

D. Lemos · M. P. Hernández-Cortés · A. Navarrete
F. L. García-Carreño · V. N. Phan

Ontogenetic variation in digestive proteinase activity of larvae and postlarvae of the pink shrimp *Farfantepenaeus paulensis* (Crustacea: Decapoda: Penaeidae)

Received: 24 September 1998 / Accepted: 20 August 1999

Abstract Proteinase (endopeptidase), trypsin-like and chymotrypsin-like activities were examined throughout the ontogenetic development of cultured *Farfantepenaeus paulensis*. Whole individuals from different larval and postlarval stages, and the hepatopancreas of adults were homogenized and assayed to quantify the enzyme activities of specific substrates. Proteinase activity was identified by substrate-SDS-polyacrylamide gel electrophoresis. Specific inhibitors for trypsin (TLCK), chymotrypsin (TPCK) and serine proteinases (PMSF) were used to identify activity zones of these enzymes in gels. Protein-specific activity of total proteinases, trypsin and chymotrypsin was negligible at the egg stage and at Nauplius III, increasing in the first protozoal substage (PZ I), and reaching a peak at PZ III; it decreased again in the subsequent postlarval substages. Different patterns of proteinase activity were observed in SDS-PAGE zymograms during ontogenetic development. Active bands of 14.6, 16.4, 17.5, 19.5, 22.5, 23.9, 25.8, 28.9, 32.0, 34.4, 37.7, and 42.2 kdaltons were detected in the adult hepatopancreas. Proteolytic activity was detected on gels in PZ I, and intense activity zones of 16.4, 17.5 and 19.5 kdaltons were found up to Mysis I (M I). Intense bands of 39.1 and 53.5 kdaltons were observed only at PZ III and M I. Band-activity intensity decreased after metamorphosis to the postlarval stage (PL). The chymotrypsin inhibitor TPCK had

no effect on the proteinase bands. Active zones in gel inhibited with both TLCK and PMSF were considered to represent trypsin. The inhibitory effect of PMSF alone on proteinase extracts indicated chymotrypsin activity. TLCK and PMSF inhibition also varied during ontogenetic development. The inhibition of bands recorded between 14.6 and 21.7 kdaltons suggested the presence of low molecular weight trypsin in *F. paulensis*. The 39.1 kdaltons band observed at PZ III and M I were trypsin-like. On the other hand, bands of 28.9, 32 and 37.7 kdaltons from the adult hepatopancreas seem to represent a chymotrypsin. We conclude that the recorded variation in enzyme activity may be associated with morphological and behavioral changes during penaeid ontogenetic development. The higher enzyme activity at PZ II, PZ III and M I may reflect the increased energy turnover associated with intense swimming behavior and food ingestion.

Introduction

The ontogenetic development of penaeid shrimp is unique among decapod crustaceans in that all their larval stages are free-swimming, and the transformation to adult morphology and habits is not attained through metamorphosis to a postlarva (decapodid) or during the stages immediately preceding or succeeding metamorphosis (Pérez-Pérez and Ros 1975; Felder et al. 1985; Lovett and Felder 1989; Dall et al. 1990). After hatching, penaeid larvae develop through 5 to 6 non-feeding naupliar, 3 protozoal, and 3 mysid substages prior to metamorphosis to the postlarval stage. In the wild, larval development is generally completed in offshore waters. Within the first days of postmetamorphic life, the postlarvae penetrate into inshore, brackish, nursery grounds. During the sequential transformation of larvae and postlarvae, significant changes occur in both external and internal morphology (Lovett and Felder 1989; Dall et al. 1990; Abubakr and Jones 1992; Icelly and

Communicated by N.H. Marcus, Tallahassee

D. Lemos (✉) · V.N. Phan
Instituto Oceanográfico,
University of São Paulo, C.P. 66149,
São Paulo 05315-9370
Brazil

Fax: 55 11 2103092
e-mail: dellemos@usp.br

M.P. Hernández-Cortés · A. Navarrete · F.L. García-Carreño
Centro de Investigaciones Biológicas del Noroeste,
A.P. 128, La Paz, Baja California Sur,
23000 Mexico

Nott 1992), habits (Emmerson 1980, 1984; Jones et al. 1992), metabolic rates (Chu and Ovsianico-Koulikowski 1994), and digestive enzyme activity (Laubier-Bonichon et al. 1977; Lovett and Felder 1990a; Fang and Lee 1992; Le Moullac et al. 1992; González et al. 1994). An understanding of digestive physiology is important for studies on the nutritional requirements and feeding ecology of marine invertebrates (Biesiot and Capuzzo 1990). Because protein is the major component in the natural food of penaeids (Smith et al. 1992), proteolytic enzymes may play a key role in the assimilation processes of these species. Among the proteolytic enzymes of shrimp, trypsin may be responsible for 40 to 60% of total protein digestion (Galgani et al. 1984, 1985; Tsai et al. 1986), and it has been characterized in several penaeid species (Honjo et al. 1990 for *Fenneropenaeus indicus*; Lu et al. 1990 and Jiang et al. 1991 for *Penaeus monodon*; Klein et al. 1996 for *Litopenaeus vannamei*). The occurrence of chymotrypsin activity has been controversial because it was believed to be either absent (Lee et al. 1980; Galgani et al. 1984; Lovett and Felder 1990a) or present (Tsai et al. 1986; Fang and Lee 1992; Van Wormhoudt et al. 1992; Le Moullac et al. 1994) in the shrimp digestive tract. Although changes in digestive enzyme activity during shrimp development have been studied in some species, characterization of the enzymes has received little attention.

The present study describes the activity of proteinases, also called endopeptidases, during larval and early postlarval development of *Farfantopenaeus paulensis*, and compares it with activity in the adult shrimp. The pink shrimp *F. paulensis*, also known as the São Paulo shrimp (Pérez-Farfante 1967), constitutes one of the main fishery resources of the Southern Brazilian coast and shelf (Valentini et al. 1991), and is an important component of the benthic megafauna of this area (Pires-Vanin 1989; Wakabara et al. 1996). To characterize trypsin and chymotrypsin in various developmental stages, protein extracts were assayed using specific inhibitors and synthetic substrates. Proteinase activity was correlated with other penaeid developmental events such as morphological and behavioral changes. The generic designations in the text follow the recently proposed nomenclature for members of the family Penaeidae (Pérez-Farfante and Kensley 1997; Holthuis 1998).

Materials and methods

Experimental shrimp

Farfantopenaeus paulensis were caught along the Santa Catarina State coast, Brazil (Latitude 27°18'S; Longitude 48°23'W). After a period of acclimation, wild females of 33.9 ± 4.7 g wet weight were induced to mature sexually by unilateral eyestalk ablation, and were maintained individually isolated in 500-liter fiberglass tanks. Spawning took place during the night, and viable eggs (~200 000 per female) were transferred to cylindrical conical tanks, in which they hatched 12 to 14 h later at 26 °C ± 1 °C. *F. paulensis* has 6 naupliar (N I to VI), 3 protozoal (PZ I to III), and 3 mysid (M I to III) substages (Iwai 1978). Then nauplii were moved

to 50 000-liter tanks, and were reared following commercial practice at 26 °C ± 1 °C and 34 ± 1‰S (Vinatea et al. 1993; Beltrame et al. 1996). Exogenous food was supplied from PZ I onward, and consisted of the diatom *Chaetoceros calcitrans* at (80 000 cells ml⁻¹) and artificial plankton (Nippai Shrimp Feed Inc., Japan; 0.03 mg larva⁻¹ d⁻¹, 30 µm particle-size). Freshly hatched nauplii of *Artemia* sp. (15 nauplii larva⁻¹ d⁻¹) were added to the diet at Stage PZ III. After metamorphosis to postlarva (PL), the diet was composed of microalgae with increasing amounts of artificial plankton and brine shrimp nauplii (*Artemia* sp.).

Pools of individuals were selected throughout ontogenetic development from a tank containing 7 to 9 synchronized spawns. A homogeneous sample was defined when >80% of individuals belonged to the same substage; the remaining individuals differed by a single substage only. Eggs were chosen 10 to 12 h after spawning at the "early nauplius" stage according to Primavera and Posadas (1981). During the naupliar stage, individuals were selected of N III. From the PZ I substage, each subsequent larval substage was analyzed until metamorphosis. Postlarvae from specific instars (PL I to PL VII) were sorted according to Iwai (1978). Samples were immediately frozen at -20 °C and transferred to liquid nitrogen. The hepatopancreas of three adult, sexually immature, females were extracted, frozen at -20 °C, and freeze-dried. These females were previously fed with frozen mussel and squid, and fasted for 12 h before dissection of the hepatopancreas.

Water and protein determination

Eggs and larvae were washed in distilled water and, after removal of excess water, 3 to 6 samples of pooled individuals were weighed prior to being oven-dried at 60 to 70 °C for 48 h. Wet and dry weight were measured on a Cahn C-31 microbalance (0.1 µg precision). Water content was determined by comparing fresh and dry weights. Total soluble proteins were determined in freeze-dried samples by the Coomassie blue dye method (Bradford 1976), with bovine albumin as the standard.

Quantification of enzyme activity

Approximately 0.7 to 1.2 g wet weight of whole larvae or post-larvae, and 0.02 to 0.08 g dried adult hepatopancreas were homogenized in chilled distilled water and centrifuged at 10 000 ×g for 20 min at 5 °C. After lipid elimination, total soluble proteins were determined in the supernatants (Bradford 1976). Total proteinase activity was measured by the rate of hydrolysis of 1.5% azocasein in 50 mM Tris buffer, pH 7.5 (García-Carreño 1992). Triplicates of 10 µl enzyme extracts were mixed with 0.5 ml substrate solution at 25 °C. The reaction was stopped 10 min later by the addition of 0.5 ml of 20% trichloroacetic acid (TCA), and the mixture was centrifuged for 5 min at 6 500 ×g. The supernatants were separated from the undigested substrate, and the absorbance for the released dye was recorded at 440 nm. Trypsin and chymotrypsin activities were determined by the rate of hydrolysis of synthetic substrates (Table 1). Trypsin activity was measured in the early stages for both TAME (*N*α-*p*-toluenesulphonyl-L-arginine methyl ester) and BAPNA (*N*α-benzoyl-DL-arginine-*p*-nitroanilide) substrates. Adult hepatopancreas were assayed for trypsin activity using only BAPNA. Samples (*n* = 6 to 20) of 300 µl enzyme extract were mixed with 1.2 ml 10 mM TAME in 46 mM Tris buffer, pH 8.1, containing 11.5 mM CaCl₂ at 25 °C, and the change in absorbance at 247 nm was recorded over 3 min (Rick 1984). One millimolar BAPNA was dissolved in 1 ml dimethylsulfoxide (DMSO) and then made up to 100 ml with Tris, pH 7.5, containing 20 mM CaCl₂. Triplicates of 10 µl were added to 1 ml of substrate solution at 37 °C, and the changes at 410 nm were recorded over 5 min (Erlanger et al. 1961). Chymotrypsin activity was determined using 0.1 mM SAPFNA (*N*-succinyl-L-ala-L-ala-L-pro-L-phe-*p*-nitroanilide) in 0.1 M Tris, pH 7.8, containing 0.01 M CaCl₂. Triplicate samples of 10 µl were mixed with 0.69 ml substrate solution, and the absorbance at 410 nm was recorded over 3 min at

Table 1 Substrates and inhibitors used in proteolytic enzyme assays during ontogenetic development of *Farfantepenaeus paulensis*

Enzyme	Substrate	Inhibitor
Total proteinases	Azocasein or casein ^a	
Chymotrypsin	SAPFNA ^b (<i>N</i> -succinyl-L-ala-L-ala-L-pro-L-phe- <i>p</i> -nitroanilide)	TPCK ^c (tosyl-phenylalanine chloromethyl ketone)
Trypsin	TAME ^c (<i>N</i> α - <i>p</i> -toluenesulphonyl-L-arginine methyl ester) BAPNA ^d (<i>N</i> α -benzoyl-DL-arginine- <i>p</i> -nitroanilide)	TLCK ^e (tosyl-lysine chloromethyl ketone)
Serine proteinases		PMSF ^e (phenylmethylsulphonyl fluoride)

^a Garcia-Carreño et al. (1993)

^b Del Mar et al. (1979)

^c Rick (1984)

^d Erlanger et al. (1961)

^e Beynon and Salvesen (1990)

25 °C (Del Mar et al. 1979). Each assay included blanks in which the changes in absorbance were recorded without the enzyme extract. Total proteinase, trypsin, and chymotrypsin activities were expressed as the change in absorbance per min per mg protein of the enzyme extract used in the assays ($\Delta A \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$).

Enzyme characterization

Proteinase activity was studied throughout ontogenetic development after separation of proteins by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). The Electrophoresis was carried out using 12% acrylamide (Laemmli 1970). The 0.1% concentration of SDS used does not affect proteinase activity in PAGE (Garcia-Carreño et al. 1993). Enzyme preparations (0.02 mg protein per lane) of each substage were loaded in a temperature-controlled (4 °C) vertical electrophoresis device (Hoefer, San Francisco, California). Molecular weight standards (0.0175 mg) were included on each plate. Proteinase activity of the extract was characterized according to Garcia-Carreño et al. (1993). After electrophoresis, gels were immersed in 3% casein in 50 mM Tris, pH 7.5, for 30 min at 5 °C to allow the substrate to diffuse into the gel at low enzyme activity. The temperature was then raised to 25 °C for 90 min for the digestion of the protein substrate by the active fractions. Gels were next washed in water, and immediately fixed and stained by immersion in a solution containing 40% ethanol, 10% acetic acid, and 0.1% Coomassie brilliant blue R-250. After staining period, of at least 24 h, gels were de-stained with the same solution without the Coomassie dye, and dried using a Bio-Rad 443 slab-gel dryer. Clear zones on a blue background indicated proteinase activities and could be compared to molecular weight standard bands. A regression between the distance of molecular weight standard bands from the top border of the gel (cm) and the log of their molecular mass (kdaltons) determined the molecular weight of active bands.

Enzyme extracts were incubated with proteinase inhibitors (Table 1) to identify possible trypsin and chymotrypsin activities in different life stages (Garcia-Carreño and Haard 1993). Solutions of 20 mM TLCK (tosyl-lysine chloromethyl ketone) in 1 mM HCl, 200 mM PMSF (phenylmethylsulphonyl fluoride) in 2-propanol, and 10 mM TPCK (tosylphenylalanine chloromethyl ketone) in MeOH were separately added to enzyme extracts at a ratio of 1:10 (inhibitor:extract) and incubated at 25 °C for 1 h. Distilled water replaced inhibitors in samples for total proteinase separation. Samples were loaded onto the gel as described above. After electrophoresis, gels were separated into molecular weight standard and inhibition lanes. The former lanes were immediately stained. Control and inhibition lanes were immersed in 3% casein following the described procedures. Bands with proteinase inhibitors were compared with control proteinase lanes (without inhibitor) to identify inhibitory effects on active bands. Since TLCK, TPCK,

and PMSF are specific trypsin, chymotrypsin and serine proteinase inhibitors, respectively (Table 1), inhibited bands on PAGE could be attributed to the presence of one of these enzymes. The degree of inhibition was not measured in PAGE zymograms, and results were interpreted by the presence or absence of inhibition in the activity bands. Casein was used as substrate for substrate-gel electrophoresis because it is digested by penaeid proteinases such as trypsin and chymotrypsin (Jiang et al. 1991; Le Moullac et al. 1996; Ezquerro et al. 1997). This technique detects only proteinases (or "endopeptidases") by the production of short-chain polypeptides. Peptidases (or "exopeptidases") do not generate clear zones in the blue background and are not detected.

The substrates azocasein, casein, TAME, BAPNA, SAPFNA, the inhibitors TLCK, PMSF, TPCK, molecular weight markers, bovine albumin, and SDS-PAGE reagents were obtained from Sigma Chemical Co.

Statistical analysis

Data were expressed as mean \pm standard deviation. Differences among means were analyzed by ANOVA followed by a Tukey multicomparison test. Since trypsin activity data (TAME and BAPNA) deviated from the normal distribution, a Kruskal-Wallis rank test (non-parametric ANOVA) was applied followed by Nemenyi's test for means comparison. A Pearson product-moment analysis of correlation was applied between protein and water contents. Differences are reported as statistically significant when $p < 0.05$ (Zar 1984).

Results

The adult hepatopancreas of *Farfantepenaeus paulensis* weighed 1.43 ± 0.27 g as dry matter. Larval water content increased significantly throughout development, reaching a maximum at PZ II (94.3%, $p < 0.05$) (Table 2). A significant decrease was observed between PZ III and the postlarval substages ($p < 0.05$). Protein content as percent of dry weight also varied significantly during ontogenetic development (Table 2). There was a significant decrease between N III and PZ I ($p < 0.05$). Values were low during the following protozoal substages and M I. An increase was observed from M II through the subsequent developmental stages ($p < 0.05$). In protozoal substages and M I, lower

Table 2 *Farfantepenaeus paulensis*. Water and protein content (means \pm SD; $n = 3$ to 6) during early life stages at $26 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ (*N* nauplius; *PZ* protozoa; *M* mysis; *PL* postlarva; *roman numerals* substages; – not determined)

Substage	Age (days after spawn)	Water (%)	Protein (% dry wt)
Egg	0.5	83.1 \pm 1.05	49.7 \pm 1.12
N III	1.3	87.0 \pm 1.03	54.3 \pm 6.30
PZ I	2.5	92.3 \pm 1.37	33.5 \pm 3.30
PZ II	3.5	94.3 \pm 2.0	30.7 \pm 2.85
PZ III	4.5	92.5 \pm 1.79	27.7 \pm 7.66
M I	5.5	89.2 \pm 2.08	24.9 \pm 3.64
M II	6.5	84.5 \pm 2.4	38.08 \pm 3.92
M III	7.5	86.6 \pm 3.64	41.03 \pm 3.96
PL I	8.5	87.2 \pm 1.27	44.07 \pm 2.75
PL II	10	87.2 \pm 0.85	37.6 \pm 2.32
PL III	11.5	–	–
PL IV	13	–	–
PL V	15.5	86.1 \pm 1.31	43.2 \pm 1.57
PL VI	17.5	–	–
PL VII	20.5	–	–

protein values coincided with a higher water content. On a percentage basis, protein correlated negatively with water ($r = -0.84$, $p < 0.05$) at early ontogenetic stages (Fig. 1).

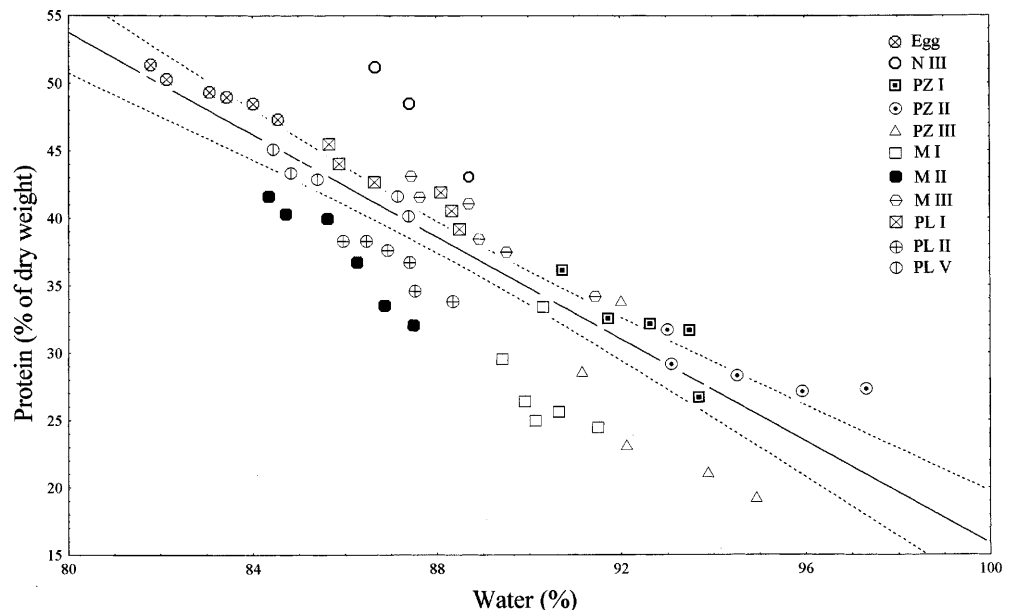
Proteinase activity varied significantly during the early life stages of *Farfantepenaeus paulensis* ($p < 0.05$). Total proteinase, chymotrypsin, and trypsin (using TAME) activities showed a similar pattern (Fig. 2a, b, c). Activity was low in the egg and N III, increased in PZ I, and peaked in PZ III. A subsequent decrease was observed between M I and PL I, which remained fairly constant until PL VII. Trypsin activity using the substrate BAPNA increased from the egg to N III, thereafter decreasing until PZ II. Activity almost doubled in

PZ III following a decrease over the course of development (Fig. 2d). Total proteinase, trypsin (BAPNA as substrate), and chymotrypsin activities in the adult hepatopancreas were 0.81 ± 0.02 , 2.6 ± 0.006 , and $7.03 \pm 0.01 \Delta A \text{ min}^{-1} \text{ mg}^{-1}$ protein, respectively.

Proteolytic activity was detected from PZ I onward, and intense activity zones of 16.2, 17.5, and 19.5 kdaltons were observed through the protozoal and mysis substages (Fig. 3). The intensity of these bands decreased after metamorphosis (PL I to PL VII). In the adult hepatopancreas, 12 active bands, distributed from 14.6 to 42.2 kdaltons, were detected (Fig. 3). Their molecular weight was 14.6, 16.4, 17.5, 19.5, 22.5, 23.9, 25.8, 28.9, 32.0, 34.4, 37.7, and 42.2 kdaltons. In PZ III and M I only, two intense bands of 39.1 and 53.5 kdaltons were found. Active bands heavier than 66 kdaltons appeared in the early stages (PZ I to M III), decreased in number and intensity during postlarval substages, and were absent from the adult hepatopancreas. Bands of 20.6 and 21.7 kdaltons appeared at M III, decreased in intensity in postlarval substages, and were absent from the adult hepatopancreas.

Specific proteinase activities were identified with synthetic inhibitors (Fig. 4). There was no inhibitory effect on activity when the extracts were incubated with TPCK. Active bands inhibited with both TLCK and PMSF were considered to represent trypsin, while the inhibition by only PMSF was evidence of chymotrypsin (Table 3). Several trypsin bands were found in larval and adult stages. Low molecular weight trypsin forms (14.6 to 21.7 kdaltons) varied in number and intensity (Fig. 3) through larval (3 forms), postlarval (4 in PL I and 2 from PL III to PL VII) and adult (4 forms) stages (Table 3). The 39.1 kdalton trypsin was found only in PZ III and M I, while the trypsin of 14.6 kdaltons was found exclusively in the adult hepatopancreas (Table 3, Figs. 3, 4). Mysis III and postlarval substages presented

Fig. 1 *Farfantepenaeus paulensis*. Correlation between relative contents of protein and water during larval and postlarval development at $26 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$. Protein = $205.44 - 1.896$ water, $r = -0.84$ ($p < 0.05$) (*N* nauplius; *PZ* protozoa; *M* mysis; *PL* postlarva; dotted lines 95% confidence intervals)



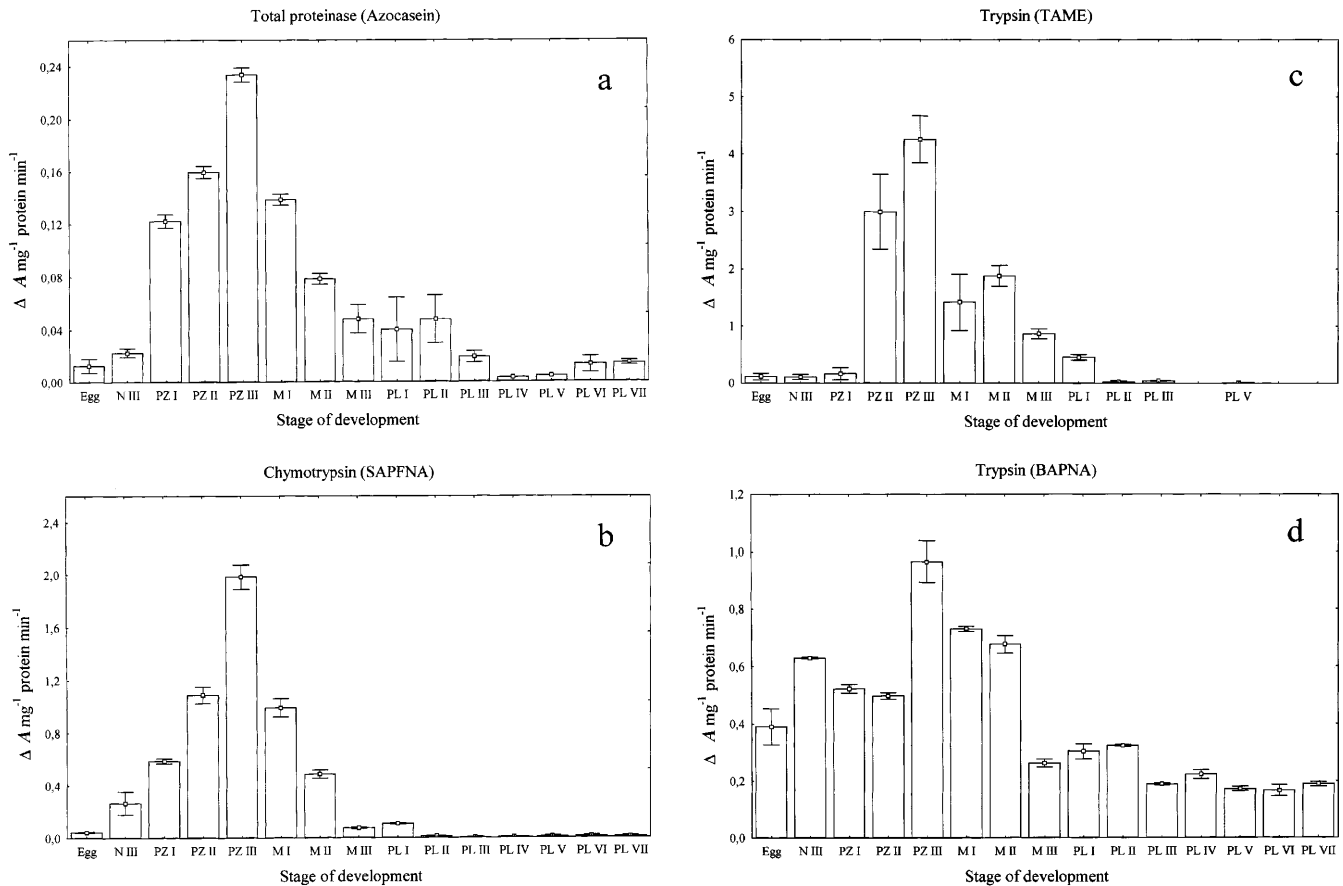


Fig. 2 *Farfantepenaeus paulensis*. Total proteinase (a), chymotrypsin (b) and trypsin (c, d) activity using azocasein, *N*-succinyl-L-ala-L-ala-L-pro-L-phe-*p*-nitroanilide (SAPFNA), *N* α -*p*-toluenesulphonyl-L-arginine methyl ester (TAME) and *N* α -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as substrates, during larval and postlarval development at 26 °C \pm 1 °C. Sample size (*n*) of means = 3, except in c, where *n* = 6 to 20 (Error bars standard deviation; abbreviations as in key to Fig. 1)

trypsins of 20.6 and 21.7 kdaltons (data not shown). In the adult stage, bands of 28.9, 32.0, and 37.7 kdaltons were inhibited by PMSF, but not by TLCK (Fig. 4). Bands heavier than 45 kdaltons were seen in postlarval substages, but identification was not possible. High molecular-mass active bands (68 to 74 kdaltons) could not be characterized with inhibitors because of their low activity.

Discussion

The protein values of *Farfantepenaeus paulensis* decreased to a minimum in Protozoa Stages I–III and M I, and thereafter gradually increased until postlarva stage; this is similar to observations of the larval development of *Marsupenaeus japonicus* (Laubier-Bonichon et al. 1977) and *Litopenaeus setiferus* (Lovett and Felder 1990a). The reduced protein content in protozoa coincides with a low RNA:DNA ratio, indicating a

decreased cellular multiplication rate and consequently less protein synthesis (Laubier-Bonichon et al. 1977). As observed in *Metapenaeus ensis*, the water content of *F. paulensis* was maximum during the larval stages.

Specific proteinase activity was higher in the adult hepatopancreas than in early stages. Nevertheless, if the hepatopancreas occupies 5% of the total body volume, as observed in postlarvae (Lovett and Felder 1990a), then adult whole-body proteinase activity would be much lower than at certain larval stages. The highest activity in early stages occurred at the PZ III–M I interval in *Farfantepenaeus paulensis*, as found for other penaeid species such as *Marsupenaeus japonicus*, *Penaeus monodon*, *Litopenaeus schmitti*, *L. setiferus* and *L. van-*

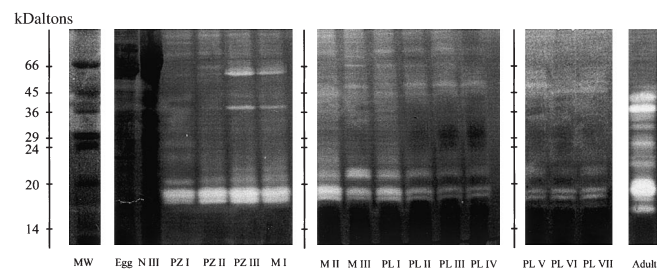


Fig. 3 *Farfantepenaeus paulensis*. Proteinase activity bands in substrate-SDS-PAGE zymograms during ontogenetic development at 26 °C \pm 1 °C (MW molecular weight; A adult; other abbreviations as in legend to Fig. 1)

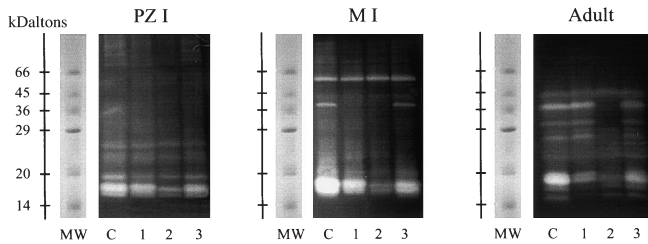


Fig. 4 *Farfantepenaeus paulensis*. Inhibition of proteinase activity in substrate-SDS-PAGE zymograms in Protozoa I (PZ I), Mysis I (M I) and adult extracts. C, 1, 2, 3 lanes denote activity pattern incubated with different inhibitors [C control (without inhibitor); 1 TLCK; 2 PMSF; 3 TPCK; MW molecular weight]

namei (Laubier-Bonichon et al. 1977; Lovett and Felder 1990a; Fang and Lee 1992; Le Moullac et al. 1992; González et al. 1994; Rodríguez et al. 1994). The egg, N III, and postlarval stages are characterized by reduced proteinase activity.

Variations in digestive-enzyme activity may be related to the characteristic life history during the early ontogenetic development of penaeids, which are unique among decapods in displaying so many morphological, metabolic, and behavioral changes during early development (Dall et al. 1990; Icely and Nott 1992; Chu and Ovsianico-Koulikowsky 1994). Anterior and lateral midgut caeca are believed to be the main enzyme-secretion sites in larvae, reaching their maximum volume at PZ III–M I (Lovett and Felder 1989; Abubakr and Jones 1992). A reduction in the size of the midgut caeca begins at the mysid stage and extends through the early postlarval substages (Lovett and Felder 1989; Icely and Nott 1992). Unlike most crustaceans, in penaeids the adult form of the gut is not achieved just until after the metamorphosis (Felder et al. 1985; Lovett and Felder 1990b). A structure similar to adult hepatopancreas derives from the lateral midgut caeca and appears only after 5 wk of postlarval life (Lovett and Felder 1989). Thus, the low enzyme activity of early postlarvae registered in the present study may have resulted from an incomplete digestive system. Reduced metabolic activity in postlarvae (Laubier-Bonichon et al. 1977; Chu and Ovsianico-Koulikowsky 1994) coincides with this low digestive-enzyme activity and may reflect an adaptation to limited food conditions during this transitional period (Lovett and Felder 1990a).

The digestive-enzyme activity of shrimp can be modulated by the quality of their food. High activity levels may maximize the assimilation of scarce compounds such as protein or carbohydrates (Ceccaldi 1986; Rodríguez et al. 1994; Lemos and Rodríguez 1998), or increase the absorption of feeds (Jones et al. 1992; Le Vay et al. 1993; Ezquerro et al. 1997). Under laboratory conditions, penaeid larvae feed on microalgae in the first protozoal substages, with the onset of omnivory at PZ III (Emmerson 1984; Yúfera et al. 1984; Rodríguez et al. 1994). Higher proteinase activity of the PZ III stage may indicate a physiological response to a different feeding

condition (Ceccaldi 1986; Harms et al. 1991). In the present experiment, the diet of larval and postlarval *Farfantepenaeus paulensis* included artificial plankton and microalgae. Thus, the food supplied during development was of the same quality throughout, and therefore the proteinase activity varied independent of diet.

This study examined digestive-enzyme activities at several transitional periods of penaeid development. Nauplii shift from endogenous feeding to herbivorous protozoae, which become omnivorous at PZ III. A change from a planktonic to a benthic existence takes place during the first postlarval substages. Proteinase activity was correlated with some of these transitional periods in *Farfantepenaeus paulensis*, since increasing activity marked the N III–PZ III interval whereas postlarvae displayed a constant low activity. Behavioral events also accompany the variety of body forms noted through the ontogenetic development of penaeids. As an exception, PZ III and M I are almost morphologically identical (Dall et al. 1990); this was also reflected by their proteinase pattern in SDS-PAGE. The profiles of enzyme activity obtained for *F. paulensis* may reflect different patterns of energy storage and use during early development (Biesiot and Capuzzo 1990). In protozoa, swimming and feeding are virtually continuous (Dall et al. 1990), and the relative ingestion rate increases through each substage, reaching its maximum during larval development in PZ III (Emmerson 1980; Loya-Javellana 1989). The retention time of food in the gut is lower than in mysid and postlarva, whereas feces production is high (Jones et al. 1992). Such strategies indicate an intense food-energy turnover achieved by higher ingestion and digestive-enzyme activity. The lower protein content of protozoal *F. paulensis* confirms a reduced accumulation of body reserves. On the other hand, the subsequent mysid and postlarval stages exhibit less-active swimming behavior, and filter-feeding efficiency declines at M II, when a shift to raptorial feeding is typical (Emmerson 1980, 1984). In late larval stages, thoracic appendices are now more specialized, enabling a better manipulation and selection of food particles (Jones et al. 1992; Marin-Magán and Cañavate 1995), leading to the ingestion of more digestible parts of food (Cushing and Vucetic 1963; Corner et al. 1972). The retention time of food in the gut is longer (Jones et al. 1992), and the development of the gastric mill may contribute to the processing of food during early postlarval stages (Lovett and Felder 1989). The morphological and behavioral changes in mysid and early postlarvae may compensate for the lower digestive-enzyme activity when the transition to the benthic life begins. The reduced metabolic activity, food uptake, and assimilation capacity of early postlarval stages accompany the search for a suitable new habitat after migration into inshore, brackish, nursery grounds.

Literature detailing the characterization of digestive enzymes during the ontogeny of marine animals is relatively scarce. Vacquier et al. (1971) found differences in

Table 4 Characteristics of trypsin and chymotrypsin from adult penaeids and other decapods (*MW* molecular weight in kdaltons; – not determined)

Species	Trypsin (MW) form	Chymotrypsin (MW) form	Source
<i>Penaeus monodon</i>	4	(–)	Fang and Lee (1992)
	2	(27, 29)	Lu et al. (1990)
	3	(18.5, 23.3, 50.1)	Jiang et al. (1991)
<i>Litopenaeus vannamei</i>			Tsai et al. (1991)
	3	(31–32)	Van Wormhoudt et al. (1992)
			Klein et al. (1996)
			Hernández-Cortés et al. (1997)
<i>Fenneropenaeus indicus</i>	7	(36)	Honjo et al. (1990)
<i>Astacus fluviatilis</i>	1	(25.03)	Titani et al. (1983)
<i>Procambarus clarkii</i>	4	(23.8, 24.8, 27.0, 31.4)	Kim et al. (1992)

(2) or *Litopenaeus vannamei* (2), while the more intense band (37.7 kdaltons) is heavier than reported for other crustaceans (Table 4).

Several forms of serine proteinases have been found in fish such as salmon (Male et al. 1995) and cod (Gudmundsdottir et al. 1996). Trypsins from salmon may present either cationic or anionic forms. The methionine content of cod elastase is similar to that recorded earlier for enzymes of psychrophilic fish enzymes (Genicot et al. 1996). In penaeids, trypsin and chymotrypsin forms have not been characterized in terms of differences in their catalytic or molecular characteristics. Moreover, the ontogenetic pattern of *Farfantepenaeus paulensis* confirms that some forms can appear in certain stages but not in others, since activity may differ between stages (Fang and Lee 1992).

Acknowledgements The authors are grateful to Professors E. Beltrame and E. Andreatta (LCM – UFSC), and all LCM staff for the supply of *Farfantepenaeus paulensis* and assistance in cultivation. We thank G. Alvarez, A. Olivera, and Professor E. Aidar for their enthusiasm in the fulfillment of this research. D. Lemos acknowledges the financial support of Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil, 96/03688-6). E. Glazier (CIBNOR) kindly edited the English text.

References

- Abubakr MA, Jones DA (1992) Functional morphology and ultrastructure of the anterior mid-gut diverticulae of larvae of *Penaeus monodon* Fabricius, 1798 (Decapoda, Natantia). *Crustaceana* 62: 142–158
- Beltrame E, Olivera A, Vinatea L, Coelho M, Alfonso E, Derner R, Petersen R (1996) Estado actual de la producción de post-larvas de *Penaeus paulensis* y *Penaeus schmitti* en el Estado de Santa Catarina, Brasil. In: Silva A, Merino G (eds) Noveno Congreso Latinoamericano de Acuicultura: Chile, Universidad Católica del Norte, Coquimbo, pp 1–4
- Beynon RJ, Salvesen G (1990) Commercially available protease inhibitors. In: Beynon RJ, Bond JS (eds) Proteolytic enzymes a practical approach. IRL Press, Oxford, pp 241–249
- Biesiot PM, Capuzzo JM (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 MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Analyt Biochem* 72: 248–254
- Ceccaldi HJ (1986) Digestion et secretions digestives chez les crustacés. *Océanis* 12: 31–49
- Chu KH, Ovsianico-Koulikowsky NN (1994) Ontogenetic changes in metabolic activity and biochemical composition in the shrimp, *Metapenaeus ensis*. *J exp mar Biol Ecol* 183: 11–26
- Corner EDS, Head RN, Kilvington CC (1972) On the nutrition and metabolism of zooplankton. V. Feeding efficiency of *Calanus finmarchicus*. *J mar biol Ass UK* 47: 260–270
- Cushing DH, Vucetic T (1963) Studies on a *Calanus* patch. III. The quantity of food eaten by *Calanus finmarchicus*. *J mar biol Ass UK* 43: 349–371
- Dall W, Hill BJ, Rothlisberg PC, Staples DJ (1990) The biology of Penaeidae. *Adv mar Biol* 27: 1–489
- Del Mar EG, Largman C, Brodrick JW, Geokas MC (1979) A sensitive new substrate for chymotrypsin. *Analyt Biochem* 99: 316–320
- Diaz M, Moyano F, García-Carreño FL, Alarcón F, Sarasquete MC (1997) Substrate-SDS-PAGE determination of protease activity through larval development in sea bream. *Aquaculture* 151: 461–471
- Emmerson WD (1980) Ingestion, growth and development of *Penaeus indicus* larvae as a function of *Thalassiosira weissflogii* cell concentration. *Mar Biol* 58: 65–73
- Emmerson WD (1984) Predation and energetics of *Penaeus indicus* (Decapoda: Penaeidae) larvae feeding *Brachionus plicatilis* and *Artemia* nauplii. *Aquaculture*, Amsterdam 38: 201–209
- Erlanger B, Kokowsky N, Cohen W (1961) The preparation and properties of two new chromogenic substrates of trypsin. *Archs Biochem Biophys* 95: 271–278
- Ezquerro JM, García-Carreño FL, Civera R, Haard NF (1997) pH-stat method to predict protein digestibility in white shrimp (*Penaeus vannamei*). *Aquaculture*, Amsterdam 157: 251–262
- Fang L-S, Lee B-N (1992) Ontogenetic change of digestive enzymes in *Penaeus monodon*. *Comp Biochem Physiol* 103B: 1033–1037
- Felder DL, Martin JW, Goy JW (1985) Patterns in early postlarval development of decapods. In: Wenner AM (ed) Crustacean issues. Vol 2. Larval growth. Balkema, Rotterdam, pp 163–225
- Galgani ML, Benyamin Y, Ceccaldi HJ (1984) Identification of digestive proteinases of *Penaeus kerathurus* (Forsk.) a comparison with *Penaeus japonicus*. *Comp Biochem Physiol* 78B: 355–361
- Galgani ML, Benyamin Y, Van Wormhoudt A (1985) Purification, properties and immunoassays of trypsin from the shrimp *Penaeus japonicus*. *Comp Biochem Physiol* 81B: 447–452
- García-Carreño FL (1992) The digestive proteases of langostilla (*Pleuroncodes planipes*, Decapoda): their partial characterization, and the effect of feed on their composition. *Comp Biochem Physiol* 103B: 575–578

- García-Carreño FL, Dimes LE, Haard NF (1993) Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Analyt Biochem* 214: 65–69
- García-Carreño FL, Haard NF (1993) Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. *J Fd Biochem* 17: 97–113
- García-Carreño FL, Hernández-Cortés MP, Haard NF (1994) Enzymes with peptidase and proteinase activity from the digestive systems of a freshwater and a marine decapod. *J agric Fd Chem* 42: 1456–1461
- Genicot S, Rentier-Delrue F, Edwards D, Vanbeeumen J, Gerday C (1996) Trypsin and trypsinogen from an antarctic fish: molecular basis of cold adaptation. *Biochim biophys Acta* 1298: 45–57
- González R, Fraga V, Carrillo O (1994) Cambios ontogenéticos en la actividad de las principales enzimas digestivas de *Penaeus schmitti*. *Revta Investes mar, La Habana* 15: 262–268
- Gudmundsdottir E, Spilliarret R, Yang Q, Craig CS, Bjarnason JB, Gudmundsdottir A (1996) Isolation and characterization of two cDNAs from Atlantic cod encoding two distinct psychrophilic elastases. *Comp Biochem Physiol* 113: 795–801
- Harms J, Anger K, Klaus S, Seeger B (1991) Nutritional effects on ingestion rate, growth, and biochemical composition of *Hyas araneus* L. (Decapoda: Majidae) larvae. *J exp mar Biol Ecol* 145: 233–265
- Hernández-Cortés MP, Whitaker J, García-Carreño FL (1997) Purification and characterization of chymotrypsin from *Penaeus vannamei* (Crustacea: Decapoda). *J Fd Biochem* 21: 497–514
- Hess R, Pearse AGE (1958) The histochemistry of indoxylesterase of rat kidney with special reference to its cathepsin-like activity. *Br J exp Path* 39: 292–299
- Holthuis, LB (1998) Book review: Pérez-Farfante, I and Kensley, B, Penaeoid and sergestoid shrimps and prawns of the world. Keys and diagnoses for the families and genera. *J Crustacean Biol* 18: p. 621
- Honjo I, Kimura S, Nonaka M (1990) Purification and characterization of trypsin-like enzyme from shrimp *Penaeus indicus*. *Bull Jap Soc scient Fish* 56: 1627–1634
- Icely JD, Nott JA (1992) Digestion and absorption: digestive system and associated organs. In: Harrison FW, Humes AG (eds) *Microscopic anatomy of invertebrates 10: decapod Crustacea*. Wiley-Liss, New York, pp 147–201
- Iwai M (1978) Desenvolvimento larval e pós-larval de *Penaeus (Melicertus) paulensis* Pérez-Farfante 1967 (Crustacea, Decapoda) e o ciclo de vida dos camarões do gênero *Penaeus* da região centro-sul do Brasil. PhD thesis. University of São Paulo, IBUSP, São Paulo, Brazil
- Jiang ST, Moody MW, Chen HC (1991) Purification and characterization of proteases from digestive tract of grass shrimp (*Penaeus monodon*). *J Fd Sci* 56: 322–326
- Jones DA, Le Vay L, Kamarudin MS (1992) Feeding and nutritional requirements of penaeid shrimp larvae. In: Calderón J, Sandoval V (eds) *Memorias del Primer Congreso Ecuatoriano de Acuicultura*. Centro Nacional de Acuicultura e Investigaciones Marinas, San Pedro de Manglaralto, Ecuador, pp 45–52
- Kim HR, Meyers SP, Godber JS (1992) Purification and characterization of anionic trypsin from the hepatopancreas of crayfish, *Procambarus clarkii*. *Comp Biochem Physiol* 103B: 391–398
- Klein B, Le Moullac G, Sellos D, Van Wormhoudt A (1996) Molecular cloning and sequencing of trypsin cDNAs from *Penaeus vannamei* (Crustacea, Decapoda): use in assessing gene expression during the moult cycle. *Int J Biochem Cell Biol* 28: 551–563
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature, Lond* 227: 680–685
- Laubier-Bonichon A, Van Wormhoudt A, Sellos D (1977) Croissance larvaire contrôlée de *Penaeus japonicus* Bate: enzymes digestives et changements de regimes alimentaires. *Publs Cent natn Exploit Océans (CNEXO) (Sér Act Colloques)* 4: 131–145
- Lee PG, Blake NJ, Rodrick GE (1980) A quantitative analysis of digestive enzymes from the freshwater prawn *Macrobrachium rosenbergii*. *Proc Wld Maricult Soc* 11: 392–402
- Lemos D, Rodríguez A (1998) Nutritional effects on body composition, energy content and trypsin activity of *Penaeus japonicus* during early postlarval development. *Aquaculture, Amsterdam* 160: 103–116
- Le Moullac G, Klein B, Sellos D, Van Wormhoudt A (1996) Adaptation of trypsin, chymotrypsin and α -amylase to casein level and protein source in *Penaeus vanammei* (Crustacea Decapoda). *J exp mar Biol Ecol* 208: 107–125
- Le Moullac G, Roy P, Van Wormhoudt A (1992) Effects of trophic prophylactic factors on some digestive enzymatic activities of *Penaeus vannamei* larvae. In: Calderón J, Sandoval V (eds). *Memorias del Primer Congreso Ecuatoriano de Acuicultura*. Centro Nacional de Acuicultura e Investigaciones Marinas, San Pedro de Manglaralto, Ecuador, pp 81–86
- 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
- Lester LJ, Cook JP (1987) Ontogenetic changes in isozyme patterns of *Penaeus* species. *Comp Biochem Physiol* 86B: 253–258
- Le Vay L, Rodríguez A, Kamarudin MS, Jones DA (1993) Influence of live and artificial diets on tissue composition and trypsin activity in *Penaeus japonicus* larvae. *Aquaculture, Amsterdam* 118: 287–297
- Lovett DL, Felder DL (1989) Ontogeny of gut morphology in the white shrimp *Penaeus setiferus* (Decapoda, Penaeidae). *J Morph* 201: 253–272
- Lovett DL, Felder DL (1990a) Ontogenetic change in digestive enzyme activity of larval and postlarval white shrimp *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). *Biol Bull mar biol Lab, Woods Hole* 178: 144–159
- Lovett DL, Felder DL (1990b) Ontogenetic changes in enzyme distribution and midgut function in developmental stages of *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). *Biol Bull mar biol Lab, Woods Hole* 178: 160–174
- Loya-Javellana G (1989) Ingestion saturation and growth responses of *Penaeus monodon* larvae to food density. *Aquaculture, Amsterdam* 81: 329–336
- Lu PJ, Liu HC, Tsai IH (1990) The midgut trypsin of shrimp (*Penaeus monodon*): high efficiency toward native protein substrates including collagens. *Biol Chem Hoppe-Seyler* 371: 851–859
- Male R, Lorens JB, Smalas AO, Torrissen KR (1995) Molecular cloning and characterization of anionic and cationic variants of trypsin from Atlantic salmon. *Eur J Biochem* 232: 677–685
- Marín-Magán V, Cañavate JP (1995) Fluorometric determination of selectivity between live and inert food by *Penaeus japonicus* larvae. *Aquaculture, Amsterdam* 134: 307–311
- Morgan RP II, Kramarsky E, Sulkin SD (1978) Biochemical changes during larval development of the xanthid crab *Rithropanopeus harrisi*. III. Isozyme changes during ontogeny. *Mar Biol* 48: 223–226
- Pérez-Farfante I (1967) A new species and two new subspecies of shrimp of the genus *Penaeus* from western Atlantic. *Proc Biol Soc Wash* 80: 83–100
- Pérez-Farfante I, Kensley BF (1997) Penaeoid and sergestoid shrimps and prawns of the world. Keys and diagnoses for the families and genera. *Mém Mus natn Hist nat, Paris* 175: 1–233
- Pérez-Pérez D, Ros RM (1975) Descripción y desarrollo de los estadios postlarvales del camaron blanco *Penaeus schmitti* Burkenroad. *Ciencias, Habana* 21: 1–114
- Pires-Vanin AMS (1989) Estrutura e dinâmica da megafauna bética da plataforma continental da região norte do Estado de

- São Paulo, Brasil. "Livro-docência" thesis. IOUSP, University of São Paulo, São Paulo, Brazil
- Primavera JH, Posadas RA (1981) Studies on the egg quality of *Penaeus monodon* Fabricius, based on morphology and hatching rates. *Aquaculture*, Amsterdam 22: 269–277
- Rick W (1984) Trypsin: measurement with N α -p-toluenesulfonyl-L-arginine methyl ester as substrate. In: Bergmeyer HU (ed) *Methods of enzymatic analysis*. Vol 2. 2nd edn. Academic Press, New York, pp 1021–1024
- Rodríguez A, Le Vay L, Mourente G, Jones DA (1994) Biochemical composition and digestive enzyme activity in larvae and postlarvae of *Penaeus japonicus* during herbivorous and carnivorous feeding. *Mar Biol* 118: 45–51
- Sellos D, Van Wormhoudt A (1992) Molecular cloning of a cDNA that encodes a serine protease with chymotryptic and collagenolytic activities in the hepatopancreas of the shrimp *Penaeus vannamei* (Crustacea, Decapoda). *Fedn eur biochem Soc (FEBS) Lett* 309: 219–224
- Smith DM, Dall W, Moore LE (1992) The natural food of some Australian penaeids. In: Allan GL, Dall W (eds) *Proceedings of the Aquaculture Nutrition Workshop*. NSW Fisheries, Salamander Bay, Australia, pp 95–96
- Titani K, Sasagawa T, Woodbury RG, Ericsson LH, Dörsam H, Kraemer M, Neurath H, Zwilling R (1983) Amino acid sequence of crayfish (*Astacus fluviatilis*) trypsin I_f. *Biochemistry*, NY 22: 1459–1465
- Tsai IH, Chuang KL, Chuang JL (1986) Chymotrypsins in digestive tracts of crustacean decapods (shrimps). *Comp Biochem Physiol* 85: 235–240
- Tsai IH, Lu P, Chuang J (1991) The midgut of shrimps *Penaeus monodon*, *Penaeus japonicus* and *Penaeus penicillatus*. *Biochim biophys Acta* 1080: 59–67
- Vacquier VD, Korn LJ, Epel D (1971) The appearance of α -amylase activity during gut differentiation in sand dollar plutei. *Devl Biol* 26: 393–399
- Valentini H, D'Incao F, Rodrigues LF, Rebelo Neto JE, Rahn E (1991) Análise da pesca do camarão-rosa (*Penaeus brasiliensis* e *Penaeus paulensis*) nas regiões sudeste e sul do Brasil. *Atlântica*, Rio Grande 13: 143–157
- Van Wormhoudt A, Le Chevalier P, Sellos D (1992) Purification, biochemical characterization and *n*-terminal sequence of a serine-protease with chymotryptic and collagenolytic activities in a tropical shrimp, *Penaeus vannamei* (Crustacea, Decapoda). *Comp Biochem Physiol* 103B: 675–680
- Vinatea L, Olivera A, Andreatta E, Beltrame E, Petersen R, Derner R (1993) Producción comercial de larvas de *Penaeus paulensis* y *Penaeus schmitti* en el sur del Brasil. In: ABCC (ed) *Anais Quarto Simpósio Brasileiro sobre Cultivo de Camarão*, MCR Aquicultura. Associação Brasileira de Criadores de Camarão (ABCC), João Pessoa, Brazil, pp 399–414
- Wakabara Y, Flynn MN, Tararam AS (1996) Ingestion and selection of suprabenthic crustaceans by small-sized fishes in a lower saltmarsh system. *Braz J Oceanogr* 44: 89–103
- Yúfera M, Rodríguez A, Lubián LM (1984) Zooplankton ingestion and feeding behaviour of *Penaeus kerathurus* larvae reared in the laboratory. *Aquaculture*, Amsterdam 42: 217–224
- Zar JH (1984) *Biostatistical analysis*. 2nd edn. Prentice Hall, Englewood Cliffs, New Jersey