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# Evaluation of fluorogenic substrates in the assessment of digestive enzymes in a decapod crustacean *Maja brachydactyla* larvae

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ABSTRACT

The potential use of fluorescent substrates for measuring digestive enzyme activities has been adapted to decapod crustacean larvae and compared to traditional analytical techniques based on spectrophotometry. The ontogeny of three digestive enzymes (trypsin, amylase, and esterase) from the hepatopancreas of *Maja brachydactyla* has been selected to illustrate the advantages and disadvantages of both spectrophotometric (SP) and fluorometric (FL) methods. The three enzyme activities showed the same pattern with either of the techniques used. Standardised discriminant function coefficients indicated that the stage of development in spider crab larvae was most strongly discriminated by the activity of amylase, whichever method of analysis was considered. The FL method allows the detection of up to three enzyme activities in a single larva (<100 μg dry weight), whereas SP needs more than 20 times this weight. The FL method allows studying the inter-individual variability, while the SP approach only permits one to evaluate the inter-population variability (pool of individuals). Nevertheless, the higher sensitivity of the FL method implies a larger variability in the results compared to SP. There are no differences in feasibility between the two methods when using kinetic analyses. Although data from different experiments assayed by SP methods are easily standardised in units of dry weight or soluble protein concentration, this standardisation is difficult when using FL substrates, since differences between the sensitivity of the equipment used for the analysis might be higher than one order of magnitude. The availability of FL substrates in the market is constantly increasing, although it is still scarce in relation to the high number and types of substrates normally used in SP methods. This may be a limitation to the study of certain enzyme activities. The cost of the analysis is based on the price of the specific substrates, FL substrates being more expensive than SP ones. Both SP and FL methods are useful to measure digestive enzymes in decapod crustacean larvae. Selection of the method used will depend on the objectives and interests of the researcher. For studies based on the characterization of enzyme activities in crustacean populations without any limitation on the number and amount of samples, the SP method is recommended. When several experimental treatments, dietary regimes, and/or moulting stages are considered, the FL analysis might be more appropriate due to the low number of individuals needed for sampling. If the study is conducted to assess the individual variability of the population, then, the FL method needs to be applied.



## 1. Introduction

Digestion is a key process in the metabolism of decapod crustaceans since it determines the availability of the nutrients needed for all their biological functions. Within this context, the study of the digestive physiology is of great importance, since net efficiency of the whole process relies on the type and function of the available enzymes (Vonk, 1960). Digestive enzymes are of particular interest, since the rate of digestion in the intestinal system limits the uptake of nutrients to the hemolymph and can potentially limit the growth of the whole organism. Therefore, the study of digestive enzymes is a major tool when studying the adaptations of any organism to fluctuations in food availability, the nutritional status of individuals, the adaptation to circadian rhythms, and the moulting cycle or developmental changes (Kanazawa, 1994; Conklin, 1995; Carrillo-Farnés et al., 2007).

These aspects are especially important during larval development, when substantial changes in the structure, physiology, size, and body shape are produced and the individuals are exposed to changes in food quantity and quality. Therefore, a great number of studies have focused on the assessment of the ontogeny of digestive enzyme activities of prawns (Galgani and Benyamin, 1985; Lovett and Felder, 1990; Fang and Lee, 1992; Lemos et al., 1999, 2000), crayfish (Kamarudin et al., 1994; Jones et al., 1997), lobster (Biesiot and Capuzzo, 1990; Kumlu and Jones, 1997; Johnston, 2003), and crabs (Hirche and Anger, 1987; Saborowski et al., 2006). In addition, several

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studies on enzymatic activities in crustacean larvae examined the effect of food deprivation (Harms et al., 1991; Jones et al., 1993; Harms et al., 1994; Johnston et al., 2004), dietary regime (MacDonald et al., 1989; Harms et al., 1991, 1994; Kumlu et al., 1992; Le Vay et al., 1993; Rodríguez et al., 1994; Le Moullac et al., 1994; Kumlu and Jones, 1995; Jones et al., 1997; Sheen and Huang, 1998; Rosas et al., 2000; Pedroza-Islas et al., 2004), and moulting process (Hirche and Anger, 1987).

All of these studies were carried out using techniques based on spectrophotometry. From a methodological point of view, some of the main constraints in this kind of analysis are the small size and low dry weight of the individuals and the extremely low activity of some of the assayed enzymes. Pooling the individuals may help to solve the problem, but in some cases the scarce number of individuals, the difficulties in the rearing process, or the complexity of the experimental design makes pooling almost impossible. Moreover, the individual variability in enzyme activity cannot be measured by spectrophotometry due to pooling, although such calculations might be essential to determine whether starvation plays a major role in the recruitment process in the case of field studies or, in the case of aquaculture, is of paramount importance to obtain the best rearing conditions in order to avoid differences in population growth. Consequently, there is a need for methodologies demanding lower amounts of biological material. In recent years, the use of fluorescent substrates for the analysis of digestive enzymes in fish larvae and adult copepods has been continuously increasing (Ueberschär, 1995; Izquierdo and Henderson, 1998; Knotz et al., 2006; Cara et al., 2007; Lazo et al., 2007), making the use of these highly sensitive fluorometric methods very attractive to perform similar studies with decapod crustacean larvae.

In the present work, the potential use of fluorescent substrates for measuring digestive enzyme activities of the spider crab *Maja brachydactyla* during early ontogeny was assessed and compared to the traditional analytical techniques based on spectrophotometry. The final objective was to obtain a general view of the potential of such techniques, considering both their advantages and limitations. This may help researchers to evaluate the suitability of such methodologies in future studies of digestive physiology in larval stages of crustaceans.

## 2. Materials and methods

### 2.1. Biological material — spider crab larvae and juvenile

Four ovigerous females of *Maja brachydactyla* were captured on the Atlantic Northwest coast of Spain and brought to IRTA (Sant Carles de la Ràpita, Tarragona, Spain) by surface transport in high humidity containers and temperatures of approximately 8 °C. Once at IRTA, they were kept in a 2000 L tank connected to a recirculation unit at a constant salinity and temperature of 36‰ and 18 °C, respectively, and fed fresh mussels and frozen crab. Active newly hatched larvae were collected from the broodstock tank immediately after hatching and transferred to 35 L mesh-bottomed (150 μm) PVC cylinders (baskets) provided with enough aeration and air-lifts to renew the water inside the baskets. The baskets were immersed in 1500 L holding tanks connected to recirculation units. Temperature and salinity were kept constant at 18 °C and 36‰, respectively; whereas photoperiod was maintained under ambient light conditions (12 h light in early spring). Larvae were fed *Artemia* sp enriched for 24 h using a commercial product (EasySelco, INVE, Belgium), and green water culture was maintained throughout the culture cycle by adding *Tetraselmis chuii* and *Isochrysys galbana* to larval tanks every second day. Larvae were sampled at each stage of development from zoea (Z) I to first juvenile (C) at hatching ($ZI_0$), and 2±1 ($ZI_2$), 8±1 ($ZII_8$), 11±1 (megalopa; $M_{11}$) and 20±1 ($C_{20}$) days post-hatching (DPH), with the aim to sample the maximum number of representative individuals at the inter-moult phase within the moulting cycle (according to Andrés et al., 2007, 2008).

Samples of larvae and juveniles for enzyme analysis were gently rinsed in distilled water, dried on filter paper, and kept frozen at −80 °C until analysis, either as a pooled sample of 500 mg for spectrophotometry (950±68 $ZI_0$ to 190±26 $C_{20}$) or kept individually for fluorometry.

### 2.2. Enzymatic analysis (Table 1)

#### 2.2.1. Spectrophotometric method (SP)

Frozen samples (500 mg fresh weight) belonging to four different spawning ($n$=4) were homogenized for 5 min in ice-cold Tris–mannitol (50 mM), HCl (2 mM), pH 7 (volume=g homogenized tissue×30) using an Ultra Turrax (Ika, Germany) and stored at −20 °C until enzymatic analysis (Table 1).

Thawed aliquots of homogenized samples were briefly centrifuged (9000×g for 15 s) prior to enzymatic analysis and assayed in triplicate. All samples for a single enzymatic assay were run in the same day. Blank controls, in which the reaction did not take place, were introduced when needed. All the enzymatic activities are expressed in international units (IU) except for the amylase activity assay, in which Wohlgemut units (WU) are used. All the enzymatic activities were read in a Sinergy HT spectrophotometer (BioTek®, Vermont, US) using either 48 (Nunclon 48 Well flat, Nunc, Roskilde, Denmark) or 96 (96 MicroWell™, Nunc, Roskilde, Denmark) flat bottom microplates.

Trypsin-like enzyme activity was assayed using 0.1 M *N*α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA, Sigma B4875) as substrate in 50 mM Tris–HCl-20 mM $CaCl_2$ buffer, pH 8.2, per 50 μl of extract. 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 cytosol extract, based on an extinction coefficient $\in_{407}$=8,200 $M^{-1}$ $cm^{-1}$.

α-Amylase activity was assayed by means of starch-iodine detection according to Metais and Bieth (1968). In brief, 50 μl of extract was mixed with the substrate (3 g $L^{-1}$ starch (Merck 101257) 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 104761), the absorbance was read at 580 nm. One WU of α-amylase activity was defined as the number of mg of starch hydrolyzed at 37 °C per mL of cytosol extract.

Non-specific esterase activity was analyzed using the Mckellar and Cholette (1986) method as modified by (Versaw et al., 1989). We used 100 mM β-naphtyl caprilate (Sigma, 4483) as substrate dissolved in dimethyl sulfoxide (DMSO, Merck 102952) in 50 mM Tris–HCl. The substrate and 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 F3378) was added, and the mixture was again incubated for 5 min. The reaction was stopped with 12% trichloroacetic acid

**Table 1**
List of enzymes, substrates and assay conditions for spectrophotometric and fluorometric enzyme analysis methods

| Enzyme Spectrophotometric analysis | Substrate | Buffer | pH | T (°C) | Absorbance (nm) | |
|---|---|---|---|---|---|---|
| Trypsin | BAPNA in DMSO | 50 mM Tris–HCL 20 mM CaCl2 | 8.2 | 25 | 407 | |
| Amylase | Starch | $NaH_2PO_4$M/15 | 7.4 | 37 | 580 | |
| Esterase | 100 mM β-Naphthyl caprylate (in DMSO) | Tris–HCl 50 mM | 7.5 | 37 | 510 | |
| Fluorometric analysis | | | | | Ext (nm) | Emi (nm) |
| Trypsin | Boc-Glan-Ala-Arg-MCA | 50 mM Tris–HCL | 8.0 | 30 | 380 | 440 |
| Amylase | Corn starch | 10 mM $CaCl_2$ Reaction buffer (kit) | 6.9 | 30 | 485 | 538 |
| Esterase | 4-methylumbelliferyl butyrate | Phosphate buffer | 7.0 | 30 | 355 | 460 |

Fluorescence was measured at excitation (Ext) and emission (Emi).

(TCA, Sigma T9159) 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 cytosol extract, based on an extinction coefficient $\in_{510}$=20,000 $M^{-1}$ $cm^{-1}$.

Specific activity was defined as enzyme activity per mg of larval protein (IU $mg^{-1}$), and total activity was defined as enzyme activity per individual (IU $individual^{-1}$). 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.

### 2.2.2. Fluorometric method (FL)

A single individual was homogenized in 100 μL distilled water and sonicated in an ice bath with three short pulses of 2 s (Vibra-cell., Sonics, USA). The homogenate was then centrifuged for 5 min at 13,000×g at 4 °C and the extract used for the analysis of trypsin, amylase, and esterase. For trypsin, homogenates were diluted to 1:50 in the case of zoea I (ZI) and crabs (C), to 1:100 in zoea II (ZII), and up to 1:200 in megalopa (M). The fluorogenic substrate Boc-Glan-Ala-Arg-methyl coumarin hydrochloride (SIGMA B4153) was diluted in dimethyl sulfoxide (DMSO), to a final concentration of 20 μM. Five μL of this substrate were mixed with 195 μL of 50 mM Tris–HCl, 10 mM $CaCl_2$ buffer, and 10 μL of the diluted homogenate were added to the microplate for analysis. Fluorescence was measured at 380 nm (excitation) and 440 nm (emission) for 5 min at 30 °C. For amylase, homogenates were diluted to 1:10 in Z I and C, and to 1:50 in ZII and M. Ultra Amylase Assay Kit (E33651) from Molecular Probes was used for the analysis. Fluorescence was measured at 485 nm (excitation) and 538 nm (emission) for 5 min at 30 °C. For non-specific esterase, larvae and juvenile homogenates were diluted to 1:10. The fluorogenic substrate 4-methylubelliferyl butyrate (MUB-Fluka 19362) was used following a modified method from Vaneechoutte et al. (1988). In brief, a stock solution was prepared by dissolving 100 mg of MUB in 10 mL DMSO, to which 100 μL Triton X-100 was added, and stored at −80 °C. This stock solution was diluted in phosphate buffer pH 7.0 to a final concentration of 0.4 mM MUB. Ten μL of the diluted homogenate were added to the microplate and mixed with 250 μL of 0.4 mM MUB for the analysis. Fluorescence was measured at 355 nm (excitation) and 460 nm (emission) for 2 min at 30 °C.

## 2.3. Protein determination

Protein content was measured in the homogenates as described by Bradford (1976) using Bio-Rad Protein Assay dye reagent (BioRad 500-0205) and bovine serum albumin (BSA, Sigma A7906) as the standard. Samples were assayed in triplicates in 96 flat bottom microplates and read at 495 nm.

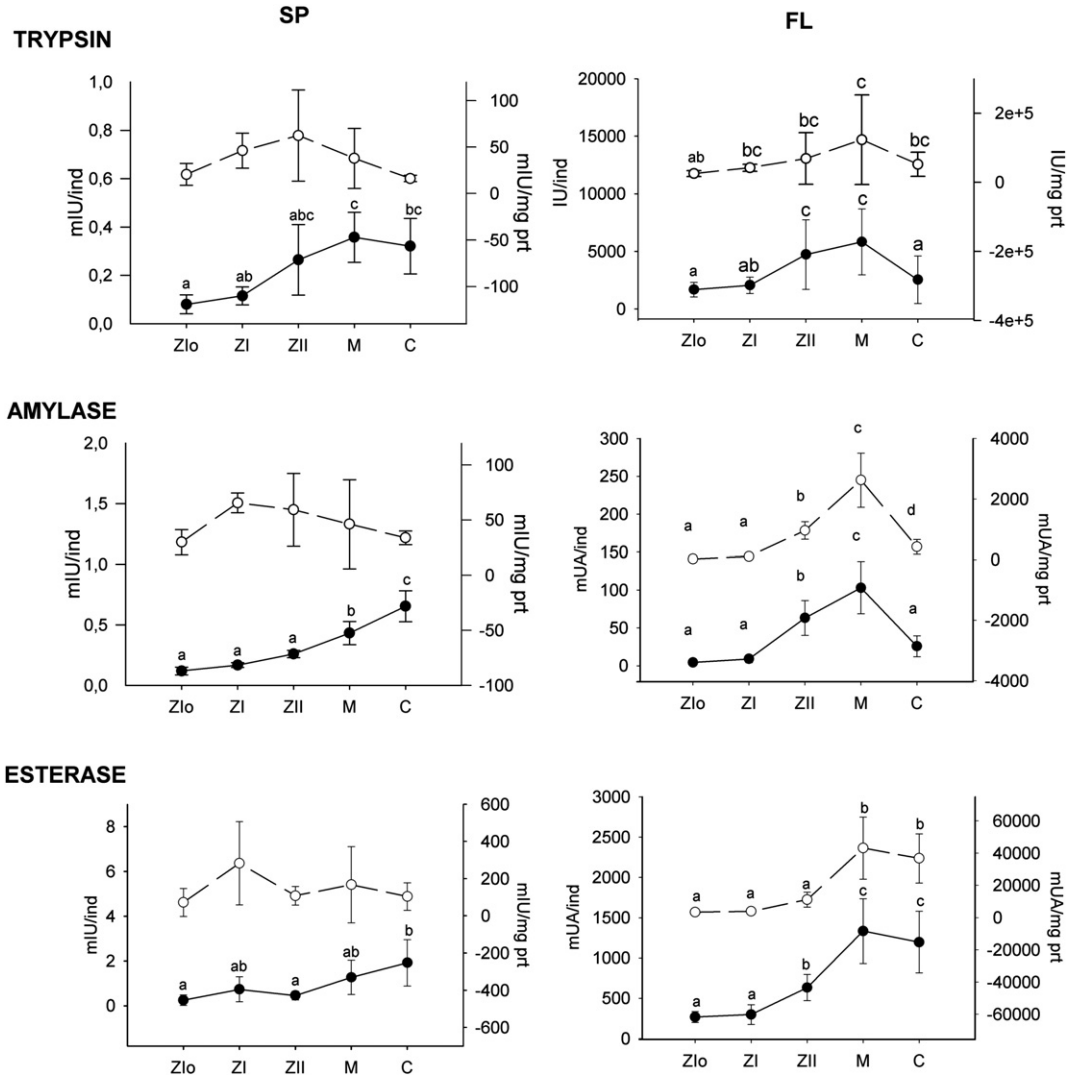


**Fig. 1.** Total (●) and specific (○) activity of selected hepatopancreatic enzymes (trypsin, amylase, and esterase) in the spider crab *Maja brachydactyla* during early life stages of development measured by means of spectrophotometric (SP) and fluorometric (FL) methods. Data presented as mean values±SD. Different letters denote statistically significant differences (ANOVA, $P<0.05$).

*2.4. Definition of the criteria used for comparison between the two methods*

- Sensitivity: the ability to register small amounts of enzyme activity.
- Resolution: capacity to measure slight variations in the enzyme activity throughout ontogenesis, moulting cycle, or variable rearing conditions (environmental or nutritional).
- Interference: the act of distorting, inhibiting, or hindering an enzyme reaction due to the presence of other substances while reading the sample.
- Precision: degree of similarity in the enzyme activity (reproducibility) when measured under the same conditions and equipment and its ability to be consistently reproduced. This parameter represents a good measure of its reliability.
- Feasibility: quality of being accomplished using the least difficult method of enzyme analysis.
- Standardisation: capacity of comparing enzyme activities among different stages, environmental conditions, or species from the same or different experiments and analysed in different equipments and laboratories.
- Cost: the expenditure of time and labour necessary to complete the analysis.

  Coefficient of variation (CV) has been use to estimate the variability between samples and reproducibility.

*2.5. Statistics*

Statistical analysis of the data was performed using a graph-statistical software package (SigmaPlot 9 and SigmaStat 3, Systat Software Inc., USA). Homoscedasticity and normality were tested (using Levene's test and Shapiro–Wilk's test, respectively) before analysing the data with an ANOVA. Differences among stages were tested by one-way ANOVA. Comparisons among groups after finding significant differences were performed by either Holm–Sidak or Tukey tests, depending on the data, with an overall significance level of 0.05.

The canonical discriminant analysis (CDA) was used for classifying a set of observations into predefined classes (e.g. spider crab stages of development — $ZI_0$, $I_2$, $II_7$, $M_{12}$, $C_{20}$). The purpose is to determine the class of an observation based on a set of variables known as predictors or input variables (e.g. enzyme activity). Prior to running the CDA, data were checked for multivariate normality, homogeneity of variances and covariances, and correlations between means and variances. Computationally, the test performs a canonical correlation analysis that determines the successive functions and canonical roots (the term root refers to the eigenvalues that are associated with the respective canonical function). Eigenvalues can be interpreted as the proportion of variance accounted for by the correlation between the respective canonical variates. The maximum number of functions will be equal to the number of groups minus one, or the number of variables in the analysis, whichever is smaller. The first function maximizes the differences between the values of the dependent variable, while the second function is orthogonal to it (uncorrelated with it) and maximizes the differences between values of the dependent variable, controlling for the first factor (StatSoft, 2007). Differences among developmental stages were measured directly by means of the Wilk's λ-criterion. The $\chi^2$-test for Wilk's λ was used to test the significance of the overall difference between the centroids in feeding regime. The relative importance of the original variables in separating the developmental stages of the groups was gauged by standardised values.

## 3. Results and discussion

The ontogeny of three hepatopancreatic enzymes (trypsin, amylase, and esterase) of *Maja brachydactyla* was selected to illustrate the advantages and disadvantages of the SP and FL methods. All enzyme activities (units per individual) increased during larval development (Fig. 1), showing the same pattern with both SP and FL techniques and confirming the results obtained for trypsin and amylase activities in another spider crab species (*Hyas araneus*) by Hirche and Anger (1987).

At the first crab stage, trypsin and amylase activities measured by FL decreased, while SP analysis revealed an increase in these activities between megalopa and crab. Such a difference may be explained considering that the substrates used for the analysis might not be measuring exactly the same enzyme activities. In crustaceans, especially at early developmental stages, dramatic physiological changes are taking place, which determine variations not only in the type but also in the amount of enzymes present in the digestive system of the larvae, as has been demonstrated for proteases in *Lithodes santolla* by Saborowski et al. (2006). Such different types of proteases, although being closely related, may show a different affinity for a given substrate. Hence, possible changes in the type of protease identified as "trypsin" might be taking place in the larvae during the transition from megalopa to crab, resulting in a variation in their ability to hydrolyze the highly sensitive fluorescent substrate and lowering the value of the activity. The effect was even more apparent when taking into account that isolated individuals were used for the FL analysis. In contrast, the use of pooled samples and a substrate with a different specificity (BAPNA) in the SP analysis might not be sensitive enough to reveal such subtle changes in the larvae, with the net result being that enzyme activity seemed to be maintained.

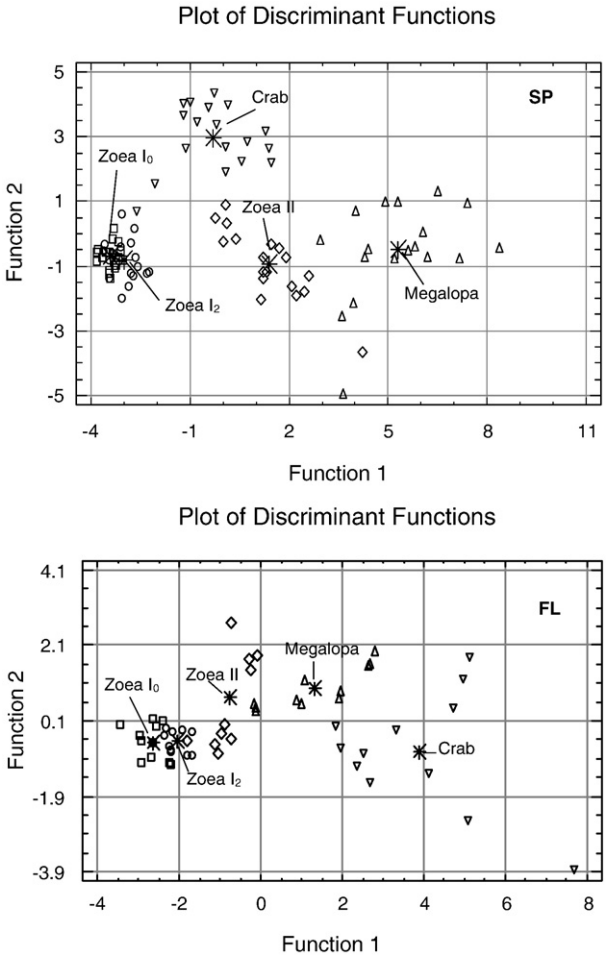


**Fig. 2.** Plots of the first two axes for the canonical discriminant analysis of the developmental stages in the spider crab *Maja brachydactyla* using hepatopancreatic enzyme activities measured by spectrophotometric (SP) and fluorometric (FL) methods. Symbols: Zoea $I_0$ (□), zoea $I_2$ (○), zoea II (◇), megalopa (Δ), crab (▽), and centroid of each developmental stage (✳).

**Table 2**
Summary of the results of the canonical discriminant analysis (only significant discriminant functions are shown)

| Method | Function | Eigenvalue | Percentage of variance | Canonical correlation | Wilks Lambda | Chi-square | d.f. | *P* |
|---|---|---|---|---|---|---|---|---|
| SP | 1 | 6.49 | 91.92 | 0.931 | 0.084 | 126.29 | 12 | <0.0001 |
| | 2 | 0.53 | 7.55 | 0.590 | 0.629 | 23.64 | 6 | 0.0006 |
| FL | 1 | 11.46 | 83.21 | 0.959 | 0.023 | 297.23 | 12 | <0.0001 |
| | 2 | 2.24 | 16.32 | 0.832 | 0.289 | 97.96 | 6 | <0.0001 |

FL = fluorometric method; SP = spectrophotometric method.

The use of fluorescent substrates resulted in no difference in the activity profile when the results were expressed either per individual or in terms of soluble protein. In contrast, when the assays were performed using SP, values per individual increased parallel with age, whereas no significant difference could be observed if the results were expressed in a soluble protein basis. Although homogenization of the samples preceded both methods, the use of SP substrates required a higher amount of biological material. Therefore, more variability in protein analysis were found in the SP samples (replicates of pooled larvae), which masked the putative differences between stages of development; while the FL showed lesser variation between samples (individuals) that resulted in significant differences.

A canonical discriminant analysis (CDA) was used to find the linear combination of the variables (enzyme activities) that best described the developmental stage in spider crab according to the both enzyme assay methods used (Fig. 2). In both cases, the CDA produced two discriminant functions that accounted for 99.5% of the variance (Table 2). Standardised discriminant function coefficients indicated that the stage of development in spider crab larvae was most strongly discriminated by the activity of amylase, whichever method of analysis was considered (Table 3). Discriminant functions were also successful in re-assigning cases to their stage of development, with 86 and 76% of cases correctly classified when data were analysed using FL and SP methods. Unsuccessful classification was only observed within the same stage of development ($ZI_0$ and $ZI_2$), when digestive enzyme activities between zoeae were still conspicuous (Fig. 2).

The overall evaluation of both analytical methods is summarized in Table 4. In particular, FL analyses allowed detecting up to three enzyme activities in a single larva from hatching (<100 μg dry weight). This high sensitivity is clearly supported by the results shown in Table 5, where the amount of biological material used in each analysis is detailed. The amount of tissue used for each analysis depends on the activity assayed, being particularly favourable for FL substrates, especially for trypsin or amylase activities analysed at zoea and megalopa stages. The individual assay of each enzyme is linked to the higher resolution of the FL method, since the substrates used for the analysis are more unstable and require less quantity of enzyme for their hydrolysis. Nevertheless, the higher sensitivity of the FL method implies a larger variability in the results compared to SP in which data were obtained from pooled samples of individuals (Table 6). However, this could be an advantage, since it facilitates studying the inter-individual variability, whereas the use of the SP method only evaluates the variability between samples, composed in most cases of a high number of individuals. The FL results also revealed that the intra-population variability (individuals from the same maternal origin) was higher than the inter-population variability (individuals from different females) (Table 7). These results suggest that special caution needs to be taken when evaluating the quality of the offspring from different spawning and maternal origin when using enzyme activity at early stages of development.

**Table 3**
Standardised discriminant function coefficients for each of the significant discriminant functions

| | Discriminant functions | | | |
|---|---|---|---|---|
| | FL | | SP | |
| Enzyme | 1 | 2 | 1 | 2 |
| Trypsin | 0.450 | −0.591 | 0.112 | 1.036 |
| Amylase | 0.917 | −0.219 | 0.979 | −0.341 |
| Esterase | 0.527 | 0.910 | −0.0576 | −0.257 |

Functions 1 and 2 account for the 99.5% of the variance in both methods. FL = fluorometric method; SP = spectrophotometric method.

**Table 4**
Global evaluation of enzyme analysis in decapod crustacean larvae by spectrophotometry (SP) and fluorometry (FL)

| | SP | FL |
|---|---|---|
| Sensitivity | 1 | 3 |
| Resolution | 2 | 3 |
| Interference | 2 | 1 |
| Repetitiveness | 2 | 2 |
| Feasibility | 2 | 3 |
| Standardisation | 3 | 1 |
| Cost | 1 | 3 |

Range of evaluation: high = 3, medium = 2 and low = 1.

A similar reproducibility (up to 12% CV) was obtained when trypsin and amylase activities were assayed by either FL or SP method (Table 8). In the case of esterase, when the FL analysis was used, a maximum of 5.7% CV was observed, compared to 26% CV obtained by the SP method.

Another important aspect to consider in this evaluation is how the results were affected by interferences. Although homogenization of the samples precedes both methods, the use of SP substrates requires a higher amount of biological material; therefore, the extracts contain larger quantities of cell debris and other compounds (e.g. enzyme inhibitors, proteins of different molecular weight) that might show the same absorbance (wavelength) as the hydrolysis products. Although blanks are used to correct this background noise, the problem arises when the extracts show very low activity. In contrast, turbidity does not affect the excitation of the FL substrates during the reading. Background noise in this case should be produced only by the presence of natural fluorescent substances like some pigments, which are widespread in samples of vegetable origin but are rare in animal tissues.

No differences in feasibility between either method were found when kinetic analyses were used to evaluate enzyme activity, as in this

**Table 5**
Sensitivity of enzyme analysis by spectrophotometry (SP) and fluorometry (FL) expressed by enzyme (trypsin, amylase, and esterase) and by stage of development [newly hatched larvae, day 0 – zoea ($ZI_0$); zoea I at day 2 ($ZI_2$); zoea II at day 7 ($ZII_7$); megalopa at day 11 ($M_{11}$); first crab at day 20 ($C_{20}$)]

| | | SP | | FL | | SP/FL |
|---|---|---|---|---|---|---|
| Enzyme | Stage | No. larvae | Dry weight sample (μg) | No. larvae | Dry weight sample (μg) | Dry weight sample (μg) |
| Trypsin | $ZI_0$ | 2.8±0.7 | 245.2±96.3 | 0.5 | 47.4±1.7 | 5.2 |
| | $ZI_2$ | 2.8±0.4 | 311.8±48.8 | 0.5 | 57.8±2.3 | 5.4 |
| | $ZII_7$ | 1.8±0.2 | 332.9±43.5 | 0.1 | 19.2±1.4 | 17.3 |
| | $M_{11}$ | 1.2±0.3 | 403.6±24.8 | 0.05 | 18.0±0.8 | 22.4 |
| | $C_{20}$ | 0.6±0.1 | 297.9±90.7 | 0.5 | 249.1±39.4 | 1.2 |
| Amylase | $ZI_0$ | 1.7±0.4 | 147.1±57.7 | 0.1 | 9.5±0.4 | 15.5 |
| | $ZI_2$ | 1.7±0.3 | 187.1±29.3 | 0.1 | 11.6±0.5 | 16.1 |
| | $ZII_7$ | 1.1±0.1 | 199.7±26.1 | 0.5 | 96.1±7.2 | 2.1 |
| | $M_{11}$ | 0.7±0.2 | 242.2±14.9 | 0.5 | 180.0±8.4 | 1.3 |
| | $C_{20}$ | 0.3±0.1 | 178.7±10.6 | 0.1 | 49.8±7.9 | 3.6 |
| Esterase | $ZI_0$ | 0.6±0.1 | 49.0±19.2 | 0.1 | 9.5±0.3 | 5.2 |
| | $ZI_2$ | 0.6±0.1 | 62.4±9.8 | 0.1 | 11.6±0.5 | 5.4 |
| | $ZII_7$ | 0.4±0.0 | 66.6±8.7 | 0.1 | 19.2±1.4 | 3.5 |
| | $M_{11}$ | 0.2±0.1 | 80.7±5.0 | 0.1 | 36.0±1.7 | 2.2 |
| | $C_{20}$ | 0.1±0.0 | 59.6±3.5 | 0.1 | 49.8±7.9 | 1.2 |

**Table 6**
Comparison of the variability of enzyme data analysed by spectrophotometry (SP) and fluorometry (FL) expressed as a coefficient of variation (CV) by enzyme (trypsin, amylase, and esterase) and by stage of development [newly hatched larvae, day 0 – zoea ($ZI_0$); zoea I at day 2 ($ZI_2$); zoea II at day 7 ($ZII_7$); megalopa at day 11 ($M_{11}$); first crab at day 20 ($C_{20}$)]

| | | SP | FL |
|---|---|---|---|
| Enzyme | Stage | CV (%) | CV (%) |
| Trypsin | $ZI_0$ | 4.37 | 80.26 |
| | $ZI_2$ | 17.40 | 87.21 |
| | $ZII_7$ | 4.79 | 79.48 |
| | $M_{11}$ | 9.13 | 92.34 |
| | $C_{20}$ | 26.55 | 99.94 |
| Amylase | $ZI_0$ | 17.83 | 88.39 |
| | $ZI_2$ | 5.52 | 87.39 |
| | $ZII_7$ | 12.08 | 71.47 |
| | $M_{11}$ | 18.98 | 76.68 |
| | $C_{20}$ | 0.66 | 87.56 |
| Esterase | $ZI_0$ | 37.00 | 64.69 |
| | $ZI_2$ | 9.55 | 84.96 |
| | $ZII_7$ | 9.64 | 67.39 |
| | $M_{11}$ | 19.12 | 82.25 |
| | $C_{20}$ | 19.81 | 65.47 |

The CV for the SP and FL methods was calculated using data from four and two different spawning, respectively.

**Table 8**
Reproducibility of enzymatic activity measured by spectrophotometry (SP) and fluorometry (FL) expressed as the coefficient of variation (CV) of replicates ($n$=2–3) from the same samples ($n$=4 for SP and $n$=15–20 for FL) for each enzyme (trypsin, amylase, and esterase) and stage of development [newly hatched larvae, day 0 – zoea ($ZI_0$); zoea I at day 2 ($ZI_2$); zoea II at day 7 ($ZII_7$); megalopa at day 11 ($M_{11}$); first crab at day 20 ($C_{20}$)]

| | | SP | FL |
|---|---|---|---|
| Enzyme | Stage | CV (%) | CV (%) |
| Trypsin | $ZI_0$ | 3.0±3.5 | 2.8±1.9 |
| | $ZI_2$ | 8.3±5.9 | 4.1±3.8 |
| | $ZII_7$ | 5.3±2.5 | 2.8±2.1 |
| | $M_{11}$ | 5.1±4.3 | 3.8±3.0 |
| | $C_{20}$ | 12.6±3.2 | 4.4±4.2 |
| Amylase | $ZI_0$ | 5.6±3.4 | 7.8±8.4 |
| | $ZI_2$ | 5.7±4.6 | 7.5±6.7 |
| | $ZII_7$ | 5.6±6.6 | 8.7±8.1 |
| | $M_{11}$ | 1.7±1.8 | 8.8±7.7 |
| | $C_{20}$ | 6.8±5.9 | 5.7±5.8 |
| Esterase | $ZI_0$ | 18.7±13.7 | 5.7±5.1 |
| | $ZI_2$ | 15.6±13.6 | 4.6±3.6 |
| | $ZII_7$ | 26.0±16.5 | 3.0±1.7 |
| | $M_{11}$ | 13.8±7.0 | 2.4±2.0 |
| | $C_{20}$ | 12.0±5.5 | 2.0±1.4 |

study with trypsin and esterase. The assay of other digestive enzymes, such as amylase, by SP methods that are not based on a kinetic reaction (end point absorbance measurement) increases the duration of the analysis in comparison to FL assays. Depending on the experimental conditions, this might be a limitation regarding the amount of samples processed per unit of time. Nevertheless, FL assays have the inconvenience that substrates are very unstable, and therefore the analysis has to be performed in a shorter time.

As previously described in crustacean larvae by Le Vay et al. (2001), results of enzyme activities obtained using SP assays are easily comparable between experiments using different spectrophotometers. This comparison can be done with data within the same order of magnitude, and the results can be standardised in units of dry body weight (Le Vay et al., 2001) or soluble protein concentration (Saborowski et al., 2006). Standardisation of units is difficult when using FL substrates, since differences between equipment sensitivity could be larger than one order of magnitude. In addition the assay of activity using SP methods is routinely based on molecular extinction coefficients, but their equivalent in FL assays are not fixed or defined in the same manner. Thus, the expression of activity in units (e.g. μmol/min) must be established previously for each piece of equipment after preparing standard curves using the fluorogen attached to each specific substrate. This greatly restricts the comparison among the results obtained by different authors.

From a practical point of view, availability and cost of either FL or SP substrates must be also considered. Although availability of FL substrates in the market is constantly increasing, it is still scarce in relation to the high number and types of substrates normally used in SP methods. This may be a limitation to the study of certain enzyme activities. The cost of the analysis is mainly based on the price of the specific substrates rather than the cost of the reaction buffers and the labour time, except for the amylase end-point reaction analysed by the SP assay. In general, FL substrates are more expensive than SP ones due to their biochemical properties (e.g. trypsin and amylase substrates are 30- and 1000-fold more expensive for FL than SP, respectively). Although the esterase substrate is cheaper in FL analyses (1000-fold), the short life-time of the FL substrate increases the reaction cost.

**Table 7**
Inter-population variability (individuals from different maternal origin) of enzymatic activity measured by fluorometry

| | | Female # 1 | | Female # 2 | |
|---|---|---|---|---|---|
| Enzyme | Stage | IU·ind$^{-1}$ | CV (%) | IU·ind$^{-1}$ | CV (%) |
| Trypsin | $ZI_0$ | 1566.84±634.98 | 80.26 | 1426.55±620.96 | 77.21 |
| | $ZII_7$ | 4436.55±1682.57 | 99.35 | 3460.11±1243.35 | 94.45 |
| | $M_{11}$ | 6955.45±2613.02 | 92.39 | 4143.27±1856.37 | 83.94 |
| | $C_{20}$ | 1965.84±1462.94 | 99.94 | 1981.17±1387.15 | 94.63 |
| Amylase | $ZI_0$ | 2.27±1.11 | 88.34 | 6.78±2.02 | 71.68 |
| | $ZII_7$ | 69.54±22.83 | 71.47 | 56.42±22.28 | 76.11 |
| | $M_{11}$ | 104.88±31.30 | 76.68 | 100.98±38.84 | 80.47 |
| | $C_{20}$ | 21.57±13.74 | 96.54 | 30.48±12.78 | 82.21 |
| Esterase | $ZI_0$ | 280.80±86.69 | 64.69 | 250.00±29.50 | 34.39 |
| | $ZII_7$ | 704.62±152.28 | 67.39 | 549.19±134.94 | 59.22 |
| | $M_{11}$ | 1308.71±478.41 | 82.25 | 1364.89±300.61 | 53.90 |
| | $C_{20}$ | 1391.53±294.62 | 65.47 | 976.20±353.59 | 98.23 |

Mean, standard deviation and coefficient of variation (CV) for two different spawning for each enzyme (trypsin, amylase, and esterase) and stage of development [newly hatched larvae, day 0 – zoea ($ZI_0$); zoea II at day 7 ($ZII_7$); megalopa at day 11 ($M_{11}$); first crab at day 20 ($C_{20}$)].

## 4. Conclusions

Both SP and FL methods are useful to measure digestive enzymes in decapod crustacean larvae and can be used to provide new insights into the digestive physiology of larvae and to evaluate the nutritional condition of individuals. Depending on the interest and the experimental design of each study, one method or the other may be more appropriate. For studies based on the characterization of enzyme activities in crustacean populations without any limitation on the number and amount of samples, such as the studies already conducted with different species of prawns, lobsters, and crabs, the SP method is recommended, since it is less labour demanding and cheaper. When several experimental treatments, such as different zootechnical parameters (temperature, salinity, oxygen concentration, tank colour, larval/prey density, etc.), dietary regimes, and/or moulting stages are considered, the FL analysis might be more appropriate due to the low number of individuals needed for sampling, reducing the experimental facilities to a minimum. If the study is conducted to assess the individual variability of the population, then, the FL method needs to be applied.

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## References

Andrés, M., Estévez, A., Rotllant, G., 2007. Growth, survival and biochemical composition of spider crab *Maja brachydactyla* (Blass, 1922) (Decapoda:Majidae) larvae reared under different stocking densities, prey:larva ratios and diets. Aquaculture 273, 494–502.

Andrés, M., Estévez, A., Anger, K., Rotllant, G., 2008. Growth and biochemical composition during larvar and early juvenile development of the spider crab, *Maja brachydactyla* (Decapoda: Majidae). J. Exp. Mar. Biol. Ecol. 357, 35–40.

Biesiot, P.M., Capuzzo, J.M., 1990. Changes in digestive enzyme-activities during early development of the American lobster *Homarus americanus* Milne-Edwards. J. Exp. Mar. Biol. Ecol. 136, 107–122.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cara, B., Moyano, F.J., Zambonino Infante, J.L., Fauvel, C., 2007. Trypsin and chymotrypsin as indicators of nutritional status of post-weaned sea bass larvae. J. Fish Biol. 70, 1798–1808.

Carrillo-Farnés, O., Forrellat-Barrios, A., Guerrero-Galván, S., Vega-Villasante, F., 2007. A review of digestive enzyme activity in Penaeid shrimps. Crustaceana 80, 257–275.

Conklin, D.E., 1995. Digestive physiology and nutrition. In: Factor, J.R. (Ed.), Biology of the Lobster Homarus americanus. Academic Press, Boston, pp. 441–463.

Fang, L.S., Lee, B.N., 1992. Ontogenic change of digestive enzymes in *Penaeus monodon*. Comp. Biochem. Physiol. 103B, 1033–1037.

Galgani, F., Benyamin, Y., 1985. Radioimmunoassay of shrimp trypsin — application to the larval development of *Penaeus japonicus* Bate, 1888. J. Exp. Mar. Biol. Ecol. 87, 145–151.

Harms, J., Anger, K., Klaus, S., Seeger, B., 1991. Nutritional effects on ingestion rate, digestive enzyme activity, growth, and biochemical composition of *Hyas araneus* L (Decapoda, Majidae) larvae. J. Exp. Mar. Biol. Ecol. 145, 233–265.

Harms, J., Meyerharms, B., Dawirs, R.R., Anger, K., 1994. Growth and physiology of *Carcinus maenas* (Decapoda, Portunidae) larvae in the field and in laboratory experiments. Mar. Ecol., Prog. Ser. 108, 107–118.

Hirche, H.J., Anger, K., 1987. Digestive enzyme activities during larval development of *Hyas araneus* (Decapoda, Majidae). Comp. Biochem. Physiol. 87B, 297–302.

Izquierdo, M.S., Henderson, R.J., 1998. The determination of lipase and phospholipase activities in gutcontents of turbot (Scophthalmus maximus) by fluorescence-based assays. Fish Physiol. Biochem. 19, 153–162.

Johnston, D.J., 2003. Ontogenetic changes in digestive enzyme activity of the spiny lobster, *Jasus edwardsii* (Decapoda; Palinuridae). Mar. Biol. 143, 1071–1082.

Johnston, D., Ritar, A.J., Thomas, C., Jeffs, A., 2004. Digestive enzyme profiles of spiny lobster *Jasus edwardsii* phyllosoma larvae. Mar. Ecol., Prog. Ser. 275, 219–230.

Jones, D.A., Kamarudin, M.S., Le Vay, L., 1993. The potencial replacement of live feeds in larval culture. J. World Aquacult. Soc. 24 (2), 199–210.

Jones, D.A., Yule, A.B., Holland, D.L., 1997. Crustacean nutrition. In: D'Abramo, L.R, Conklin, D.E., Akiyama, D.M. (Eds.), Larval nutrition, vol. 6. Academic Press, New York, USA, pp. 353–389.

Kamarudin, M.S., Jones, D.A., Levay, L., 1994. Ontogenic change in digestive enzyme activity during larval development of *Macrobrachium rosenbergii*. Aquaculture 123, 323–333.

Kanazawa, A., 1994. Nutrition and food something is wrong here. In: Philips, B.F., J.S.C., J., Kittaka (Eds.), Spiny Lobster Management. Fishing New Books. Blackwell, London, pp. 483–494.

Knotz, S., Boersma, M., Saborowski, R., 2006. Microassays for a set of enzymes in individual small marine copepods. Comp. Biochem. Physiol. 145, 406–411.

Kumlu, M., Jones, D.A., 1995. Feeding and digestion in the caridean shrimp larva of *Palaemon elegans* Rathke and *Macrobrachium rosenbergii* (De Man) (Crustacea: Palaemonidae) on live and artificial diets. Aquac. Nutr. 1, 3–12.

Kumlu, M., Jones, D.A., 1997. Digestive protease activity in planktonic crustaceans feeding at different trophic levels. J. mar. biol. Assoc. U.K. 77, 159–165.

Kumlu, M., Sarihan, E., Tekelioglu, N., 1992. Trypsin activity in larvae of *Penaeus monodon* Fabricius, 1789 (Crustacea, Decapoda, Penaeidae) in relation to their diet. Isr. J. Aquac.-Bamidgeh 44, 103–110.

Lazo, J.P., Mendoza, R., Holt, G.J., Aguilera, C., Arnold, C.R., 2007. Characterization of digestive enzymes during larval development of red drum (*Sciaenops ocellatus*). Aquaculture 265, 194–205.

Lemos, D., Hernández-Cortés, M.P., Navarrete, A., Garcia-Carreño, F.L., Phan, V.N., 1999. Ontogenic variation in digestive proteinase activity of larvae and postlarvae of the pink shrimp *Farfantepenaeus paulensis* (Crustacea: Decapoda: Penaeidae). Mar. Biol. 135, 653–662.

Lemos, D., Ezquerra, J.M., Garcia-Carreño, F.L., 2000. Protein digestion in penaeid shrimp: digestive proteinases, proteinase inhibitors and feed digestibility. Aquaculture 186, 89–105.

Le Moullac, G., Van Wormhoudt, A., AQUACOP, 1994. Adaptation of digestive enzymes to dietary protein, carbohydrate and fibre levels and influence of protein and carbohydrate quality in *Penaeus vannamei* larvae (Crustacea, Decapoda). Aquat. Living Resour. 7, 203–210.

Le Vay, L., Rodríguez, A., Kamarudin, M.S., Jones, D.A., 1993. Influence of live and artificial diets on tissue composition and trypsin activity in *Penaeus japonicus* larvae. Aquaculture 118, 287–297.

Le Vay, L., Jones, D.A., Puello-Cruz, A.C., Sangha, R.S., Ngamphongsai, C., 2001. Digestion in relation to feeding strategies exhibited by crustacean larvae. Comp. Biochem. Physiol. Part A 128, 623–630.

Lovett, D.L., Felder, D.L., 1990. Ontogenetic change in digestive enzyme activity of larval and postlarval white shrimp *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). Biol. Bull. 178, 144–159.

MacDonald, N.L., Stark, J.R., Keith, M., 1989. Digestion and nutrition in the prawn Penaeus monodon. World Aquaculture Soc., p. 20.

Mckellar, R.C., Cholette, H., 1986. Determination of the extracellularlipases of Pseudomonas fluorescens spp. in skim milkwith the beta-naphthyl caprylate assay. J. Dairy Res. 53, 301.

Metais, P., Bieth, J., 1968. Determination of alpha amylase by a microtechnic. Ann. Biol. Clin. 26, 133–142.

Pedroza-Islas, R., Gallardo, P., Vernon-Carter, E.J., García-Galano, T., Rosas, C., Pascual, C., Gaxiola, G., 2004. Growth, survival, quality and digestive enzyme activities of larval shrimp fed microencapsulated, mixed and live diets. Aquac. Nutr. 10, 167–173.

Rodríguez, C., Pérez, J.A., Lorenzo, A., Izquierdo, M.S., Cejas, J.R., 1994. n-3 HUFA requirement of larval gilthead seabream *Sparus aurata* when using high levels of eicosapentaenoic acid. Comp. Biochem. Physiol. 107A, 693–698.

Rosas, C., Cuzon, G., Gaxiola, G., Arena, L., Lemaire, P., Soyez, C., Van Wormhoudt, A., 2000. Influence of dietary carbohydrate on the metabolism of juvenile *Litopenaeus stylirostris*. J. Exp. Mar. Biol. Ecol. 249, 181–198.

Saborowski, R., Thatje, S., Calcagno, J.A., Lovrich, G.A., Anger, K., 2006. Digestive enzymes in the ontogenetic stages of the southern king crab, *Lithodes santolla*. Mar. Biol. 149, 865–873.

Sheen, S.S., Huang, H.T., 1998. The effects of different protein sources on the survival of Grass Shrimp, *Penaeus monodon* (Fabricius, 1798) larvae from zoea to postlarva (Decapoda, Natantia). Crustaceana 71, 909–924.

StatSoft, Inc. (2007). Electronic Statistics Textbook. Tulsa, OK: StatSoft. WEB:http://www.statsoft.com/textbook/stathome.html.

Ueberschär, B., 1995. The use of tryptic enzyme activity measurement as a nutritional condition index: laboratory calibration data and field application. ICES Mar. Sci. Symp. 201, 119–129.

Vaneechoutte, M., Verschraegen, G., Claeys, G., Flamen, P., 1988. Rapid identification of *Branhamella catarrhalis* with 4-methylumbelliferyl butyrate. J. Clin. Microbiol. 26, 1227–1228.

Versaw, W.K., Cuppett, S.L., Winters, D.D., Williams, L.E., 1989. An improved colorimetric assay for bacterial lipase in nonfat dry milk. J. Food Sci. 54, 1557.

Vonk, H.J., 1960. Digestion and metabolism. In: Waterman, T.H. (Ed.), The physiology of crustacea I: Metabolism and growth. Academic Press, New York, USA, pp. 291–316.