sampling of their gastric juice (GJ) and hepatopancreas (HP) as follows. A syringe connected to a flexible cannula was introduced into the mouth of living adults in order to obtain 0.5 mL of fresh GJ from the digestive system. After the extraction of GJ, adults were anesthetized on ice for at least 10 min, dissected, and then a sample of HP was extracted. Samples were immediately frozen (−80 °C) and lyophilized prior to enzymatic analysis (SDS-PAGE).

2.2. Larval rearing and sampling

Active newly hatched larvae (ZI_0, subscript number indicating days post-hatch) were collected from broodstock tanks immediately after hatching and transferred to 500-L rearing tanks provided with aeration (initial stocking density: 60–100 larvae L^{-1}). Temperature and salinity were kept constant by means of recirculation units at 18 °C and 36‰, respectively, whereas photoperiod was natural (12 h light in early spring). Larvae were fed enriched *Artemia* metanauplii (EG strain and EasySelco, INVE, Belgium) throughout development and green water conditions were maintained throughout the culture cycle by adding *Tetraselmis chuii* and *Isochrysys galbana* to larval tanks every second day.

At every larval stage, including newly hatched larvae and first juvenile, a sample of 500 mg wet weight (between 120 and 1100 individuals depending on the stage of development) was collected, as follows: 0 days post-hatch (DPH) (ZI_0), 3 ± 1 DPH (ZI_3), 7 ± 1 DPH (ZII_7), 12 ± 1 DPH (M_{12}) and 18 ± 2 DPH (first juvenile crab, C_{18}). Larvae were then gently rinsed with distilled water and dried on filter paper and kept frozen (−80 °C) in 1.5 mL tubes until processed for enzyme analyses. In addition (due to the different analytical technique used for the detection of chitinase activity; see Section 2.3.10 in the enzymatic activity methodology), 16 individuals of each representative larval or first juvenile stage were collected individually in separate tubes (one individual per tube) for each of the above-mentioned sampling dates. Tubes containing the larvae were immediately frozen (−80 °C) and later lyophilized previous to the performance of fluorometric analysis (following Rotllant et al., 2008).

Measures of larval and first juvenile weight were performed at the same sampling time as enzymatic assays. For weight determination, six larvae per replicate ($n = 5$) were rinsed in distilled water and dried on filter paper for wet weight and water content estimation. After 24 h at 60 °C, dry weight (DW) was determined to the nearest 0.01 mg.

All analyses were carried out from 4 different hatches belonging to different females to get 4 independent replicates for every developmental stage.

2.3. Enzyme quantification

Two different groups of enzymes were assayed: a) extracellular enzymes and b) brush border enzymes linked to cell membranes. The extracellular enzymes assayed were total proteases, trypsin, chymotrypsin, pepsin-like (acid proteinases like enzymes belonging to the aspartic peptidases family, such as cathepsin D and E), amylase and esterase. Brush border (BB) enzymes assayed were aminopeptidase N, alkaline phosphatase and maltase.

Preparation of samples for both groups of enzymes was as follows: frozen samples were homogenized 5 min in 30 volumes (v/w) of ice-cold Tris-Mannitol (50 mM), HCl (2 mM) buffer at pH = 7 using an Ultra turrax T-25 (IKA® WERKE, Germany). Each homogenate was then divided in two different aliquots of 1.5 mL that were processed differently. Aliquots for extracellular enzyme assays were centrifuged for 5 min at 13,000 *g* (4 °C) and the supernatant stored after homogenization at −20 °C until enzymatic analyses were performed. Brush border quantification was described in Crane et al. (1979).

The aliquots were thawed and briefly centrifuged (10,000 rpm for 15 s) prior to enzymatic analysis and every sample was assayed in triplicate. All samples for a single enzymatic assay were run the same day. Blank controls in which reaction did not take place were introduced when needed. All the enzymatic activities except the chitinase were read in a Sinergy HT, BIO-TEK® spectrophotometer using either 48 (Nunclon 48, Nunc, Denmark) or 96 (F96 MicroWell™ Plates, Nunc) well flat bottom microplates. Chitinase activity was read

in a Fluoroskan reader (ThermoFisher Scientific; U.S.A.) using 96-well CLINIPLATE black flat bottom microplates (Thermo Scientific).

Total activity was defined as enzyme activity per individual (IU ind^{-1}). The number of larvae/juveniles in the homogenates was estimated by means of individual dry weight and water content calculations, considering the 500 mg original sample as a pool of individuals. Water-soluble protein content (WSP) was measured in both type homogenates (extracellular and BB) by the Bradford method (1976) using its assay dye reagent (Sigma) and bovine serum albumin (BSA, Sigma) as a standard. A great source of variation was found in total WSP analyzed in the last larval stage (M_{12}) and first juvenile (C_{18}) resulting in high standard deviations (SD) (Table 1). Despite this variation in total WSP, no significant differences were found in brush border (BB) extracts, which presented less variability across stages of larval development and first juvenile. Because of this variation, specific enzymatic activities analyzed in the extracellular extracts were normalized by DW (expressed as activity per mg DW^{-1}), whereas specific enzymatic activities analyzed in the BB extracts were normalized by the WSP (expressed as activity per mg prt^{-1}).

Samples were assayed in triplicate in 96-well flat bottom microplates and absorbance was read at 495 nm. In addition to the enzyme quantification, some activity ratios (amylase/protease, protease/esterase and amylase/esterase) were calculated.

2.3.1. Protease

Total protease activity was estimated in crude homogenates using azocasein 0.5% as substrate in 50 mM Tris–HCl buffer, pH 8.0, following the method by García-Careño and Haard (1993). In brief, 20 µL of enzyme extract was incubated with 500 µL substrate for 10 min at room temperature. Reaction was stopped with 500 µL 20% TCA (trichloroacetic acid). Samples were centrifuged at 10,000 *g* for 5 min and absorbance of the supernatant was measured at 366 nm. One unit of protease activity corresponded to a 1 µmol of substrate hydrolyzed in 1 min per mL of extracellular enzymatic extract ($\varepsilon_{366} = 900\ M^{-1}\ cm^{-1}$).

2.3.2. Trypsin

Trypsin-like enzyme activity was assayed using 0.1 M N α-benzoyl-DL-arginine p-nitroanilide (BAPNA) as substrate in 50 mM Tris–HCl–20 mM $CaCl_2$ buffer, pH 8.2, for 50 µL extract (Holm et al., 1988). The change in absorbance was measured at room temperature over 2 min at 407 nm. One unit of trypsin activity corresponded to 1 µmol of 4-nitroaniline liberated in 1 min per mL of extracellular enzymatic extract, based on the extinction coefficient of the substrate ($\varepsilon_{407} = 8200\ M^{-1}\ cm^{-1}$).

2.3.3. Chymotrypsin

Chymotrypsin activity was assayed using Suc-Phe-4-nitroanilide (Sigma) as substrate in 200 mM triethanolamin (TEA), 20 mM $CaCl_2$ buffer pH 7.8. Change in absorbance was measured at room temperature over 2 min at 405 nm. One unit of chymotrypsin activity corresponded to 1 µmol of substrate hydrolyzed in 1 min per mL of extracellular enzymatic extract, based on the extinction coefficient of the substrate ($\varepsilon_{405} = 10{,}200\ M^{-1}\ cm^{-1}$) (Geiger, 1988).

Table 1
Dry weight (DW) and protein content (EC = extracellular extracts; BB = brush border extracts) of *M. brachydactyla* larval stages (Zl_0 = non-fed newly hatched zoea I; Zl_3 = fed zoea I; ZlI_7 = zoea II; M_{12} = megalopa) and first juvenile (C_{18}). Subscripts indicate days post-hatch. Data are shown as mean ± SD ($n = 4$). Different letters in superscripts indicate significant statistical differences among ontogenetic stages ($P<0.05$).

	DW (µg ind^{-1})	Water-soluble protein (µg ind^{-1})	
		EC	BB
Zl_0	86 ± 16^{a}	$4.18 \pm 1.21^{a,b}$	1.51 ± 0.65
Zl_3	112 ± 10^{a}	2.35 ± 0.42^{a}	1.69 ± 0.53
ZlI_7	183 ± 23^{a}	$5.06 \pm 1.75^{a,b}$	2.86 ± 1.92
M_{12}	355 ± 72^{b}	$13.39 \pm 6.60^{b,c}$	4.65 ± 2.43
C_{18}	542 ± 129^{c}	20.18 ± 6.61^{c}	3.30 ± 0.87

2.3.4. Pepsin-like

Pepsin-like activity quantification followed Anson's (1938) method as modified by Worthington (1982). In brief, the enzymatic extract was mixed with the substrate (2% hemoglobin solution in 0.3 N HCl at pH 2.0) and incubated for 10 min at 37 °C. The reaction was stopped with 5% TCA and the assay tubes were centrifuged at 4000 *g* for 6 min at 4 °C. The absorbance of the supernatant was read at 280 nm. One unit of pepsin activity was defined as the µg of tyrosine released at 37 °C $min^{-1}\ mL^{-1}$, considering the extinction coefficient ($\varepsilon_{280} = 1250\ M^{-1}\ cm^{-1}$).

2.3.5. Amylase

α-Amylase activity was assayed by means of a starch-iodine detection following Metais and Bieth (1968). In brief, 50 µL of enzymatic extract was mixed with the substrate (3 g L^{-1} starch (starch soluble, Merck, Merck KGaA, Germany) in M/15 Na_2PO_4, pH 7.4) and incubated for 30 min at 37 °C. The reaction was stopped with 20 µL of 1 N HCL and, after the addition of 2 mL of N/3000 iodine solution (Merck) the absorbance was read at 580 nm. One unit (UW = Unit Wohlgemut) of α-amylase activity was defined as the mg of starch hydrolyzed per min at 37 °C per mL of extracellular enzymatic extract.

2.3.6. Esterase

Non-specific esterase activity was analyzed using 100 mM β-naphtyl caprilate as a substrate dissolved in dimethyl sulfoxide (DMSO, Merck) and in 50 mM Tris–HCl pH 7.5. Substrate and enzymatic extract were mixed with 100 mM sodium taurocholate and incubated for 30 min at 37 °C. Fast Blue Salt (100 mM dissolved in DMSO, Sigma) was added and the mixture was again incubated for 5 min. Reaction was stopped with 12% TCA and 1:1 ethyl acetate: ethanol and absorbance of the supernatant was read at 510 nm. One unit of esterase activity corresponded to the release of 1 µmol of naphtol in 1 min per mL of extracellular enzymatic extract, based on the extinction coefficient of the substrate ($\varepsilon_{510} = 20{,}000\ M^{-1}\ cm^{-1}$) (Versaw et al., 1989).

2.3.7. Aminopeptidase N

Aminopeptidase N activity was analyzed using 0.1 M L-leucine-p-nitroanilide dissolved in DMSO as a substrate in 80 mM NaH_2PO_4–H_2O buffer, pH 7.0 (Maroux et al., 1973). The change in absorbance was recorded at room temperature over 2 min at 410 nm. Aminopeptidase N activity was defined as the amount (µmol) of substrate hydrolyzed in 1 min per mL of the BB enzymatic extract, ($\varepsilon_{410} = 8200\ M^{-1}\ cm^{-1}$).

2.3.8. Alkaline phosphatase

Alkaline phosphatase activity was assayed using 5 mM p-nitrophenyl phosphate (Merck) as a substrate in 30 mM Na_2CO_3–H_2O, 1 mM $MgCl_2$–$6H_2O$ buffer, pH 9.8 (Bessey et al., 1946; Hausamen et al., 1967). The enzymatic extract was mixed with the substrate solution and the change in absorbance was measured at 37 °C over 2 min at 407 nm. One unit of phosphatase alkaline activity corresponded to a 1 µmol of the substrate hydrolyzed in 1 min per mL of the BB enzymatic extract ($\varepsilon_{407} = 18{,}300\ M^{-1}\ cm^{-1}$).

2.3.9. Maltase

Maltase activity was assayed using 56 mM maltose in 100 mM sodium maleate buffer, pH 6.0 (Dahkqvist, 1970). The BB enzymatic extract was incubated with the substrate at 37 °C for 30 min. After the first incubation, 0.125 mM glucose oxidase in 500 mM Tris–HCl buffer,

pH 7.0, 0.11 mM peroxidase, 3 mM Triton X-100 (Merck) in 95% ethanol and 0.4 mM o-diadisine (Sigma) in absolute ethanol were added and the reaction was incubated again at 37 °C for 20 min. Absorbance was read at 420 nm, using blank controls in which the reaction did not take place to correct values. Maltase activity was defined as the amount (μmol) of glucose released in 1 min per mL of BB enzymatic extract.

2.3.10. Chitinase

Freeze-dried individual specimens ($n=16$) were each homogenized in 100 μL distilled water and sonicated (Vibra-cell, Sonics, USA) on an ice bath using three short pulses of 2 s at high power. The homogenate was then centrifuged for 5 min at 13,000 *g* (4 °C) and supernatant used for the enzymatic fluorimetric assay. Chitinase activity was measured with a Chitinase Assay Kit using 4-methylumbelliferyl N-acetate-β-D-glucosaminide dissolved in DMSO as a substrate. The extract was incubated with the substrate solution for 30 min at 37 °C and then, fluorometry was read every 20 s at 360 nm (excitation)/450 nm (emission) over a period of 5 min at 30 °C. The chitinase activities were expressed as international units (IU = μmol of substrate hydrolyzed in 1 min per mL of enzymatic extract) per individual.

2.4. Zymograms of digestive enzymes

2.4.1. Alkaline endopeptidases

Substrate-gel electrophoresis of alkaline proteinases was performed following the technique of Garcia-Carreño et al. (1993). In brief, sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) in semi-denatured conditions and discontinuous system: stacking gel (4% T; 2.6% C) and separating gel (12% T; 2.6% C).

Enzyme extracts (prepared as described in Section 2.3.10) were mixed with equal parts of sample buffer (0.125 M Tris–Cl, 2% SDS, 20% v/v glycerol, 0.04% bromophenol blue, pH 6.8) (without 2-mercaptoethanol) and were not heated. Volumes of each sample, containing 5–10 μg of soluble protein, were loaded into individual gel wells at 4 °C. Electrophoresis running conditions and measurement of proteinase activities were carried out according to Perera et al. (2008a).

2.4.2. Amylases

Two techniques were used for amylase zymograms. In one of them the amylase substrate was copolymerized with the gel prior, while in the other gels were included in a solution of the substrate. The substrate containing gel-SDS-PAGE was performed in two phases. Proteins were separated by SDS-PAGE, performed according to Laemmli (1970) in semi-denatured conditions and discontinuous system: stacking gel (4% T; 2.6% C) and separating gel (6.9–8% T; 2.6% C) copolymerized with 0.25% w/v starch. Electrophoresis was run in a vertical device (Mini Protean II, BioRad Laboratories) partially submerged in a water-cooled bath at -9 ± 1 °C. Before starting electrophoresis, the running buffer was iced and hailed, being introduced in the lower buffer chamber to ensure a low temperature in the gels. This reduced the activity of amylase of the samples during electrophoresis and limited clear streaks on the lanes. Running conditions were 100 V per gel, constant voltage. After electrophoresis, gels were immersed in 0.05 M citric acid, 0.1 M Di-basic sodium phosphate buffer at pH 6.0 and 37 °C for 60 min and then fixed with 12% TCA (15 min) and stained with 1% I_2–2% KI solution. Bands with amylase activity appeared as light yellow zones or non-stained bands over a dark brown background of non-hydrolyzed stained starch. Since the staining is not permanent gels were immediately recorded by image scanning.

The substrate-SDS-PAGE for amylases was performed as above with modifications. Proteins were separated as for alkaline proteinases, using a separating gel (11% T; 2.6% C) with the same vertical device and running conditions as above.

Bands of amylase activity were revealed after electrophoresis when gels were immersed in starch solution (1% w/v) buffered with 0.05 M citric acid, 0.1 M di-basic sodium phosphate, at pH 6.0 and 37 °C for 60 min. Reaction was stopped with 12% TCA (15 min) and gel stained with 1% I_2–2% KI solution.

2.5. Statistics and data analysis

Data sets were analyzed using the SigmaPlot 9 and SigmaStat 3 software package (Systat Software Inc., USA). Ontogenetic changes in DW, protein content, enzymatic activities and ratios were evaluated by means of one-way ANOVA analysis (data normally distributed, Kolmogorov–Smirnov test) and comparisons between groups (using stages of ontogenic development as an independent variable) after finding statistical significances were performed by Bonferroni test. Statistically significant differences ($P<0.05$) were indicated by different letters.

Jaccard similarity coefficient (*J*) was used for comparing the similarity between proteinase zymograms at different development stages. Electromobility of proteinase bands was considered as a binary variable. For two sets (*i* and *k*), it was calculated as follows in accord with Härdle and Simar (2007):

$$J_{ik} = \left[\frac{a}{a+b+c}\right] \times 100$$

where:

- a = number of variables (proteinases with the same electromobility) that are positive for both samples, *i* and *k*
- b = number of variables that are positive in sample *i* and not in *k*
- c = number of variables that are positive in sample *k* and not in *i*.

3. Results

3.1. Extracellular enzymes

Total activity of proteases increased significantly during larval development ($ZI_0 = 0.88\pm0.12$ mIU ind^{-1}; $M_{12} = 3.66\pm0.17$ mIU ind^{-1}), with a slight decrease after metamorphosis to first juvenile ($C_{18} = 3.36\pm1.00$ mIU ind^{-1}) (Fig. 1a). This trend was reflected by the activities of trypsin and pepsin-like acid proteinases (Fig. 1b and c). Chymotrypsin presented a different pattern during ontogeny (Fig. 1d), showing a slightly (but not significantly different) increase throughout larval development ($ZI_0 = 2.45\pm0.17$ mIU ind^{-1}; $M_{12} = 5.56\pm2.10$ mIU ind^{-1}) that continued after metamorphosis ($C_{18} = 9.74\pm2.32$ mIU ind^{-1}). No significant differences were found in any of the proteolytic activities analyzed between ZI_0 and fed ZI_3.

Although specific activity of proteolytic enzymes was not significantly different in any of those enzymes, activity pattern shown by total proteases and trypsin (Fig. 1a and b) was the same throughout ontogeny, with a maximum peak of activity found in ZII_7 stage (total proteases $= 12.65\pm3.94$ mIU mg DW^{-1}; trypsin $= 1.41\pm0.68$ mIU mg DW^{-1}). On the contrary, chymotrypsin and pepsin-like enzyme showed a decreasing trend in specific activity up to ZII_7 (chymotrypsin: $ZI_0 = 29.46\pm7.24$ mIU mg DW^{-1}, $ZII_7 = 11.28\pm6.22$ mIU mg DW^{-1}; pepsin-like: $ZI_0 = 0.02\pm0.01$ mIU mg DW^{-1}, $ZII_7 = 0.002\pm0.001$ mIU mg DW^{-1}), with chymotrypsin increasing slightly in M_{12} and C_{18}. Pepsin-like increased in M_{12}, to decrease again in the C_{18} (Fig. 1c and d).

Proteinase activity was evident in ZI_0 (Fig. 2). No differences were found between the pattern of alkaline proteinases of ZI_0 and fed ZI_3. Total number of caseinolytic bands increased from ZI_0 to M_{12} and

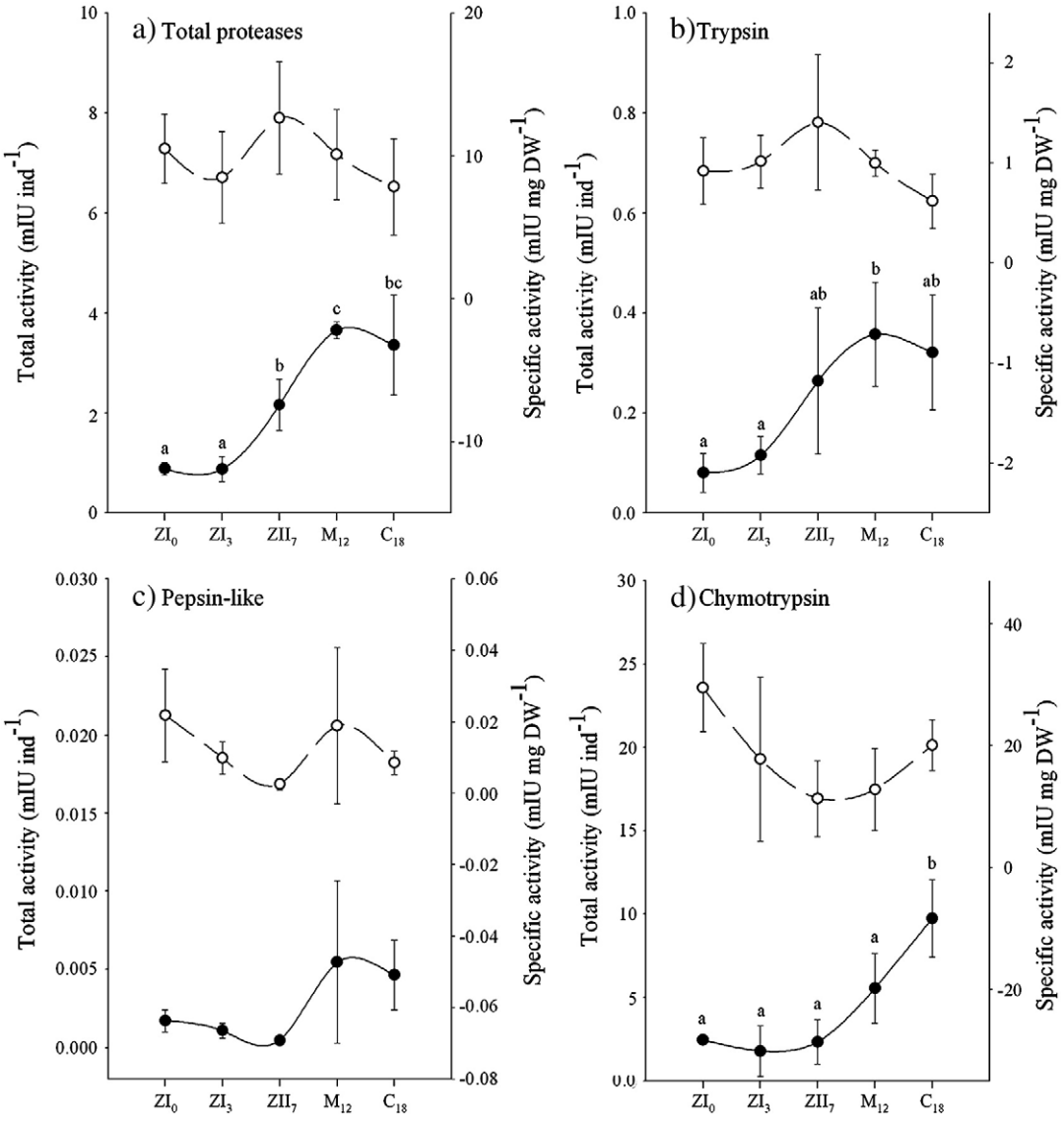

Fig. 1. Proteolytic enzyme activities (● = total in left *Y*-axis; ○ = specific in the right *Y*-axis) from different developmental stages of *Maja brachydactyla* (mean ± SD). Subscript indicates days post-hatch (DPH). ZI_0 = newly hatched zoea I (0 DPH); ZI_3 = intermolt zoea I (3 ± 1 DPH); ZII_7 = intermolt zoea II (7 ± 1 DPH); M_{12} = intermolt megalopa (12 ± 1 DPH) and C_{18} = first juvenile (18 ± 1 DPH). Different letters indicate significant differences among stages ($P<0.05$, $n=4$).

decreased in C_{18}. The most complex set of bands was observed in megalopa stage. A group of bands with high molecular mass (ranging from 75 to 83 kDa) was specific to larval stages (Table 2). Two groups of high intensity bands (ranging from 49.8 to 64 kDa and from 40 to 43 kDa) appeared in larval stages.

In adults, the main group of bands ranged from 12.2 to 42.0 kDa (Fig. 2). Two of these bands appeared from ZI_0 (18 and 16 kDa) while most of the others appeared from ZII_7. The trend for these bands was to increase in intensity with age. Pattern of alkaline proteinases of GJ and HP extracts in adults was practically the same (with a Jaccard similarity coefficient of 93.75%). Similarity coefficient between juvenile and adult was 52.94%, being 50% between C_{18} and M_{12}. Despite these differences the proteinases that appeared in juveniles are those that later appeared in adults with higher intensity (Table 2).

Total activity of amylase increased significantly as development advanced (Fig. 3a), being the minimum activity recorded at hatching ($ZI_0 = 0.12 \pm 0.03$ UW ind^{-1}) and the maximum peak activity reported after metamorphosis ($C_{18} = 0.65 \pm 0.13$ UW ind^{-1}). Despite slight variations, specific activity of amylase showed no significant changes with development. Chitinase showed significant changes in both total and specific activity with age (Fig. 3b). Both activities showed the same pattern of increase during larval development, being the minimum activity recorded in first zoea (ZI_0 total = 10.44 ± 0.60 mIU ind^{-1}/specific = 0.99 ± 0.06 mIU mg DW^{-1}; ZI_3 total = 11.07 ± 1.24 mIU ind^{-1}/specific = 1.28 ± 0.14 mIU mg DW^{-1}) and the maximum in M_{12} (total = 29.88 ± 5.06 mIU ind^{-1}/specific = 10.75 ± 1.82 mIU mg DW^{-1}). However, total chitinase activity decreased after metamorphosis ($C_{18} = 18.44 \pm 6.42$ mIU ind^{-1}) to a level of activity comparable with that found in ZII_7, whereas specific activity remained at the same level as M_{12} in the C_{18} (9.20 ± 3.20 mIU mg DW^{-1}). Total esterase activity also increased significantly during development (Fig. 3c). Fed ZI_3 showed a higher esterase activity (0.73 ± 0.56 mIU ind^{-1}) than ZI_0 (0.25 ± 0.24 mIU ind^{-1}), this level being similar to that found in ZII_7 and M_{12}. The maximum level of total activity was recorded after metamorphosis ($C_{18} = 1.92 \pm 1.04$ mIU ind^{-1}). Specific activity of esterase showed no significant changes during ontogeny.

Both substrate-SDS-PAGE and substrate containing gel-SDS-PAGE showed high sensitivity, allowing amylase activity to be revealed even though they were not detected as protein bands (Figs. 4 and 5). Although substrate-SDS-PAGE, has a lower resolution and capacity to reveal amylase activity, it was used to determine the molecular mass

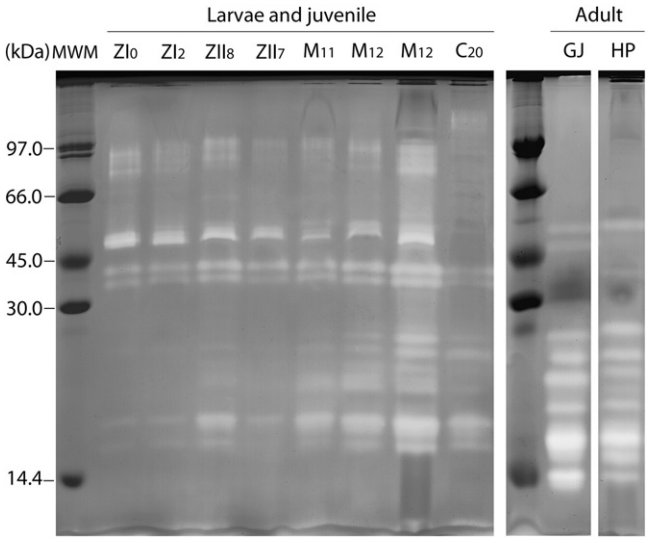

Fig. 2. Alkaline proteinase activity bands in substrate-SDS-PAGE zymograms during ontogenic development of *Maja brachydactyla*. Gels of 12% polyacrylamide (PAA) were revealed at pH = 7.7 and 26 °C. Developmental stage abbreviations: zoea I (ZI), zoea II (ZII), megalopa (M) and first juvenile (C). Zymograms of adults include: GJ = gastric juice, HP = hepatopancreas extracts, obtained from the same specimen. MWM = molecular weight markers (4 μL): phosphorylase b (molecular weight, Mr 97,000), albumin (Mr 66,000), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 30,000) and α-lactalbumin (Mr 14,400).

of amylases from *M. brachydactyla*. Four bands were detected, with molecular masses of 68.4 kDa, 59 kDa, 49 kDa and 27.8 kDa. First two appeared from ZI_0, being the only ones detected in larval stages. The 27.8 kDa band appeared only in some juveniles and the 49 kDa band appeared in adults, with the same pattern for both GJ and HP extracts.

Substrate containing gel-SDS-PAGE could not be used to determine molecular mass of amylases (Fig. 5) but provides a much higher resolution and capacity to detect the different patterns of amylase bands in larvae stages, juveniles and adults (Fig. 6). These patterns were the same in ZI_0 and ZI_3. The most complex set of bands was observed in ZII_7. Patterns of bands did not differ in juvenile stages, and they were similar to those shown in GJ of adults.

Although no significant differences were found in any of the ratios of amylase/protease, protease/esterase and esterase/amylase, some trends were observed from the results (Table 3). All ratios showed high variation between newly hatched ZI_0 and fed ZI_3; thus, amylase/protease ratio increased as first zoea developed ($ZI_0 = 140.2 \pm 58.3$; $ZI_3 = 197.8 \pm 74.9$), whereas protease/esterase and amylase/esterase ratios decreased. A decrease in amylase/protease ratio was observed in the transition from ZI_3 to ZII_7 and M_{12}, concomitant with an increase in the protease/esterase and amylase/esterase proportions of activity. As larval development advanced, all ratios became stabilized, showing similar values between ZII_7 and M_{12} (i.e. protease/esterase $ZII_7 = 6.2 \pm 3.9$; $M_{12} = 5.0 \pm 4.6$). The beginning of juvenile life implied a new relative increase of the amylase/protease ratio ($M_{12} = 128.8 \pm 38.0$; $C_{18} = 174.7 \pm 66.9$), and a remarkable decrease in the protease/esterase ratio ($C_{18} = 2.4 \pm 0.8$), whereas only a slight decrease in the amylase/esterase ratio ($M_{12} = 558.7 \pm 495.0$; $C_{18} = 428.2 \pm 216.9$) was observed.

Table 2
Schematic representation of proteinase activity bands in substrate-SDS-PAGE zymograms (Fig. 2) during ontogenetic development of *M. brachydactyla* larval stages, first juvenile and adult (GJ = gastric juice, HP = hepatopancreas extract) (Mr = molecular mass range, in kiloDaltons; the number of (+) is directly related with the intensity band; other abbreviations as in legend to Table 1).

Mr (kDa)	Larval stages and first juvenile					Adult		Mr (kDa)
	ZI_0	ZI_2	ZII_7	M_{12}	C_{20}	GJ	HP	
83.74	+	+	+	++				
79.26	+	+	+	++				
75.24	+	+	+	++				
						+	+	72.57
						+	+	64.41
63.98			+	+				
54.58			+	+		++	++	53.60
52.61	+	+	+	+				
51.03	+	+	+	+				
49.79	+	+	+	+		++	+	48.45
43.00	++	++	++	+++	+	+	+	42.00
40.32	++	++	++	+++	+	+	+	39.00
31.00				+				
29.16				++	+	++	+++	29.07
26.85			+	++	+	+++	+++	26.11
24.27			+	++	+			
22.97			+	++	+	++++	++	22.41
21.74			+	++	+	+	+	21.21
19.77				+		+++	++	19.29
18.32	+	+	++	++	++	+	++	17.44
17.08				+	+	++++	++++	16.00
15.77	+	+	+	+	+	++	+++	14.60
						++++	++	13.00
						++		12.23

3.2. Brush border enzymes

The digestive enzymes analyzed in BB extracts showed similar trends in total activity to those found in the extra cellular enzymes. Aminopeptidase N total activity increased during larval development, reaching its peak activity at M_{12} (0.20 ± 0.03 mIU ind^{-1}) and decreasing again after metamorphosis ($C_{18} = 0.13 \pm 0.06$ mIU ind^{-1}) (Fig. 7a). Specific activity of aminopeptidase N showed an increasing trend from ZI_0 and ZI_3 to the rest of stages, but the great variation recorded in the latter prevented detection of any significant differences. Alkaline phosphatase showed no significant changes throughout ontogeny (Fig. 7b), despite higher levels of both total and specific activity found in C_{18}. Both activities (total and specific) showed the same pattern during development. Maltase displayed an increasing pattern of total activity (Fig. 7c) similar to that found in amylase (Fig. 3a). First juveniles showed the maximum maltase activity (209.32 ± 74.41 mIU ind^{-1}), whereas no significant differences were found between newly hatched larvae ($ZI_0 = 38.65 \pm 21.36$ mIU ind^{-1}) and ZI_3 (49.21 ± 29.86 mIU ind^{-1}). Specific activity of maltase showed no significant changes among the stages of development, due to high variation among replicates.

4. Discussion

The activity of all assayed enzymes observed in larvae of *M. brachydactyla* confirms their ability to digest protein, carbohydrates and lipids as soon as they hatch. Total enzyme activity (mIU ind^{-1}) increased during larval development, reflecting the increase of midgut gland (MDG) complexity and volume with development. These results agree with those reported in a previous work on *M. brachydactyla* by Rotllant et al. (2008) as well as with the ontogenetic digestive patterns reported in another brachyuran species such as *Carcinus maenas* (Harms et al., 1994). However, the end of larval life in *M. brachydactyla* implies a change in the increasing trend of proteolytic (total proteases, trypsin, pepsin-like and aminopeptidase N) and chitinase activities, this resulting in a decrease from last larval stage (M_{12}) to first juvenile. A similar pattern has been also reported in the American lobster, *Homarus americanus* by Biesiot and Capuzzo (1990). Such effect cannot be explained by the increasing volume of the MDG from larvae to juvenile, which is expected to be accompanied by increases in total enzyme activities (Anger, 2001). Nor is it explained by a change in the diet, since in culture, *Artemia* was the only prey provided during the entire developmental period. Rather it appears due to a specific regulation of the enzymes, possibly

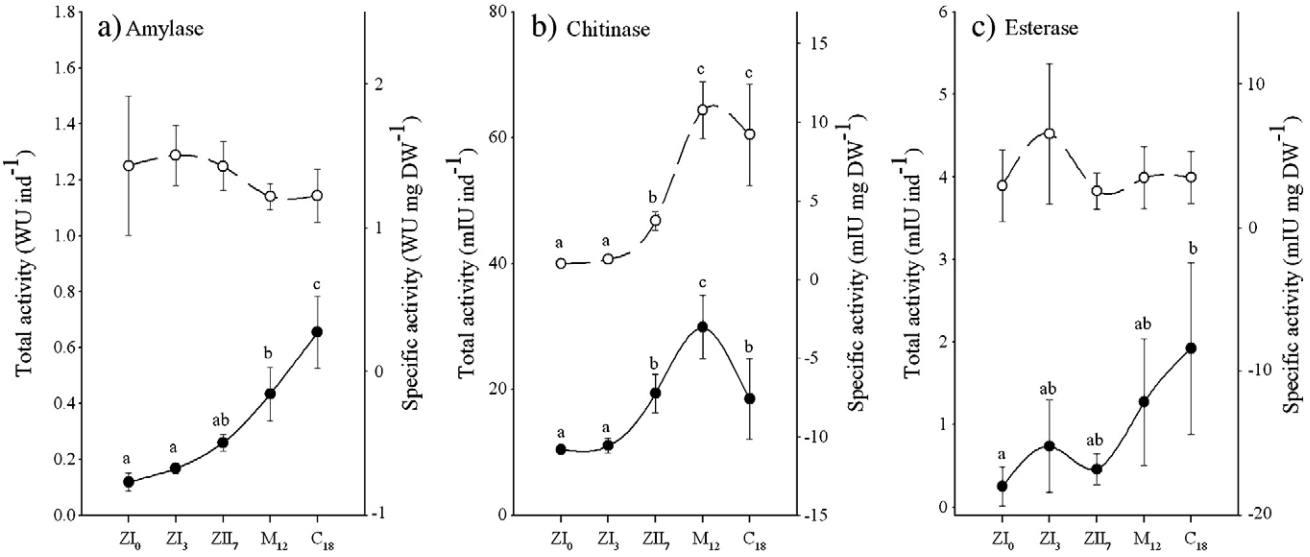

Fig. 3. Carbohydrase and esterase enzymatic activities (● = total in left *Y*-axis; ○ = specific in the right *Y*-axis) from different developmental stages of *Maja brachydactyla* (mean ± SD). For abbreviations see Fig. 1. Different letters indicate significant differences among stages ($P<0.05$, $n=4$).

related to the change from planktonic to a fully benthic life (change in the nutritional requirements or feeding behavior like feeding frequency). Moreover, Bermudes et al. (2008) attributed the increase in oxygen consumption in planktonic larvae of the spiny lobster *Jasus edwarsii* to increased locomotor activity and suggested that larvae might use protein as a metabolic substrate to fuel the muscular activity involved in swimming.

Despite statistical differences found in total activity of most enzymes, no significant changes in specific activities (mg prt^{-1} or mg DW^{-1}) of early larval stages were observed, except for chitinase. The high production of all types of digestive enzymes throughout early development, which has been also reported for other decapod larvae (Biesiot and Capuzzo, 1990; Harms et al., 1991; Johnston et al., 2004) may represent an adaptation of the larvae to variability in biochemical composition of their planktonic food. Such a pattern characterizes an omnivore predator and may result in a high survival potential at early life stages of *M. brachydactyla*.

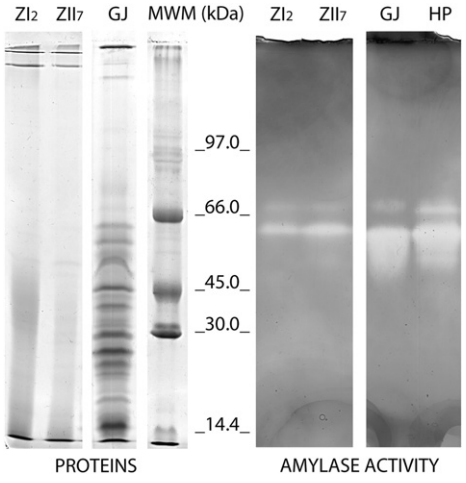

Fig. 4. Protein-gel after SDS-PAGE and amylase activity after substrate-SDS-PAGE of ontogenic development stages and gastric juice and hepatopancreas extracts from adults of *Maja brachydactyla*. Both electrophoresis runs were carried out in twin gels with a common molecular mass standard (as in legend of Fig. 2). Bands of amylase activity were revealed after electrophoresis: 11% PAA gels were immersed in starch solution (1% w/v), pH 6.0 at 37 °C during 60 min. For abbreviations of developmental stages see Fig. 2.

On the other hand, a decreasing – but not significant – trend was observed in the main specific proteolytic activities (trypsin and total proteases) after the two first zoeal stages. Lemos et al. (2002) suggested that similar changes occurring in specific enzymatic activities during the ontogeny of *Litopenaeus schmitti* (especially in proteases) were related to developmental events. The intense swimming activity and metabolic rates of planktonic naupliar and protozoeal shrimp stages require an increased capacity for food assimilation, decreasing throughout the transition to benthic habitats in late mysis and postlarval stages. Therefore, the raptorial feeding in late larval (megalopa) and juvenile stages of *M. brachydactyla* would allow individuals to better manipulate and select food particles complementing their digestive capacities.

The ingestion of live prey has been suggested to contribute to enzymatic activities (exogenous enzymes) in early life stages of decapods (Chen and Lin, 1992; Kurmaly et al., 1990). However, in the present study no differences in enzymatic activities (neither total nor specific) between ZI_0 (non-fed) and ZI_3 (fed on *Artemia* sp.), were found, indicating that newly hatched larvae of *M. brachydactyla* do not rely on external live prey enzymatic activities to complete digestion. These results are also confirmed by SDS-PAGE zymograms of amylase and protease activities that show the same pattern of activity bands in ZI_0 and ZI_3. The only exception to this pattern was found in the esterase activity, which increased significantly from ZI_0 to fed ZI_3. The observed pattern seemed to be dietary-induced as suggested by the intense accumulation of lipids (increase in the lipid/protein and carbon/nitrogen ratios) observed throughout larval ontogeny (Andrés et al., 2008). The relative importance of esterase activity at the beginning of ontogeny is also suggested by the protease/esterase and amylase/esterase ratios, which appeared to decrease from hatch to fed ZI_3 though not at statistically significant levels. The importance of lipids as an energy reserve has also been confirmed by the presence and variation of lipases and non-specific esterases in other decapod larvae (Johnston, 2003; Jones et al., 1997; Kamarudin et al., 1994; Perera et al., 2008b).

In the present study, digestive proteolytic capacities of *M. brachydactyla* larvae and first juveniles were described using five different enzymatic activities, one exopeptidase (aminopeptidase N) and four endopeptidases (total proteases, trypsin, chymotrypsin and pepsin-like). To date, trypsin-like activity has been considered as the main endopeptidase activity in all decapod larvae studied so far (Anger, 2001; Jones et al., 1997; Lovett and Felder, 1990). In *M. brachydactyla*, total protease and trypsin activities present parallel

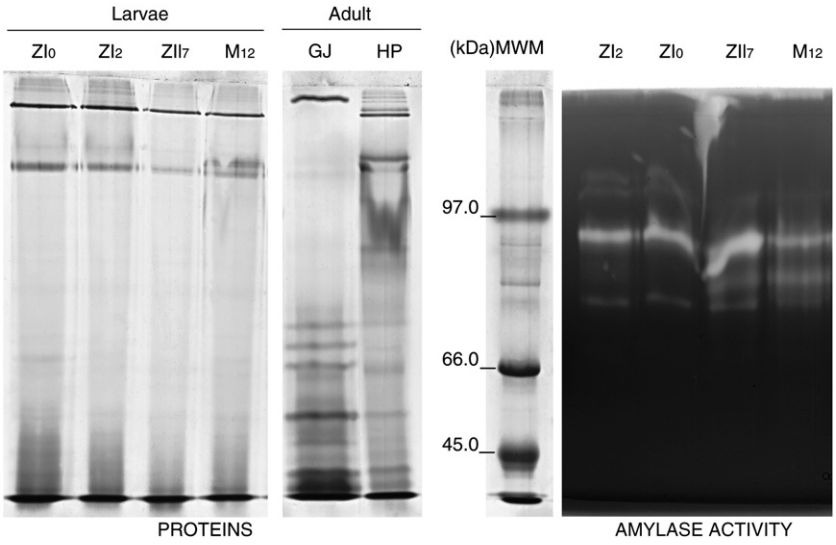

Fig. 5. Protein-gel after SDS-PAGE and amylase activity after substrate containing gel-SDS-PAGE of larval stages, and gastric juice and hepatopancreas extracts from adults of *Maja brachydactyla*. Both electrophoresis runs were carried out in twin gels, prepared by copolymerizing with 7% PAA with starch (0.25% in gel), using common molecular mass standard (as in legend of Fig. 2). Bands of amylase activity were revealed after electrophoresis: gels were immersed in citrate-phosphate buffer, pH 6 at 37 °C during 60 min. For abbreviations of developmental stages see Fig. 2.

patterns of variation throughout the ontogeny, suggesting that variations in total protease activity are mainly caused by trypsin, rather than by the other proteinases studied, which showed different patterns of variation. These data might confirm the important role of trypsin in the digestive proteolysis of *M. brachydactyla* larvae. Chymotrypsin also seems to have a relatively high importance during ontogeny, but it is clearly subjected to a specific regulation different from trypsin, since its basal activity is constant among larval stages increasing significantly only after metamorphosis.

To our knowledge, the presence of acid proteinases (pepsin-like) has never been detected in the digestive systems of any of the decapod larval stages studied (Jones et al., 1997), and has only been recently reported in some adult commercial lobsters and crabs (Celis-Guerrero et al., 2004; Navarrete del Toro et al., 2006). Our data support that larvae of *M. brachydactyla* have the capacity of digesting proteins at low pH throughout the ontogeny in a similar pattern of activity to that of other proteases. The role that these acid proteases play in crustacean digestion remains unclear, although Navarrete del Toro et al. (2006) suggested these enzymes might either be used for protein digestion or remain as a vestigial evolutionary character with a limited physiological function. The activity of exopeptidases such as alanine-aminopeptidase or leucine- and valine-arylamidase has been recently reported in crustacean larval stages (Saborowski et al., 2006), being related to the ability of using intra-cellular yolk reserves during lecitotrophy. Since larvae of *M. brachydactyla* are considered planktotrophic, the activity of aminopeptidase N throughout ontogeny might not be associated with digestion of yolk reserves but be considered as part of the proteolytic set of enzymes that is present in the larval stages of this species.

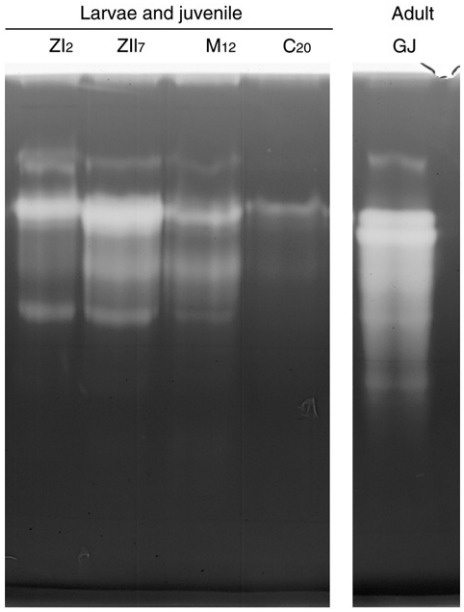

Fig. 6. Amylase activity bands after higher resolution substrate containing gel-SDS-PAGE (8% PAA + 0.25% starch) of ontogenic development stages and gastric juice extract from adults of *Maja brachydactyla*. For abbreviations of developmental stages see Fig. 2.

SDS-PAGE of alkaline proteinases revealed, in adult *M. brachydactyla*, 16 activity bands in gastric juice extracts and 15 in hepatopancreas, having a similar pattern of distribution and molecular weight to those reported in shrimps (Lemos et al., 2000), spiny lobster (Perera et al., 2008a) and crabs (Saborowski et al., 2006). Low molecular weight proteinases (14 to 20 kDa), which were identified as trypsin in shrimps (Lemos et al., 1999), were present throughout development of *M. brachydactyla*, increasing in number of active bands and intensity as development advanced. High molecular weight bands (>66 kDa) were exclusive of larval stages and could not be detected in adults. Similarly, in *Farfantepenaeus paulensis* those bands appeared in early developmental stages, decreased in number and intensity in postlarval stages and disappeared in adults (Lemos et al., 1999). Moreover, juveniles of *M. brachydactyla* presented a relatively simple pattern of bands, as occurred in *F. paulensis* postlarvae. Thus, recently

Table 3
Digestive enzymatic activity ratios of *M. brachydactyla* larval stages (ZI_0 = non-fed newly hatched zoea I; ZI_3 = fed zoea I; ZII_7 = zoea II; M_{12} = megalopa) and first juvenile (C_{18}). Subscripts indicate days post-hatch. Data is shown as mean ± SD ($n = 4$).

	Amylase/protease	Protease/esterase	Amylase/esterase
ZI_0	140.2 ± 58.3	5.8 ± 4.1	832.4 ± 549.3
ZI_3	197.8 ± 74.9	1.7 ± 0.8	367.2 ± 255.3
ZII_7	123.3 ± 46.5	6.2 ± 3.9	665.0 ± 310.0
M_{12}	128.8 ± 38.0	5.0 ± 4.6	558.7 ± 495.0
C_{18}	174.7 ± 66.9	2.4 ± 0.8	428.2 ± 216.9

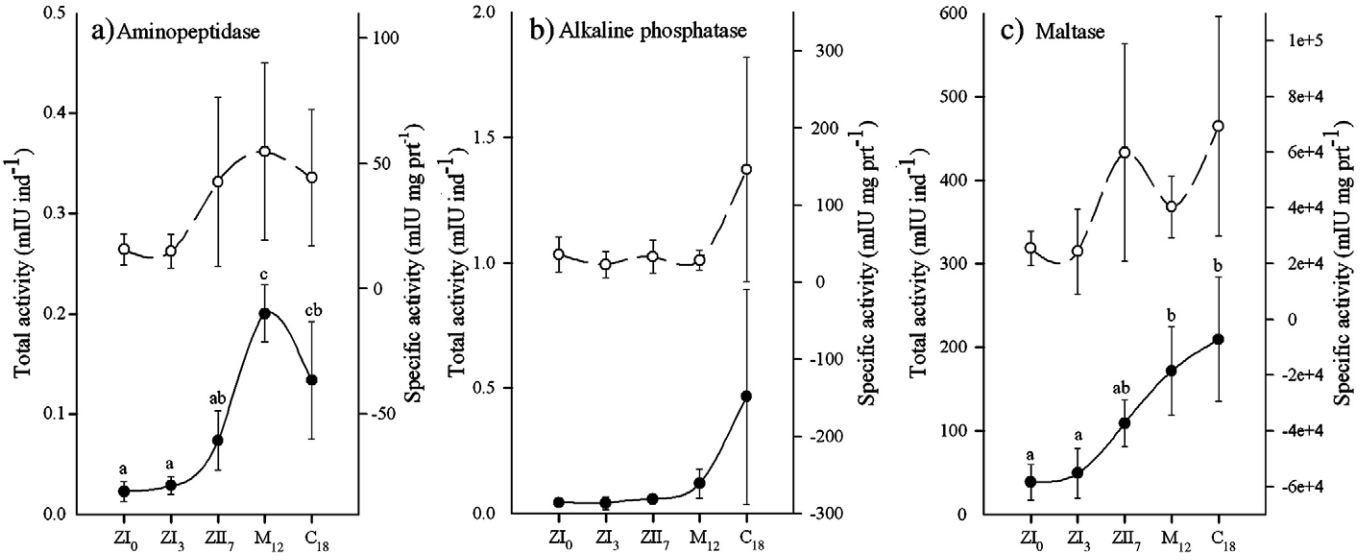

Fig. 7. Enzymatic activities (● = total in left *Y*-axis; ○ = specific in the right *Y*-axis) analyzed in the brush border extracts of the different developmental stages of *Maja brachydactyla* (mean ± SD). For abbreviations of developmental stages see Fig. 1. Different letters indicate significant differences among stages ($P<0.05$, $n=4$).

metamorphosed juveniles seem to present an intermediate proteolytic capacity between larvae and adult.

Carbohydrate digestion has been shown to be important for larvae of decapods, irrespectively of their feeding habits, being present either in herbivorous penaeids (Carrillo-Farnés et al., 2007), omnivorous crabs (Harms et al., 1994; Saborowski et al., 2006) or carnivorous lobsters (Biesiot and Capuzzo, 1990) and carideans (Kamarudin et al., 1994). Digestion of dietary polysaccharides, such as starch and glycogen, usually demands amylase and other glucosidases, maltase being an essential enzyme for their complete digestion (Aguilar-Quaresma and Sugai, 2005). Our results show that *M. brachydactyla* displayed high carbohydrase activities in the form of α-amylase, maltase and chitinase throughout ontogeny. Total activities of amylase and maltase reflected the increase in complexity and volume of the MDG with development, as might be deduced by the increasing amount of activity from newly hatched larvae to first juvenile. However, specific activities did not show variations throughout ontogeny.

The activity of maltase was very high in *M. brachydactyla* larvae and first juveniles when compared to the values obtained in juveniles of the shrimp *F. paulensis* (Aguilar-Quaresma and Sugai, 2005), where maltase specific activity was found to be around 10 times lower than in spider crab larvae. Despite the major importance given to the α-amylase in the evaluation of the carbohydrase activity in all the decapod larvae studied so far (for references, see Jones et al., 1997), some authors have suggested that other enzymes may also play important roles during digestion of dietary carbohydrates. Taking into account that starch is not a common component in their natural food (Anger, 2001), it has been suggested that the high amylase activities reported in larvae of other majoids (*Hyas araneus*) could be the result of a co-regulation with other enzymes such as trypsin or laminarinase (Hirche and Anger, 1987). In this context, the high maltase activity (considered as the last step in carbohydrate assimilation due to its capacity to break down disaccharides) found in *M. brachydactyla* might be the consequence of the activity of several carbohydrases acting together to transform complex dietary glucides into simple disaccharides, and hence, might be considered as an important indicator for the evaluation of carbohydrate digestion in decapod larvae. Chitinase also represents a complement in carbohydrate digestion during *M. brachydactyla* development, as occurs in other decapod larvae such as the spiny lobster, *J. edwarsii* phyllosoma (Johnston et al., 2004). Since chitinase was the only enzyme showing a significant increase in its specific activity from hatching to metamorphosis, with maximum activity in megalopa and first juvenile stages, it seems to be physiologically modulated during ontogeny. The increased ability to digest chitin found in later larval stages and juveniles could be related to a shift in their dietary habits, changing from the ingestion of planktonic non-chitinose available preys to benthic chitinose captures, dead counterparts that settle to the bottom, and own old exuviae, which might be eaten after a molt.

In the present work, two techniques were combined for qualitative assessment of the amylase activity in *M. brachydactyla*. Even though substrate containing gel-SDS-PAGE provided much higher resolution and capacity to detect different patterns of amylase bands in *M. brachydactyla*, it could not be used to determine the molecular masses of the amylase bands. This was because protein migration under semi-denaturing conditions interacts with the starch-PAA matrix, retarding proteins, making the molecular mass appear higher than it actually is (Martínez Moya et al., 2002). Zymograms revealed four starch degrading enzymes with a similar pattern but a higher molecular weight than those found in adults of spiny lobsters (Perera et al., 2008a). In contrast, Van Wormhoudt et al. (1995) found only a single molecular form of amylase in adults of *M. brachydactyla* (as *M. squinado*) and several species of shrimps. To date, no work has been published concerning variations in amylase polymorphism during decapod development. As occurred for alkaline protease activity, amylase-active bands increased in number and intensity as larval development advanced. However juveniles showed a simple and weak activity compared to both larvae and adults. Further studies might consider the assessment of those changes occurring after metamorphosis, from early first juveniles onward, in order to determine the moment in which *M brachydactyla* presents the adult type isoenzyme pattern for both alkaline proteases and amylases.

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