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Ontogenetic variation in digestive proteinase activity, RNA and DNA content of larval and postlarval white shrimp *Litopenaeus schmitti*

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Abstract

Proteinase (endopeptidase), trypsin and chymotrypsin activities, RNA, and DNA contents were examined throughout the ontogenetic development of cultured *Litopenaeus schmitti*. Whole individuals from larval and postlarval stages, and adult hepatopancreas were homogenized and assayed for quantification of enzyme activity. Proteinase activity of different life stages was characterized by substrate-SDS-PAGE. Specific inhibitors for trypsin (TLCK) and serine proteinases (PMSF) were used to identify activity zones of these enzymes in gels. Nucleic acids were also quantified in larval and postlarval stages. Protein-specific activity of total proteinases and trypsin presented higher values in nauplius IV (N IV), protozoa I (PZ I), and PZ III, compared to the remaining stages, while chymotrypsin activity peaked in N IV and PZ III. Enzyme activity was lower in egg, early nauplius, mysis, and postlarval stages. Different proteinase patterns were observed in SDS-PAGE during ontogenetic development. Active bands of 15.5, 16.0, 19.9, 21.8, 24.0, 27.9, 30.3, 33.5, 35.5, 38.2, and 48.1 kDa were detected in the adult hepatopancreas. In the course of development, proteolytic activity was detected in N IV, and intense bands of 17.3, 19.9, and 20.9 kDa were found up to mysis (M III). A band at 38.2 kDa was present between N IV and M I, and between PL II and PL IV. Most bands were serine proteinases, and only two bands in adult (15.5 and 15.9 kDa) were inhibited by TLCK. Both RNA and DNA contents ($\mu\text{g mg}^{-1}$ of fresh weight) reduced with development from egg to PZ III, increasing afterwards until PL IV. RNA/DNA ratios increased from egg, reaching a peak in PZ I, and decreased in the following stages until a minimum was reached in M II. A secondary peak was observed in PL III followed by reduction in PL IV. The variation in enzyme activity indicates different strategies of energy use throughout development. Higher enzyme activity combined with increased RNA/DNA in protozoal stages

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denotes intense food energy use and accumulation into growth. The shift in energy strategy during the ontogeny of *L. schmitti* is accompanied by variation in proteinase characteristics as observed in other penaeid species.

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1. Introduction

Shrimp farming is a growing industry and an important source of income for many countries (Barg et al., 2000). The supply of healthy postlarval shrimp to be stocked in growth systems is a crucial factor for the whole process and accounts for a considerable part of the total costs of production. Efficient postlarval harvest depends on adequate culture conditions and survival during larval and early postlarval stages, resulting in better growth in the remaining developmental stages (Castille et al., 1993). The improvement of routines for rearing early life stages of penaeid shrimp has been mostly attempted by the optimization of feeds (Jones et al., 1979, 1997; Le Vay et al., 1993), feeding schedules (Emmerson, 1980, 1984; Loya-Javellana, 1989; Gallardo et al., 1995), and alternative nutrient sources (Rodríguez et al., 1994; Thompson et al., 1999). Food digestion in larval and postlarval penaeids has been assessed by digestive enzyme activities (Fang and Lee, 1992; González et al., 1994; Jones et al., 1997) and the characterization of enzyme isoforms was recently reported (Lemos et al., 1999). The understanding of energy metabolism associated with growth and development in early stages is also necessary for the optimization of culture techniques and can be achieved by energy budgets (Kurmaly et al., 1989; Lemos and Phan, 2001a) and through the quantification of RNA/DNA ratios (Buckley, 1980; Anger and Hirche, 1990; Clemmesen, 1996), among other methods.

Though a large number of penaeid species appears apt for culture (Fast and Lester, 1992), few have proven suitable for the bulk volume needed to sustain the worldwide demand of aquaculture (Pillay, 1990). The search for rearing alternative species may contribute, in many cases, to a sustainable expansion of farming activity as well as for business diversification. Moreover, autochthonous species are often highly appreciated in local markets and can generate revenue by exportation. The white shrimp *Litopenaeus schmitti* is an important fishery resource endemic to tropical Atlantic waters of the Americas (Pérez-Farfante and Kensley, 1997) and has been cultivated in the central and southern parts of the continent (Nascimento et al., 1991; Beltrame et al., 1996). In spite of its high farming potential, expansion of *L. schmitti* cultivation, like other native species, will depend on profitable results derived from adequate rearing protocols. In the present study, protein digestion was assessed throughout the larval and postlarval development of mass cultured *L. schmitti* by the quantification and characterization of proteinase activity. Cellular and metabolic activities associated with growth in the various life stages were analyzed by the quantification of RNA and DNA. Results could be related to previous findings in the literature on digestion and energetics of early stages of penaeid shrimp.

2. Material and methods

2.1. Experimental shrimp

Male and female specimens of *L. schmitti* were caught along the coast of the State of Santa Catarina, Brazil (27°18' S, 48°23' W) at 1–5 m depth. After acclimation in the laboratory, females of ~ 30 g wet weight were induced to mature sexually by unilateral eyestalk ablation and were maintained individually isolated in 500-l fiberglass tanks. Spawning took place during the night, and viable eggs (~ 130 000 per female) were transferred to cylindrical conical tanks, in which they hatched 12 to 14 h later at 26 ± 1 °C. The larval development of *L. schmitti* comprises 5 naupliar (N I to N V), 3 protozoal (PZ I to PZ III), and 3 mysid (M I to M III) stages (Garcia, 1972). Nauplii were moved to 50 000-l tanks, and reared following commercial techniques at 26 ± 1 °C and 34 ± 1 ‰ salinity (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, Japan; 0.03 mg larva⁻¹ day⁻¹, 30 µm particle-size). Freshly hatched nauplii of *Artemia* sp. (Great Salt Lake, USA) were added to the diet at PZ III at 5 nauplii larva⁻¹ day⁻¹, increasing to 15 nauplii larva⁻¹ day⁻¹ from M I onward. After metamorphosis to postlarval stage (PL), the diet included 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 10 synchronized spawns. A homogeneous sample was defined when >80% of individuals belonged to the same stage; the remaining individuals differed by a single stage. Eggs were chosen 10 to 12 h after spawning at the “early nauplius” embryonic stage, according to Primavera and Posadas (1981). During the naupliar stage, individuals were selected in N I, N II, and N IV. After the PZ I stage, each subsequent larval stage was analyzed until metamorphosis. Postlarvae from specific instars (PL I to PL IV) were sorted according to the procedure of Iwai (1978), where roman numbers denote the numbers of molts of postlarvae, including metamorphosis. Samples were immediately frozen at -20 °C and transferred to liquid nitrogen. The digestive glands of two, sexually immature female adults were extracted, frozen at -20 °C, and freeze-dried. The specimens were previously fed frozen mussel and squid, and fasted for 12 h before dissection of the digestive gland. Individuals were chosen in intermolt that comprise from 7% to 30% of the total molt cycle since the last ecdysis (Sasaki et al., 1986; Dall et al., 1990).

2.2. Quantification of enzyme activity

Approximately 0.5 to 1.3 g wet weight of whole larvae or postlarvae samples were homogenized in chilled 10 mM Tris-HCl buffer at pH 7.5 and centrifuged at $12\,000 \times g$ for 20 min at 5 °C. After lipid elimination, total soluble protein was determined in the supernatants, using bovine serum albumin as standard (Bradford, 1976). Trypsin and chymotrypsin activity was measured by the rate of hydrolysis of the synthetic substrates BAPNA (*N*α-benzoyl-DL-arginine-*p*-nitroanilide) and SAPFNA (*N*-succinyl-L-ala-L-ala-L-pro-L-phe-*p*-nitroanilide), respectively. BAPNA (1 mM) was dissolved in 1 ml dimethylsulphoxide (DMSO) and then made up to 100 ml with 50 mM Tris-HCl at pH 7.5,

containing 20 mM CaCl_2 . Triplicate samples of 10 μl from replicate extracts 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 in 0.1 M Tris–HCl at pH 7.8, containing 0.01 M CaCl_2 . Samples ($n = 3$ to 6) of 10 μl were mixed with 0.75 substrate solution, and the absorbance at 410 nm was recorded over 3 min at 25 °C (Del Mar et al., 1979). Each assay included blanks without the enzyme extract. The enzyme activities were expressed as the change in absorbance per minute per milligram of protein of the enzyme extract used in the assays ($\Delta \text{ABS min}^{-1} \text{mg}^{-1}$ protein). The expression of body weight based activity was avoided due to possible underestimation of enzyme activity under malnutrition of shrimp (Lovett and Felder, 1990; Lemos and Rodríguez, 1998).

2.3. Enzyme characterization

Proteinase activity was studied in various stages of early development after separation of proteins by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE), using 12% acryl amide (Laemmli, 1970). Enzyme preparations (0.028 mg protein) for each stage were loaded in a temperature-controlled (4 °C) vertical electrophoresis device (Hofer, San Francisco, CA). Molecular mass standards (0.0175 mg) were included on each plate. Proteinase activity of the extracts was characterized according to Garcia-Carreño et al. (1993). After electrophoresis for protein separation, gels were immersed in 3% casein in 50 mM Tris at pH 7.5, for 30 min at 5 °C, allowing 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 at least 24 h, gels were de-stained with the same solution without Coomassie dye, then dried in a slab-gel dryer (Bio-Rad 443). Clear zones on the blue background indicated proteinase activities and could be compared to molecular mass standard bands. The 0.1% concentration of SDS does not affect proteinase activity in PAGE (Garcia-Carreño et al., 1993). At this SDS concentration, proteins are negatively charged, they have the same charge per unit of length (Walker, 2000), and therefore, the mobility is due to the weight of the protein. Previous results showed that the inclusion of SDS at concentrations used in the gels did not affect the proteolytic activity of crustacean, fish, and some commercial enzyme preparations (Garcia-Carreño et al., 1993). Moreover, commercial trypsin and chymotrypsin migrate according to their molecular weights under this condition. A regression between the distance of molecular mass standard bands from the top border of the gel (measured in cm) and the log of their molecular mass (kDa) determined the molecular mass of active bands.

Enzyme extracts were incubated with proteinase inhibitors in order to identify possible trypsin and serine proteinase activities in the developmental stages (Garcia-Carreño and Haard, 1993; Lemos et al., 1999). Solutions of 20 mM tosyl-lysine chloromethyl ketone (TLCK) in 1 mM HCl, and 200 mM phenylmethylsulphonyl fluoride (PMSF) in 2-propanol were used to inhibit trypsin and serine proteinase active bands, respectively. Chymotrypsin inhibitors such as TPCK (tosylphenylalanine chloromethyl ketone) were

not used because they do not affect penaeid proteinases (Lemos et al., 1999). Inhibitor solutions 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. After electrophoresis, gels were separated into molecular mass standard and inhibition lanes. The former lanes were immediately stained. Control and inhibition lanes were immersed in 3% casein as described earlier. Bands with proteinase inhibitors were compared with control proteinase lanes (without inhibitor) to identify inhibitory effects on active bands. Inhibited bands on PAGE could be attributed to the presence of trypsin or other serine proteinase from extracts. The degree of inhibition was not measured in PAGE zymograms. Results were interpreted by the presence or absence of inhibition in the activity bands. The substrate casein was used for substrate-gel electrophoresis because it is digested by penaeid proteinases (Jiang et al., 1991; Le Moullac et al., 1996; Ezquerro et al., 1997). This technique detects only proteinases (or “endopeptidases”), especially serine proteinases (e.g. trypsin and chymotrypsin most important for shrimp protein digestion), by the production of short chain polypeptides. Peptidases (or “exopeptidases”) do not generate clear zones on the blue background and are not detected. Whole body homogenates were used due to the small size of individuals analyzed. Bands displayed in SDS-PAGE gels were mainly digestive proteinases from the serine class. Even though other proteinases may be present in muscle and other organs of crustaceans, it occurs in minimal quantities compared to hepatopancreas or other digestive structures as midgut caeca (Klein et al., 1996).

2.4. *Quantification of nucleic acids*

RNA and DNA were separately extracted in duplicate samples for each developmental stage. Frozen samples of pooled individuals (± 60 mg fresh weight) were weighed in microtubes free of RNase and DNase and homogenized with a Teflon pestle. RNA was processed at room temperature and DNA was processed in an ice bath. All reagents were free of RNase and DNase, and all containers and material were sterilized. Samples for RNA determination were homogenized in 750 μ l Trizol LS reagent (Gibco BRL, New York), a monophasic solution of phenol and guanidine isothiocyanate, for 5 min. The reagent does not destroy the integrity of the RNA during homogenization while disrupting tissue and dissolving cell components (Chomczynski and Sacchi, 1987). RNA is separated after adding 200 μ l chloroform, shaking vigorously, and followed by standing for 15 min. The treated samples were then centrifuged at $12\,000 \times g$ for 15 min at 5 °C (Chomczynski, 1993). The aqueous phase, containing the RNA, was transferred to a new tube and the RNA was precipitated with 500 μ l isopropyl alcohol. After 10 min, the RNA samples were centrifuged at $12\,000 \times g$ for 10 min at 5 °C. The pellets containing RNA were treated with 1 ml 75% ethanol and centrifuged at $7\,500 \times g$ for 5 min at 5 °C to eliminate the residual isopropyl alcohol that can interfere in some analyses. The ethanol was then discarded and the pellets were air-dried. The RNA was dissolved in 100 μ l distilled water and incubated for 10 min at 55 °C.

DNA was extracted following standard procedures (Sambrook et al., 1989). Samples were homogenized in 450 μ l extraction buffer (SDS 0.5%, 100 mM EDTA, 100 mM NaCl, 10 mM Tris at pH 8.0) in ice bath. The homogenate was incubated for 2 h at 37 °C then 1

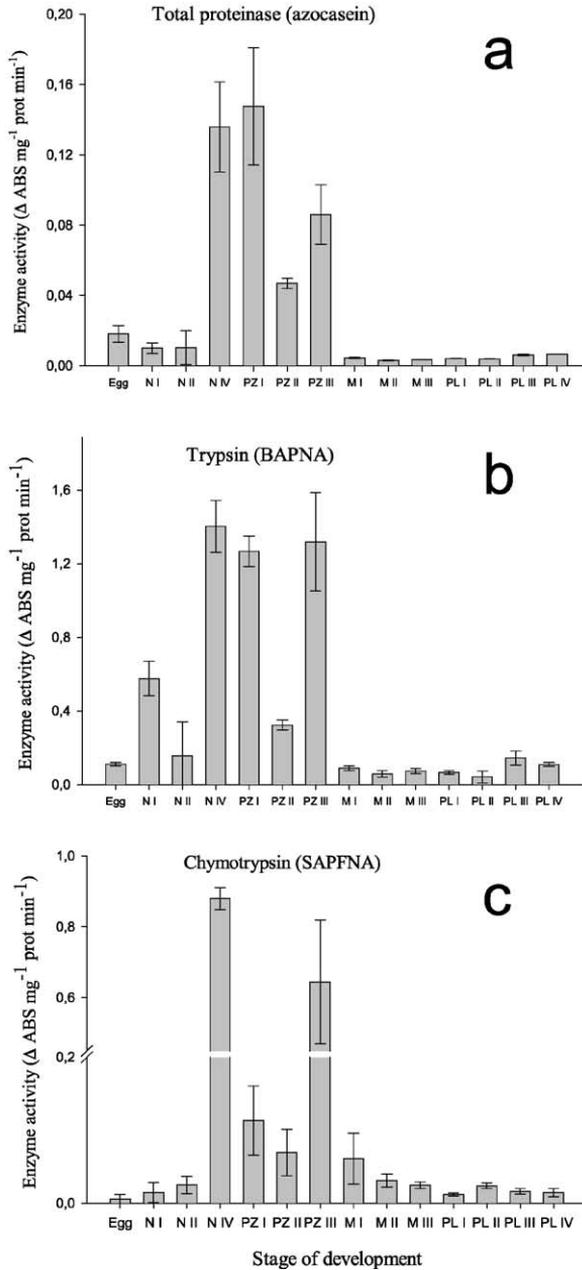


Fig. 1. (a) Total proteinase, (b) trypsin, and (c) chymotrypsin activity of *L. schmitti* using azocasein, BAPNA and SAPFNA as substrates during the early developmental stages at 26 ± 1 °C. Sample size (*n*) of means 3–6 (error bars: standard deviation; N=nauplius; PZ=protozoa; M=mysis and PL=postlarva).

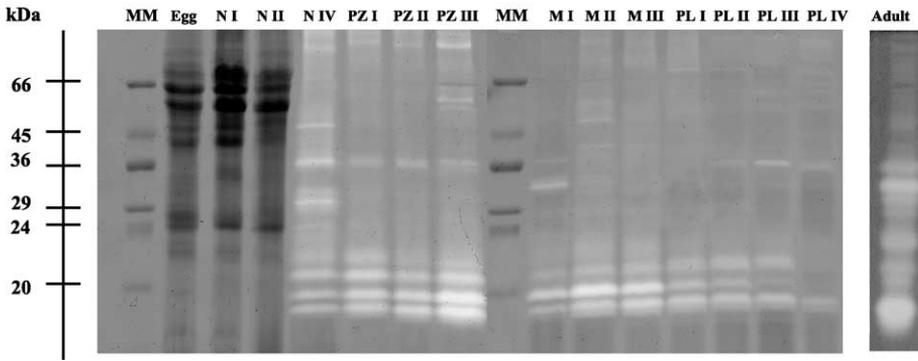


Fig. 2. Proteinase activity bands in substrate-SDS-PAGE zymograms during the ontogenetic development of *L. schmitti* at 26 ± 1 °C. (MM=molecular mass; other abbreviations as in legend to Fig. 1).

ml of phenol/chloroform/isoamyl alcohol (P/C/I) (at 24:25:1 ratio) was added to the DNA and extraction buffer homogenate and incubated for 5 min on ice. The treated sample was then centrifuged at $12\,000 \times g$ for 5 min at 4 °C. The aqueous phase was recovered and treated one more time with P/C/I solution and treated as above. The treated homogenate was transferred to new tubes. Then, 100 µl of 95% cold ethanol was added and the homogenate incubated at -20 °C for 20 min following centrifugation at $15\,000 \times g$ for 20 min at 4 °C. The ethanol was carefully decanted. The gel-like pellet containing DNA was washed twice with 100 µl of 70% ethanol and centrifuged. After each centrifugation, the ethanol was decanted. The DNA was dissolved in 250 µl of TE buffer (10 mM Tris, 1 mM EDTA at pH 8.0), and maintained at 60 °C for 5 min. Five microliters of RNase stock

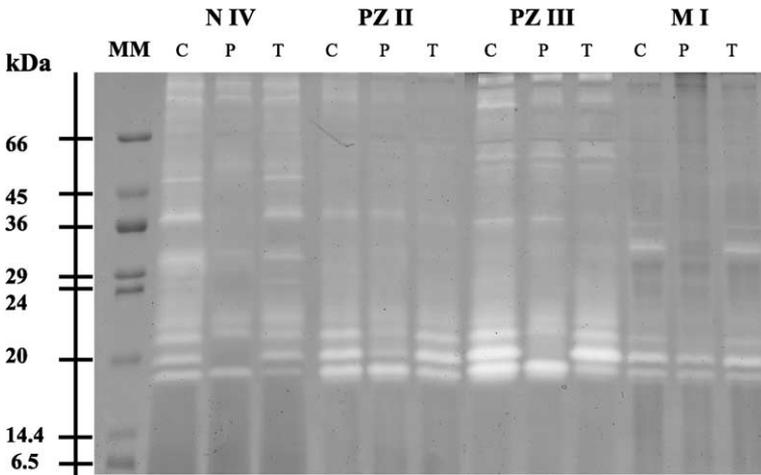


Fig. 3. Inhibition of proteinase activity in substrate-SDS-PAGE zymograms of N IV, PZ II, PZ III, and M I of *L. schmitti* at 26 ± 1 °C. C, P, T lanes denote activity pattern incubated with different inhibitors [C=control (without inhibitor); P=PMSF; T=TLCK; MM=molecular mass; other abbreviations as in legend to Fig. 1].

solution (10 mg ml^{-1}) was added to hydrolyze RNA and incubated at 37°C for 30 min. Then $500 \mu\text{l}$ of P/C/I was added and mixed slowly. The samples were incubated at 25°C for 5 min and centrifuged at $12\,000 \times g$ for 5 min at 4°C . The aqueous phase was carefully recovered and treated two more times with P/C/I. Residual protein was eliminated from the aqueous phase by adding $400 \mu\text{l}$ of chloroform, mixed slowly, and centrifuged at $12\,000 \times g$ for 15 min at 4°C . The upper aqueous phase was recovered, and the DNA precipitated with $10 \mu\text{l}$ of 4 M ammonium acetate and $500 \mu\text{l}$ cold absolute ethanol, then incubated at -20°C for 20 min. The samples were centrifuged at $15\,000 \times g$ for 15 min at 4°C . The ethanol was decanted and the pellets were air-dried. Pellets containing DNA were dissolved in $100 \mu\text{l}$ of TE buffer at 42°C .

The strong absorbance at 260 nm for nucleic acids was used for quantitative determination. One unit of optical density (OD) corresponds to approximately $40 \mu\text{g ml}^{-1}$ of RNA and $50 \mu\text{g ml}^{-1}$ of DNA. The ratio between the readings at 260–280 nm provided an estimate of the purity of the nucleic acids (Chomczynski, 1993). Most of RNA measured in this way belong to the ribosomal pool (Clemmesen, 1989).

Substrates, inhibitors, and SDS-PAGE reagents were purchased from Sigma if no other company is indicated.

Table 1

Schematic representation of proteinase activity by tosyl-lysine chloromethyl ketone (TLCK) and phenylmethylsulphonyl fluoride (PMSF) in substrate-SDS-PAGE zymograms during the ontogenetic development of *L. schmitti*

MM (kDa)	N IV	PZ I	PZ II	PZ III	M I	M II	M III	PL I	PL II	PL III	PL IV	Adult	MM (kDa)
78.0													78.0
75.1	*	*	*	P–T									75.1
69.1							*						69.1
67.0								*					67.0
55.8				*									55.8
55.2						*							55.2
52.9				*									52.9
49.0						P							49.0
48.1	P											*	48.1
42.3						P	P						42.3
38.2	P	*	P–T	P–T					*	*	*	*	38.2
35.5					P							*	35.5
33.5					P							P	33.5
30.3	P											P	30.3
27.9						*						P	27.9
25.6	P												25.6
24.0												P	24.0
21.8	P	P	P	P	P	P	*					*	21.8
19.9	P	P	P	P	P	P	P	*	*	*		*	19.9
17.3	P–T	P–T	P–T	P–T	P–T	P–T	P–T	*	*	*	*		17.3
16.0	*	*	*	*	*	*	*					P–T	16.0
15.5												P–T	15.5

MM: molecular mass; T, P, and P–T: inhibition by TLCK, PMSF, and by both, respectively; *: no inhibition detected; other abbreviations as in legend to Fig. 1.

2.5. Statistical analysis

One-way ANOVA followed by Tukey's multicomparison test was applied when data followed normal distribution. Otherwise, differences were detected by the nonparametric Kruskal–Wallis, analysis followed by the mean comparison of Nemenyi. Differences were considered significant at $P < 0.05$ (Zar, 1984).

3. Results

The activity of digestive proteinases showed significant variation throughout the early stages of *L. schmitti*, with higher values in late nauplius and protozoal stages (Fig. 1a–c). Total proteinase was reduced in egg, N I, and N II and followed by a strong increase in N IV and PZ I (Fig. 1a). Activity decreased in PZ II, rising again in PZ III ($P < 0.05$), and remained reduced in the subsequent mysid and postlarval stages. Trypsin increased significantly between egg and N I, and declined in N II ($P < 0.05$) (Fig. 1b). Similarly to total proteinase, trypsin activity presented elevated values in N IV and PZ I, reduction in PZ II, another peak in PZ III, and low values in subsequent stages. Chymotrypsin showed a similar pattern of activity throughout development as total proteinase and trypsin, except for significant lower activity in PZ I (Fig. 1c).

Proteolytic activity was detected in SDS-PAGE from N IV onward. Intense activity bands of 17.3, 19.9, and 21.8 kDa were found up to mysis (M III) (Fig. 2). The intensity of bands decreased after metamorphosis (PL I to PL VI). N IV also presented intense bands of 30.3, 38.2, and 48.1 kDa. A band of 38.2 kDa was present between N IV and M I, appearing again between PL II and PL IV. A 33.5-kDa band was observed only in M I and

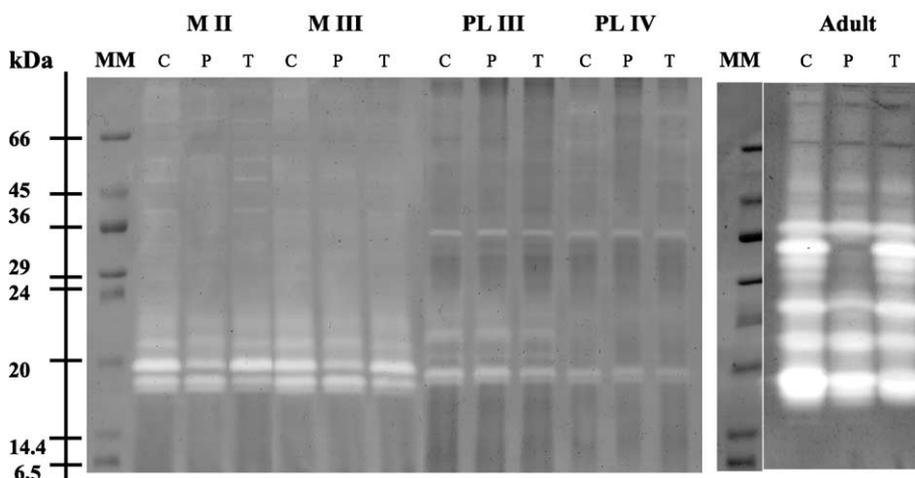


Fig. 4. Inhibition of proteinase activity in substrate-SDS-PAGE zymograms of M II, M III, PL III, PL IV, and adult of *L. schmitti* at 26 ± 1 °C. C, P, T lanes denote activity pattern incubated with different inhibitors [C = control (without inhibitor); P = PMSF; T = TLCK; MM = molecular mass; other abbreviations as in legend to Fig. 1].

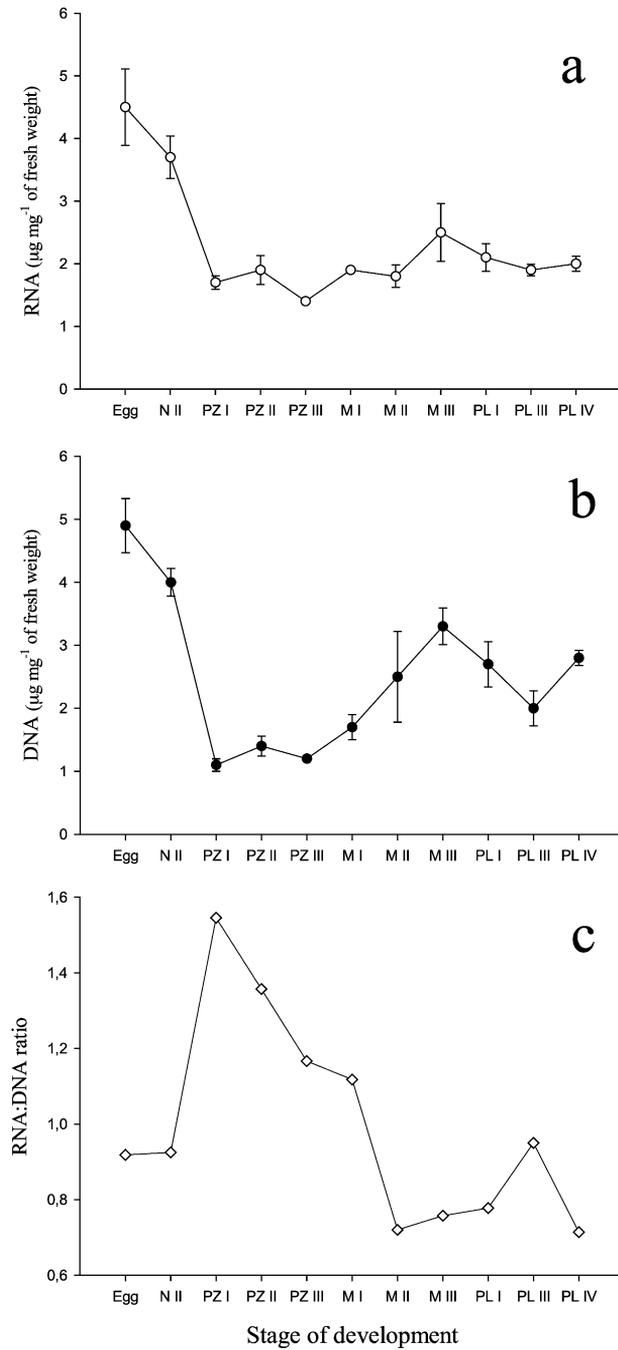


Fig. 5. (a) RNA and (b) DNA contents, and (c) RNA/DNA ratios during the early life stages of *L. schmitti* at 26 ± 1 °C. Sample size (*n*) of means 3–6 (error bars: standard deviation; other abbreviations as in legend to Fig. 1).

adult tracks. Heavier bands were seen in PZ III with 52.2 and 55.8 kDa, and a 75.1-kDa band that occurred between N IV and PZ III. The adult proteinase pattern showed 11 active bands, distributed from 15.5 to 48.3 kDa (Fig. 2). Their molecular mass was 15.5, 16.0, 19.9, 21.8, 24.0, 27.9, 30.3, 33.5, 35.5, 38.2, and 48.1 kDa.

After incubation with enzyme inhibitors, most inhibited bands were identified as serine proteinases, having reacted to PMSF (Figs. 3 and 4; Table 1). Exceptions are listed here. The 38.2-kDa band in PZ II was inhibited only by TLCK. In PZ III (38.2 kDa) and adult (15.5 and 16.0 kDa), bands were inhibited by TLCK and PMSF. In the course of development, the 19.9-kDa band was inhibited by PMSF from N IV to M III (Figs. 3 and 4). In N IV, bands of 30.3, 38.2, and 48.1 kDa were inhibited by PMSF (Fig. 3). Stages M I (33.5 and 38.2 kDa) and M II (42.3 and 48.1 kDa) also presented bands heavier than 19.9 kDa, which were inhibited by PMSF. Adults showed bands of 27.9, 30.3, and 33.5 kDa inhibited by PMSF (Fig. 4; Table 1).

Nucleic acid varied significantly in concentration throughout the early life stages of *L. schmitti* (Fig. 5a,b). Relative concentration of RNA ($\mu\text{g mg}^{-1}$ fresh weight) decreased significantly between egg and PZ III ($P < 0.05$) and showed a slight increase in the remaining stages (Fig. 5a). Similar to RNA, values of DNA declined drastically after egg until reaching minimum in PZ I (Fig. 5b). Concentration of DNA showed an increasing trend after PZ I and peaked in M III, which was followed by lower values in the remaining stages. The ratio between concentrations of RNA and DNA (RNA/DNA) was higher between PZ I and M I (Fig. 5c). In the course of development, RNA/DNA increased from egg and reached the maximum value in PZ I. Values declined in the following ontogenetic stages until the minimum in M II. An increasing trend was observed between M I and PL III, followed by another decrease in PL IV.

4. Discussion

The results of this study of enzyme activity in cultured *L. schmitti* indicate variation in the digestive potential during the stages of development. Early larval stages showed higher capacity for protein digestion than mysis and postlarval stages. In spite of digestive proteinase activities associated with attainment of carnivory in the course of larval development (Van Wormhoudt, 1973; Laubier-Bonichon et al., 1977), proteinase activity in *L. schmitti* was relatively independent of the trophic state of individuals. The addition of *Artemia* sp. nauplii to the diet from PZ III onwards was not accompanied by rise in enzyme activity (Fig. 1a–c). Lecitotrophic N IV and herbivorous PZ I presented higher activity than omnivorous stages from PZ III onwards. The high digestive capacity of planktonic filter-feeding protozoal stages seem to be reached in the last naupliar stages, but a possible role in the use of yolk protein was not verified. The profile of enzyme activity in the course of development of *L. schmitti* coincides with reports for other penaeid species, such as *Marsupenaeus japonicus* (Laubier-Bonichon et al., 1977), *Litopenaeus setiferus* (Lovett and Felder, 1990), *Penaeus monodon* (Fang and Lee, 1992), *Litopenaeus vannamei* (Le Moullac et al., 1992) and *Farfantepenaeus paulensis* (Lemos et al., 1999). The dynamics of enzyme synthesis and activity regulation is still unclear, but the variation seems to be related to developmental events (Lovett and Felder,

1989, 1990; Lemos et al., 1999). Penaeids are unique among decapods in exhibiting so many morphological, behavioral, and metabolic changes during early development (Dall et al., 1990; Icely and Nott, 1992; Chu and Ovsianico-Koulikowsky, 1994; Lemos and Phan, 2001b). Higher enzyme activity in protozoal stages occurs when enzyme secretion sites (anterior and lateral midgut caeca) reach their maximum volume during larval development (Lovett and Felder, 1989; Icely and Nott, 1992). Intense swimming activity and metabolic rates of planktonic naupliar and protozoal stages require an increased capacity for food assimilation, decreasing throughout the transition to benthic habits in late mysis and postlarval stages. Continuous swimming and feeding of protozoa indicate an intense food-energy turnover achieved by higher ingestion and digestive enzyme activities (Lemos et al., 1999; Lemos and Phan, 2001b). With the adoption of raptorial feeding behavior (Emmerson, 1980, 1984), late larval stages are able to better manipulate and select food particles (Marín-Magán and Cañavate, 1995) that may compensate for the lower digestive enzyme activity. Reduction in digestive enzyme activity after protozoa was also observed in previous studies of larval development in *L. schmitti* (González et al., 1994; Lemos et al., 2000). However, present results showed unexpected lower trypsin activity in PZ II (Fig. 1b) if compared with results reported by Lemos et al. (2000). The reason for differences in the proteinase activity profile is unclear, but it may be possibly be related to the use of different substrates to measure the activity of the enzyme (Lemos et al., 1999).

The characterization of enzymes is essential to determine catalytic properties of purified fractions and for greater understanding of the regulation of enzyme activity. During the larval development of *L. schmitti*, proteinase activity was first manifested by SDS-PAGE in N IV, bearing a singular pattern with seven intense bands distributed from 16 to 48.1 kDa (Fig. 2). PZ III and adult gel patterns also presented a high number of bands. The elevated number of bands observed in the adult proteinase pattern may be attributed to the assay of pure hepatopancreas extracts containing greater quantity of enzymes than whole body homogenates from larval and postlarval stages. On the other hand, the higher number of bands may suggest increased cellular and physiological complexity attained in the hepatopancreas of adult specimens (Morgan et al., 1978; Biesiot and Capuzzo, 1990). The presence of active bands heavier than 30 kDa in N IV and PZ III coincided with peaks of enzyme activity during early development (Fig. 1a–c). The relationship between the occurrence of such bands and increased digestive capacity appears to be a relevant topic for further studies. Accordingly, the reduction in the number and intensity of active bands after metamorphosis to the postlarval stage corresponded to low enzyme activity.

Most of the digestive proteinases of decapod crustaceans belong to the serine class (García-Carreño et al., 1994) that includes trypsin and chymotrypsin, being inhibited by PMSF (Kim et al., 1992). These enzymes seem to be the main ones responsible for protein digestion in penaeid shrimp (Galgani et al., 1985; Tsai et al., 1986). Bands affected by both TLCK (specific for trypsin) and PMSF correspond to trypsin while the inhibition only by PMSF may indicate chymotrypsin bands (Lemos et al., 1999). Though the high number of bands inhibited by PMSF coincided with increased chymotrypsin activity in N IV, a correlation between the number of bands identified as trypsin or chymotrypsin and their respective activity ($\Delta \text{ABS mg}^{-1} \text{prot min}^{-1}$) was not verified. Low molecular mass trypsin (15.5–17.3 kDa) characterized the majority of stages while

protozoal stages presented heavier bands. The variation in proteinase forms appears to be typical during the ontogenetic development of penaeids as observed in *P. monodon* (Fang and Lee, 1992) and *F. paulensis* (Lemos et al., 1999). Lower molecular mass trypsin (17.3 kDa) marked the majority of early stages and the adult pattern of *L. schmitti* (Table 1). The exceptions were the N IV (30.3 kDa) and protozoal stages (38.2 and 75.1 kDa), which exhibited heavier trypsin bands. In early life stages and adults of *L. schmitti*, most bands in SDS-PAGE behaved like chymotrypsin, in contrast with *F. paulensis* where trypsin predominated throughout development (Lemos et al., 1999). In adult penaeids, trypsin weight varies from 14.6 to 50.1 kDa, while chymotrypsin presents a narrower range of 20.9 to 37.7 kDa (Table 2). Curiously, adults of species with a closed thelycum such as *P. monodon* and *F. paulensis* display higher number of trypsin than chymotrypsin forms. The opposite is observed in species with an open thelycum species such as *L. vannamei* and *L. schmitti* (Table 2). Further examination of proteinase bands in many other penaeid species may show if these findings can be generalized to these morphological features.

The analysis of nucleic acids can be an index of tissue metabolic activity. DNA amount is constant per cell in different tissues (Leslie, 1955), providing an estimate of cell number (Geise, 1967). RNA coordinates the transfer of encoded information between DNA and ribosomes, and varies as a function of protein synthesis rate (Brachet, 1960; Otta and Landry, 1984). Thus, the RNA/DNA ratio becomes an indicator of the relative synthesis activity of growth-like cells of an organism (Buckley, 1979; Lemmens, 1995). The decrease in RNA/DNA in a certain tissue can be attributed to reduced protein synthesis or increased DNA content by cell division (hyperplasia). On the other hand, increments in cell size produce higher RNA/DNA since the amount of DNA is constant (hypertrophy). Growth in protozoal *L. schmitti* is associated with increasing cell size, rather than cell multiplication, as denoted by the exponential rise in estimated cell number throughout

Table 2
Characteristics of trypsin and chymotrypsin from adult penaeid shrimps

Species	Trypsin forms	(MM)	Chymotrypsin forms	(MM)	Reference
<i>Fenneropenaeus indicus</i>	7	36			Honjo et al. (1990)
<i>Penaeus monodon</i>	2	27, 29			Lu et al. (1990)
	3	18.5, 23.3, 50.1	1	20.9	Jiang et al. (1991)
	4	–	2	–	Fang and Lee (1992)
<i>Litopenaeus vannamei</i>			2	27	Van Wormhoudt et al. (1992)
	3	31–32			Klein et al. (1996)
			1	33.2	Hernández-Cortés et al. (1997)
<i>Farfantepenaeus paulensis</i>	4	14.6, 16.2, 17.5, 19.5	3	28.9, 32, 37.7	Lemos et al. (1999)
<i>Litopenaeus schmitti</i>	2	15.5, 16.0	3	27.9, 30.3, 33.5	Present study

MM: molecular mass in kDa; (–): not determined.

larval development (Fig. 6a). In the course of development, the rate of cell multiplication seems to increase after PZ III, which may be necessary for the specialization of certain organs (Morgan et al., 1978; Biesiot and Capuzzo, 1990). Reduced energy content in

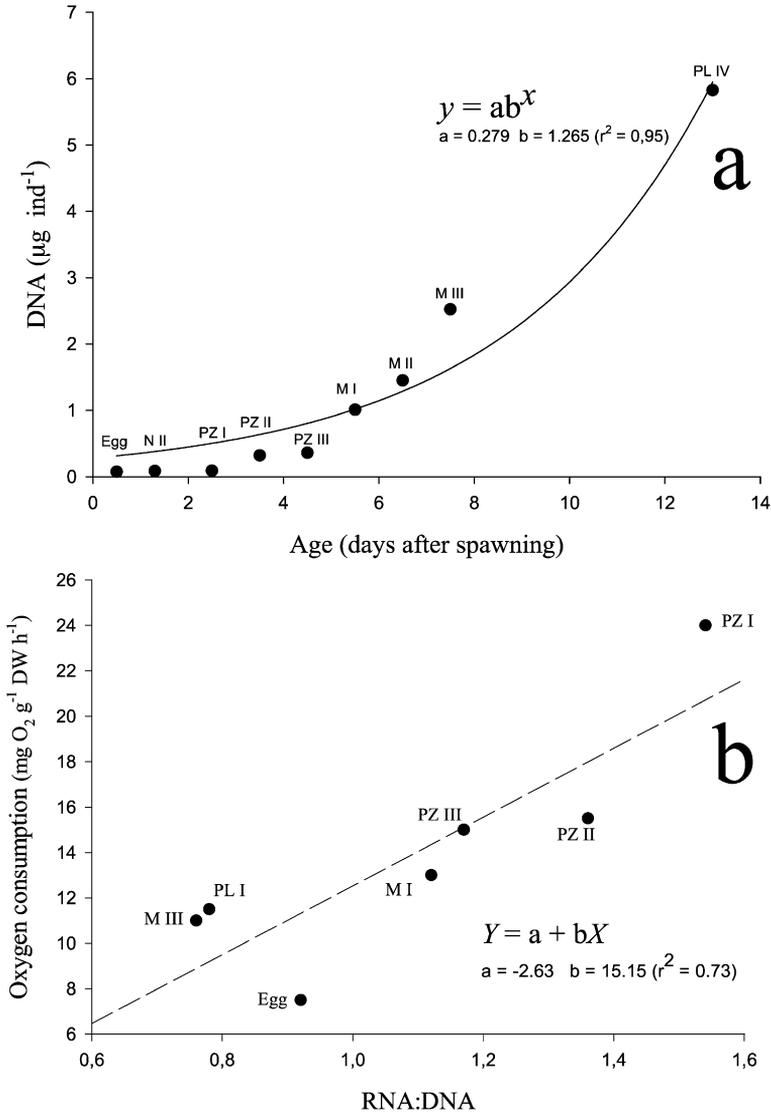


Fig. 6. Estimated DNA content of *L. schmitti* (a) and the relationship between RNA/DNA ratios and weight-specific oxygen consumption during the early life stages of penaeids (b). DNA content (a) and the relationship RNA/DNA versus oxygen consumption (b) were calculated based on individual weight and oxygen consumption rates of *F. paulseni* early stages at 26 ± 1 °C, respectively (data from Lemos and Phan, 2001b). Oxygen consumption of larval shrimp was determined after incubation in sealed respirometers. Abbreviations as in legend to Fig. 1.

protozoal stages and M I (Lemos and Phan, 2001b) suggests increased protein growth with limited accumulation of energy reserves during this period of development.

The relative quantity of RNA and DNA in *L. schmitti* was similar to the quantity reported in other decapods during their early stages of development (Otta and Landry, 1984; Anger and Hirche, 1990; Lemmens, 1995). The marked reduction in the proportion of RNA and DNA ($\mu\text{g mg}^{-1}$ of fresh weight) between egg and PZ I (Fig. 5a,b) may be attributed to increased water in these stages (Lemos and Phan, 2001b). Plotting present RNA/DNA ratios against direct measurements of oxygen consumption of other penaeid species, larval *F. paulensis*, resulted in a positive relationship of RNA/DNA to aerobic cellular activity (Fig. 6b). It is worth speculating that higher growth rates in the protozoa and early mysis of penaeids (Lemos and Phan, 2001a) involve efficient protein digestion to convert elevated amounts of ingested food (Emmerson, 1980; Loya-Javellana, 1989) into biomass. Bioenergetics of larval development of penaeid shrimp includes early planktonic larval stages with high cellular activity, increased cell size, and greater digestive enzyme activity. Through the transition to the benthic existence between mysis and the first postlarvae, growth rate and cellular activity are reduced, accompanied by decreased rates of food utilization (Lemmens, 1995; Jones et al., 1997; Lemos et al., 1999). Accordingly, a re-evaluation of food quantity requirements of early postlarvae appears to be warranted for further optimization of feeding schedules in aquaculture of penaeid shrimp.

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References

- Anger, K., Hirche, H.-J., 1990. Nucleic acids and growth of larval and early juvenile spider crab, *Hyas araneus*. Mar. Biol. 105, 403–411.
- Barg, U., Subasingher, R., Willmann, R., Rana, K., Martinez, M., 2000. Towards Sustainable Shrimp Culture Development: Implementing the FAO Code of Conduct for Responsible Fisheries (CCRF). FAO Fisheries Department, Rome, Italy, 36 pp.
- 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. Universidad Católica del Norte, Coquimbo, Chile, pp. 1–4.
- 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.
- Brachet, J., 1960. The Biological Role of Ribonucleic Acids. Elsevier, New York, 144 pp.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.

- Buckley, L.J., 1979. Relationships between RNA/DNA ratio, prey density, and growth rate Atlantic cod (*Gadus morhua*). J. Fish. Res. Board Can. 36, 1497–1502.
- Buckley, L.J., 1980. Changes in the ribonucleic acid, deoxyribonucleic acid, and protein content during ontogenesis in winter flounder, *Pseudopleuronectes americanus*, and the effect of starvation. Fish. Bull. 77, 703–708.
- Castille, F.L., Samocha, T.M., Lawrence, A.L., He, H., Frelrier, P., Jaenike, F., 1993. Variability in growth and survival of postlarval shrimp (*Penaeus vannamei* Boone 1931). Aquaculture 113, 65–81.
- Chomczynski, P., 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. BioTechniques 15, 532–534, 536–537.
- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.
- Chu, K.H., Ovsianico-Koulikowsky, N.N., 1994. Ontogenetic changes in metabolic activity and biochemical composition in the shrimp, *Metapenaeus ensis*. J. Exp. Mar. Biol. Ecol. 183, 11–26.
- Clemmesen, C., 1989. RNA/DNA ratios of laboratory reared and wild herring larvae determined with a high sensitive fluorescence method. J. Fish Biol. 35A, 331–333.
- Clemmesen, C., 1996. Importance and limits of RNA/DNA ratios as a measure of nutritional condition in fish larvae. Proc. Intern. Workshop “Survival Strategies in Early Life Stages of Marine Resources. Balkema, Rotterdam, pp. 67–82.
- Dall, W., Hill, B.J., Rothlisberg, P.C., Staples, D.J., 1990. The biology of Penaeidae. Adv. Mar. Biol. 27, 1–489.
- Del Mar, E.G., Largman, C., Brodrick, J.W., Geokas, M.C., 1979. A sensitive new substrate for chymotrypsin. Anal. Biochem. 99, 316–320.
- Emmerson, W.D., 1980. Ingestion, growth and development of *Penaeus indicus* larvae as a function of *Thalassiosira weissflogii* cell concentration. Mar. Biol. 58, 65–73.
- Emmerson, W.D., 1984. Predation and energetics of *Penaeus indicus* (Decapoda: Penaeidae) larvae feeding *Brachionus plicatilis* and *Artemia* nauplii. Aquaculture 38, 201–209.
- Erlanger, B., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.
- Ezquerria, J.M., García-Carreño, F.L., Haard, N.F., 1997. Effects of feed diets on digestive proteases from the hepatopancreas of white shrimp (*Penaeus vannamei*). J. Food Biochem. 21, 401–419.
- Fang, L.-S., Lee, B.-N., 1992. Ontogenetic change of digestive enzymes in *Penaeus monodon*. Comp. Biochem. Physiol. 103B, 1033–1037.
- Fast, A.W., Lester, L.J., 1992. Marine Shrimp Culture: Principles and Practices. Elsevier, Amsterdam.
- Galgani, M.L., Benyamin, Y., Van Wormhoudt, A., 1985. Purification, properties and immunoassays of trypsin from the shrimp *Penaeus japonicus*. Comp. Biochem. Physiol. 81B, 447–452.
- Gallardo, P.P., Alfonso, E., Gaxiola, G., Soto, L.A., 1995. Feeding schedule for *Penaeus setiferus* larvae based on diatoms (*Chaetoceros ceratosporum*), flagellates (*Tetraselmis chuii*) and *Artemia* nauplii. Aquaculture 131, 239–252.
- García, T., 1972. Descripción de los estadios larvales del camarón blanco *Penaeus schmitti* Bunkeroad obtenidos en laboratorio. Ciencias 1, La Havana, 54 pp.
- García-Carreño, F.L., Haard, N.F., 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. J. Food Biochem. 17, 97–113.
- García-Carreño, F.L., Dimes, L.E., Haard, N.F., 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. Anal. Biochem. 214, 65–69.
- García-Carreño, F.L., Hernández-Cortés, M.P., Haard, N.F., 1994. Enzymes with peptidase and proteinase activity from the digestive systems of a freshwater and a marine decapod. J. Agric. Food Chem. 42, 1456–1461.
- Geise, A.C., 1967. Some methods for study of the biochemical constitution of marine invertebrates. Oceanogr. Mar. Biol. Annu. Rev. 5, 159–186.
- González, R., Fraga, V., Carrillo, O., 1994. Cambios ontogenéticos en la actividad de las principales enzimas digestivas de *Penaeus schmitti*. Rev. Investig. Mar., La Habana 15, 262–268.
- Hernández-Cortés, M.P., Whitaker, J., García-Carreño, F.L., 1997. Purification and characterization of chymotrypsin from *Penaeus vannamei* (Crustacea: Decapoda). J. Food Biochem. 21, 497–514.
- Honjo, I., Kimura, S., Nonaka, M., 1990. Purification and characterization of trypsin-like enzyme from shrimp *Penaeus indicus*. Bull. Jpn. Soc. Sci. Fish. 56, 1627–1634.

- Icely, J.D., Nott, J.A., 1992. Digestion and absorption: digestive system and associated organs. In: Harrison, F.W., Humes, A.G. (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, 138 pp.
- Jiang, S.T., Moody, M.W., Chen, H.C., 1991. Purification and characterization of proteases from digestive tract of grass shrimp (*Penaeus monodon*). *J. Food Sci.* 56, 322–326.
- Jones, D.A., Kanazawa, A., Ono, K., 1979. Studies on the nutritional requirements of the larval stages of *Penaeus japonicus* using microencapsulated diets. *Mar. Biol.* 54, 261–267.
- Jones, D.A., Yule, A., Holland, D.L., 1997. Larval nutrition. In: D'Abramo, L., Conklin, D., Akiyama, D.M. (Eds.), *Crustacean Nutrition*. World Aquaculture Society, Baton Rouge, LA, pp. 353–389.
- Kim, H.R., Meyers, S.P., Godber, J.S., 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 moult cycle. *Int. J. Biochem. Cell Biol.* 28, 551–563.
- Kurnaly, K., Yule, A.B., Jones, D.A., 1989. An energy budget for the larvae of *Penaeus monodon* (Fabricius). *Aquaculture* 81, 13–25.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* 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. *Publ. Cent. Natl. Exploit. Océans* (CNEOX), Sér. Actes Colloques 4, 131–145.
- Lemmens, J.W.T.J., 1995. Nucleic acid levels, cellular activity and growth during the puerulus stage of the western rock lobster (*Panulirus cygnus* (George); Decapoda: Palinuridae). *J. Exp. Mar. Biol. Ecol.* 194, 143–156.
- Lemos, D., Phan, V.N., 2001a. Energy partitioning into growth, respiration, excretion and exuvia during larval development of the shrimp *Farfantepenaeus paulensis*. *Aquaculture* 199, 133–145.
- Lemos, D., Phan, V.N., 2001b. Ontogenetic variation in metabolic rates, biochemical composition and energy content during the early life stages of *Farfantepenaeus paulensis* (Crustacea: Decapoda: Penaeidae). *Mar. Biol.* 138, 985–997.
- Lemos, D., Rodríguez, A., 1998. Nutritional effects on body composition, energy content and trypsin activity of *Penaeus japonicus* during early postlarval development. *Aquaculture* 160, 103–116.
- Lemos, D., Hernández-Cortés, M.P., Navarrete, A., García-Carreño, F.L., Phan, V.N., 1999. Ontogenetic 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., Ezquerro, J.M., García-Carreño, F.L., 2000. Protein digestion in penaeid shrimps: digestive proteinases, proteinase inhibitors and feed digestibility. *Aquaculture* 186, 89–105.
- 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*, San Pedro de Manglaralto, Ecuador, CENAIM. San Pedro de Manglaralto, Ecuador, pp. 81–86.
- 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 vannamei* (Crustacea, Decapoda). *J. Exp. Mar. Biol. Ecol.* 208, 107–125.
- Leslie, I., 1955. The nucleic acid content of tissues and cells. In: Chargaff, E., Davidson, J.N. (Eds.), *The Nucleic Acids: Chemistry and Biology*, vol. 2. Academic Press, New York, pp. 1–50.
- 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.
- Lovett, D.L., Felder, D.L., 1989. Ontogeny of gut morphology in the white shrimp *Penaeus setiferus* (Decapoda, Penaeidae). *J. Morphol.* 201, 253–272.
- 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.

- Loya-Javellana, G., 1989. Ingestion saturation and growth responses of *Penaeus monodon* larvae to food density. *Aquaculture* 81, 329–336.
- Lu, P.J., Liu, H.C., Tsai, I.H., 1990. The midgut trypsin of shrimp (*Penaeus monodon*): high efficiency toward native protein substrates including collagens. *Biol. Chem. Hoppe-Seyler* 371, 851–859.
- Marín-Magán, V., Cañavate, J.P., 1995. Fluorometric determination of selectivity between live and inert food by *Penaeus japonicus* larvae. *Aquaculture* 134, 307–311.
- Morgan, R.P., Kramarsky, E., Sulkin, S.D., 1978. Biochemical changes during larval development of the xanthid crab, *Rithropanopeus harrisi*: III. Isozyme changes during ontogeny. *Mar. Biol.* 48, 223–226.
- Nascimento, I.A., Bray, W.A., Leung-Trujillo, J.R., Lawrence, A., 1991. Reproduction of ablated and unablated *Penaeus schmitti* in captivity using diets consisting of fresh-frozen natural and dried formulated feeds. *Aquaculture* 99, 387–398.
- Otta, A.Y., Landry, M.R., 1984. Nucleic acids as growth rate indicators for early developmental stages of *Calanus pacificus* Brodsky. *J. Exp. Mar. Biol. Ecol.* 80, 147–160.
- Pérez-Farfante, I., Kensley, B.F., 1997. Penaeoid and sergestoid shrimps and prawns of the world. Keys and diagnoses for the families and genera. *Mém. Mus. Natl. Hist. Nat., Paris* 175, 233.
- Pillay, T.V.R., 1990. *Aquaculture: Principles and Practices*. Blackwell, London.
- Primavera, J.H., Posadas, R.A., 1981. Studies on the egg quality of *Penaeus monodon* Fabricius, based on morphology and hatching rates. *Aquaculture* 22, 269–277.
- Rodríguez, A., Le Vay, L., Mourente, G., Jones, D.A., 1994. Biochemical composition and digestive enzyme activity in larvae and postlarvae of *Penaeus japonicus* during herbivorous and carnivorous feeding. *Mar. Biol.* 118, 45–51.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory, New York.
- Sasaki, G.C., Capuzzo, J.M., Biesiot, P., 1986. Nutritional and bioenergetic considerations in the development of the American lobster *Homarus americanus*. *Can. J. Fish. Aquat. Sci.* 43, 2311–2319.
- Thompson, F., Abreu, P.C., Cavalli, R., 1999. The use of microorganisms as food source for *Penaeus paulensis* larvae. *Aquaculture* 174, 139–153.
- Tsai, I.H., Chuang, K.L., Chuang, J.L., 1986. Chymotrypsins in digestive tracts of crustacean decapods (shrimps). *Comp. Biochem. Physiol.* 85, 235–240.
- Van Wormhoudt, A., 1973. Variations des protéases, des amylases et des protéines soluble au cours du développement larvaire chez *Palaemon serratus*. *Mar. Biol.* 19, 245–248.
- 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 do Quarto Simpósio Brasileiro sobre Cultivo de Camarão, MCR Aquicultura, João Pessoa, Brazil*, pp. 399–414.
- Walker, J.M., 2000. Electrophoretic techniques. In: Wilson, K., Walker, J. (Eds.), *Principles and Techniques of Practical Biochemistry*, 5th ed. Cambridge Univ. Press, UK.
- Zar, J.H., 1984. *Biostatistical Analysis*, 2nd ed. Prentice-Hall, Englewood Cliffs, NJ.