ELSEVIER

Journal of Experimental Marine Biology and Ecology
292 (2003) 1–17

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

www.elsevier.com/locate/jembe

Differential expression of trypsin mRNA in the white shrimp (*Penaeus vannamei*) midgut gland under starvation conditions

Arturo Sánchez-Paz[a], Fernando García-Carreño[a,*], Adriana Muhlia-Almazán[a], Norma Y. Hernández-Saavedra[a], Gloria Yepiz-Plascencia[b]

[a] *Centro de Investigaciones Biológicas del Noroeste (CIBNOR), P.O. Box 128, La Paz, B.C.S. C. P. 23000, Mexico*
[b] *Centro de Investigación en Alimentación y Desarrollo (CIAD), P.O. Box 1735, Carretera a la Victoria Km. 0.6 Hermosillo, Sonora C.P. 83000 Mexico*

Received 4 November 2002; received in revised form 4 March 2003; accepted 10 March 2003

Abstract

Regulation of the expression of trypsin encoding genes in juveniles white shrimp (*Penaeus vannamei*) was studied during starvation for up to 120 h. Since molting is now recognized as a physiologically important process in penaeids, specimens were selected according to molt stage. Starvation led to immediate weight loss and a sharp decrease in hepatosomatic index. Specific DNA probes for trypsin were synthesized and trypsin mRNA concentration was evaluated by dot blot hybridization. Trypsin-encoding RNA levels were strongly influenced by starvation, resulting in an increase after 24 h of starvation, followed by a steep decline to lower level compared to those obtained in fed shrimp. A possible mechanism for regulation of trypsin activity during transcription is proposed. The biological implications of our findings for transcriptional regulation of trypsin mRNA during starvation are discussed.

Keywords: Adaptation; Molt cycle; mRNA turnover; Shrimp; Transcription; Trypsin

1. Introduction

Proteolysis refers to the biochemical degradation of protein by hydrolysis of peptide bonds (Kato, 1999), and plays two vital roles in the physiology and development of

* Corresponding author. Tel.: +52-612-123-8484; fax: +52-612-125-36-25.
E-mail address: fgarcia@cibnor.mx (F. García-Carreño).

0022-0981/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0022-0981(03)00142-4

organisms: (a) it is essential for digestion of proteins in ingested food to provide essential amino acids, and (b) the regulation of metabolism by controlling myriads of processes. The two functions must be properly controlled for efficient use of energy and to avoid internal tissue damage. Proteases and proteinases are involved in a broad repertoire of physiological processes, including blood clotting and fibrinolysis, protein hormones release from precursor molecules, transport of secretory proteins through cell membranes, assembly of molecular structures like collagen fiber and certain kinds of viruses, fertilization, and control of proteolytic digestion (Zimmerman et al., 1980; Neurath, 1984, 1986, 1989; Furie and Furie, 1988; Whitaker, 1994; Lu et al., 1996).

As protein digestion is crucial for heterotrophic organisms, it is paramount to control proteolytic activity to the right moment and place, and to avoid undesired proteolysis and tissue damage. Three principal mechanisms of proteinase activity regulation have been found: (a) synthesis of zymogens, (b) activation of inactive protease precursors (zymogens) by limited proteolysis, and (c) inactivation of proteases by forming complexes with specific proteinase inhibitors (Neurath, 1984).

The crustacean midgut gland releases huge amounts of digestive enzymes. The most abundant digestive proteinase found in the shrimp *Penaeus japonicus* is trypsin, which contributes about 6% of the total soluble protein (Galgani et al., 1985a), while in the fiddler crab (*Uca pugilator*), trypsin accounts for approximately 33% of the total hepatopancreatic protein (Eisen and Jeffrey, 1969). Some authors (Galgani et al., 1985a,b) have emphasized the important role of this enzyme in penaeids and estimate its contribution to protein digestion at 60%. However, the mechanisms by which this enzyme is regulated and released to the midgut gland lumen are still unknown (García-Carreño et al., 1998).

Synthesis of digestive enzymes in crustaceans fluctuates in response to several factors and conditions, which include age (van Wormhoudt, 1973; Lee et al., 1984), molt cycle stage (van Wormhoudt and Favrel, 1988), temperature (Galgani et al., 1985b), food, and food protein origin and quality (van Wormhoudt, 1973; Lee et al., 1984). In invertebrates like female mosquito *Aedes aegyptii*, synthesis of mRNA for one out of two isoforms of trypsin is regulated by juvenile hormone (Noriega et al., 1997; Noriega and Wells, 1999). Also, enzyme activity in penaeids seems to fluctuate under hormonal control (Tan et al., 2000; Klein et al., 1996). Ecdysteroid hormone stimulates protein and mRNA synthesis in the midgut gland (Skinner, 1965). In the white shrimp *P. vannamei*, trypsin mRNA increases almost 4-fold during the premolt stage D_1, suggesting that ecdysteroid hormones regulate concentrations of mRNA and protein, and that this regulation affects transcription (Klein et al., 1996).

Molting or ecdysis is one of the most important processes in the arthropod life cycle because shedding of the exoskeleton is an absolute requisite for growth (Chung et al., 1999). Penaeids, like other crustaceans, increase their size in sequential steps: a fast increase of the body height at each ecdysis is followed by a period of little or no growth. This process might last days or weeks, making it a continuous process, with morphological, physiological, and behavioral alterations occurring almost daily (Dall et al., 1990). During stage D_2 in proecdysis, feeding declines, and ceases by stage D_3, while water is taken up to increase body volume.

In the present study, the influence of molting and fasting over the concentration of trypsin mRNA was evaluated because the feeding behavior of *P. vannamei* changes during

molt, and reaches the most dramatic point during a period of almost 2 days of starvation during the premolt stage D_1, when the concentration of trypsin mRNA increases followed by an abrupt decline of trypsin mRNA during late premolt (D_2–D_3) (Dall et al., 1990). Thus, from energy availability and physiologic arguments, we predict that the transcription of trypsin mRNA is affected during starvation in *P. vannamei*. This might allow us to draw some conclusions on the regulation of the trypsin gene in relation to digestion physiology.

2. Materials and methods

2.1. Organisms

Juvenile white shrimp *P. vannamei*, ($n = 240$) were obtained from CIBNOR aquaculture facilities (Mexico) and distributed randomly in twelve 120-l indoor tanks, divided into four triplicate groups of 20 organisms each (average weight 10 g). Organisms were acclimated for 1 week in filtered seawater at 28 °C and 34 ppt salinity, and fed twice daily. Uneaten food and solid excreta were removed daily. After this period, shrimp were subjected to 2, 24, 72, or 120 h of starvation. Control organisms were sacrificed 2 h after feeding by decapitation.

At the end of each interval, three organisms at each molt stage (postmolt, A; intermolt, C; early premolt, DE; and late premolt, DL) were collected from each tank and decapitated. In some cases, it was not possible to collect three organisms of a certain molt stage. Specimens were selected according to molt stage by setogenesis (Chan et al., 1988), which relies on changes in seta in the inner margin of the uropods. Individual midgut glands were weighed and stored at − 80 °C until used.

To determine individual or combined effects of fasting and molting on total body weight and hepatosomatic index, statistical analyses were performed by one- and two-way ANOVA, since the data were homogeneous. Three-way ANOVA was conducted in order to consider simultaneously the effects of treatment on all three genes. For post hoc analysis, the Tukey honest significant difference (HSD) test was used. Statistical significance was considered when $p < 0.05$. Analyses were performed using Statistica v. 6.0 software.

2.2. RNA extraction

Total RNA was extracted individually from whole midgut glands using TRIzol LS (GIBCO BRL), following manufacturer instructions. In brief, approximately 200 mg of each tissue sample was homogenized into 750 μl of TRIzol LS in 2.0 ml Eppendorf tubes. Samples were incubated at room temperature for 15 min. Following incubation, 200 μl of chloroform were added to each sample and tubes were shaken vigorously. Samples were incubated for 15 min at room temperature and centrifuged at 10,000 × *g* for 15 min at 4 °C. The aqueous phase was transferred to clean tubes, and 0.5 ml of isopropyl alcohol were added to precipitate RNA. Samples were incubated for 15 min at room temperature and centrifuged at 10,000 × *g* for 15 min at 4 °C. RNA samples were washed once with 75% ethanol (prepared with DEPC water), and centrifuged at 5000 × *g* for 15 min at 4 °C.

RNA samples were air-dried and dissolved in nuclease-free water. RNA concentration and purity were measured spectrophotometrically at 260/280 nm (Lambda Bio 20, Perkin Elmer, USA). RNA intactness was detected running a 1.5% agarose-formaldehyde gel electrophoresis (Sambrook et al., 1989).

2.3. Probe preparation

Genomic DNA was extracted from abdominal muscle according to protocol described by Sambrook et al. (1989). DNA concentration was determined spectrophotometrically. Sets of oligonucleotide primers to amplify specific fragments of trypsin, actin mRNAs, and 16S rRNA genes were designed. Actin and 16S primers were used to compare expression of trypsin genes with that of other shrimp genes during starvation and throughout the molt cycle (both genes are considered constitutive). Trypsin primers were designed to amplify across exon 3 in *P. vannamei* trypsin genes *TryPv I*, *TryPv II*, and *TryPv III* (Klein et al., 1998). These primers, named TryEx3a (5′-TCCTCTCCAAGATCATCCAA-3′) and TryRev (5′-ATTGGCCTTAATCCAATCGAC-3′), annealed in *TryPv II* to positions 854–873 and 1306–1326, respectively. Actin primers Act-S (5′-TACCTGATGAAGATCCTGAC-3′) and Act-AS (5′-TAGAAGCACTTCCTGTGAAC-3′) annealed to positions 652–671 and 1197–1216 of the white shrimp β-actin gene (EMBL accession number AF300705.1), respectively. Amplification of 16S rRNA gene was performed with primers 16S-S (5′-CGCCTGTTTATCAAAAACAT-3′) and 16S-AS (5′-CCGGTCTGAACTCAGATCACGT-3′), which annealed to positions 1149–1167 and 1686–1707 of white shrimp 16S rRNA gene (EMBL accession number AJ132780), respectively, and were kindly provided by Dr. Murugan Gopal (CIBNOR). PCR amplifications were done in a 50-μl final volume containing 38.3 μl of H_2O, 5 μl of 10 × PCR buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 2 μl containing 10 pmol of dNTPs, 40 pmol of each primer, 50 ng of abdominal muscle genomic DNA, and 1 U of *Taq* DNA Polymerase (Boehringer Mannheim). Amplification was done under the following conditions: 95 °C for 5 min (1 cycle), denaturing at 95 °C for 45 s, annealing at 50 °C for 2 min, and extension at 72 °C for 1.5 min (35 cycles) and an overextension cycle step at 72 °C for 5 min. Controls containing all reagents except the template were run with each primer set. Trypsin PCR products were purified with the Concert Rapid Gel extraction kit (GIBCO), while actin and 16S PCR products were purified as described by Vogelstein and Gillespie (1979). Purified PCR products were electrophoresed in a 1.2% agarose gel.

Digoxigenine-labeled probes were synthesized using a PCR Dig probe synthesis kit (Boehringer Mannheim), and the amplicons obtained used as template. Product concentration was measured following manufacturer instructions.

2.4. Sequencing

DNA sequencing was carried out to identify PCR products. Direct sequencing of PCR products was done for both strands, using the *Taq* Dideoxy terminator cycle sequencing kit and automatic DNA sequencing system 377 (Applied Biosystems, Perkin-Elmer). Sequencing was performed either at the Biotechnology Institute at UNAM, or at the

Molecular Biology Laboratory at CIBNOR. PCR products were identified by analyzing nucleotide sequence through the FASTA3 online network service of the European Bioinformatics Institute at http://www.ebi.ac.uk/fasta33 (Pearson and Lipman, 1988).

2.5. RNA dot blot hybridization

Individual RNA samples from different treatments were adjusted to a final concentration of 1 μg/μl by dilution in the following buffer: DEPC water, 20 × SSC, and formaldehyde (5:3:2). Onto a positively charged nylon membrane (Boehringer Mannheim), 4.0 μg of each RNA sample was dotted and fixed by UV irradiation in a crosslinker (Stratagene). Prehybridization was performed with 20 ml of DIG Easy Hyb (ROCHE) for 3 h at 50 °C. The probe was denatured at 95 °C for 10 min, and then diluted in prehybridization solution (250 ng/ml). Hybridization was performed overnight at 50 °C, according to manufacturer protocol. Membranes were washed twice in 2 × SSC, and twice more in 0.5 × SSC, each for 15 min at room temperature.

mRNA–DNA hybridization was detected with a DIG nucleic acid detection kit (Boehringer Mannheim) with alkaline phophatase-conjugated anti-digoxigenin antibody and NBT/BCIP (18.75 mg/ml nitro blue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 67% dimethyl formamide) as substrate, and developed for 2 h at room temperature. Reaction was stopped by rinsing the membranes thoroughly with distilled water. Results were documented by digital scanning of the membranes (Duoscan T1200, AGFA), and quantifying dot intensity by densitometry, using Kodak Digital Science 1D software.

To determine individual or combined effects of fasting and molting on transcript levels of gene coding trypsin β-actin and 16S rRNA, statistical analyses were performed by one- and two-way ANOVA, since the data were homogeneous. Three-way ANOVA was conducted for each variable to compare interactions among factors. For post hoc analysis, the Tukey honest significant difference (HSD) test was used. Statistical significance was considered when $p < 0.05$. Analyses were performed using Statistica v. 6.0 software.

3. Results

3.1. Effect of starvation on hepatosomatic index

Statistical analysis showed no significant effect of fasting or molting on the total weight of the specimens during the period of the experiment. However, when the effect of starvation on the weight of the midgut gland was analyzed, a significant decrease ($p < 0.05$) was found (Fig. 1). No effect of molting on the organ weight was found.

3.2. Sequence analysis

All nucleotide sequences of trypsin, actin, and 16S amplicons showed significant degrees of identity with the reported nucleotide sequences. Amplification with trypsin primers yielded a fragment of the expected size (473 bp), corresponding to the last exon of

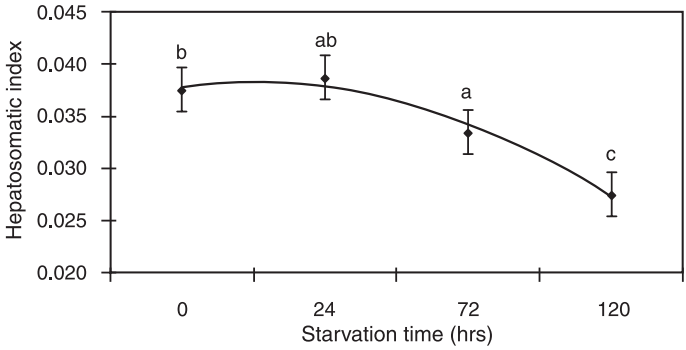

Fig. 1. Changes in hepatosomatic index during starvation in *P. vannamei*. Significant differences ($p < 0.05$) are indicated by different letters.

P. vannamei trypsin genes (Klein et al., 1998), which had 85.5% identity with *P. vannamei* exon 3 of trypsin gene 2 (GenBank Y15040). Similarly, β-actin primers Act-S and Act-AS yielded a PCR product of the expected size (566 bp), and showed 92.2% identity when compared with the reported sequence of the white shrimp β-actin gene (GenBank AF300705). Amplification of 16S rRNA genes produced a 437-bp fragment, 98.3% similar to the sequence reported (GenBank AJ132780). Therefore, our amplicons corresponded to the target genes. No effort was made to sequence 100% of both strands and to resolve ambiguities, since this high identity indicates with confidence bona fide amplification of the trypsin, β-actin, and 16S genes.

3.3. Expression patterns of β-actin, trypsin mRNAs and 16S rRNA

β-actin mRNA concentration was measured during fasting and molting. The concentration of β-actin mRNA was lower than those of trypsin and 16S rRNA (Fig. 2). In all

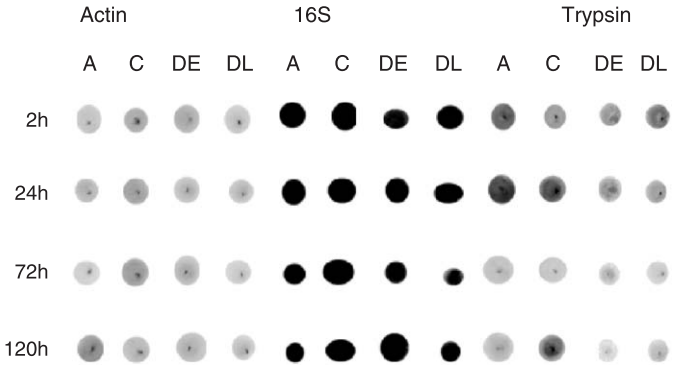

Fig. 2. A representative example of RNA dot blot hybridization analysis for β-actin and trypsin mRNA, and 16S rRNA steady-state contents in the midgut gland of *P. vannamei* during starvation and molting: ~ 4.0 μg of each individually sampled RNAs were dotted on a nylon membrane. Blots were hybridized with actin, 16S, and trypsin DIG-labeled probes, respectively. A, postmolt; C, intermolt; DE, early premolt; and DL, late premolt.

cases, the effects of molting and starvation on the expression patterns of β-actin, 16S, and trypsin genes were statistically significant ($p < 0.05$), although there was no interaction among these factors in terms of expression of the genes analyzed.

Dot signal intensity was analyzed by densitometry and statistically compared. As shown in Fig. 3, both molt stage and starvation had statistically significant effect on β-actin mRNA concentration ($p < 0.05$). There was a clear tendency of β-actin mRNA levels to decrease after 24 h of starvation, with a minimum at early premolt stage (DE). β-actin mRNA levels increased in all molt stages toward the end of the experiment. During postmolt (A) and intermolt (C), β-actin mRNA concentrations were close. Also, β-actin mRNA levels in early (DE) and late (DL) premolt were close, but different from those for A and C stages (Fig. 3), although the effect of molting on β-actin mRNA concentration was less evident (Fig. 4) than the effect of starvation. The effect of early premolt on β-actin mRNA was significant, dropping concentration to the lowest measured during the experiment.

The abundance of 16S rRNA in white shrimp during starvation and molting was analyzed. As shown in Fig. 2, the signal intensity of rRNA dot blots was the highest when compared with β-actin and trypsin mRNAs, however, there were slight differences between levels of 16S rRNA and that of control samples. The effect of starvation on 16S rRNA (Fig. 5) produced significant differences ($p < 0.05$) among signal intensities of the different molt stages. Molting affected transcription of 16S rRNA (Fig. 6), with the lowest concentration found in organisms at early premolt stage. The effect of molting on 16S rRNA levels was less evident than that of starvation. However, the similarity in the patterns of the two molecules throughout the molt stages is remarkable. At the beginning

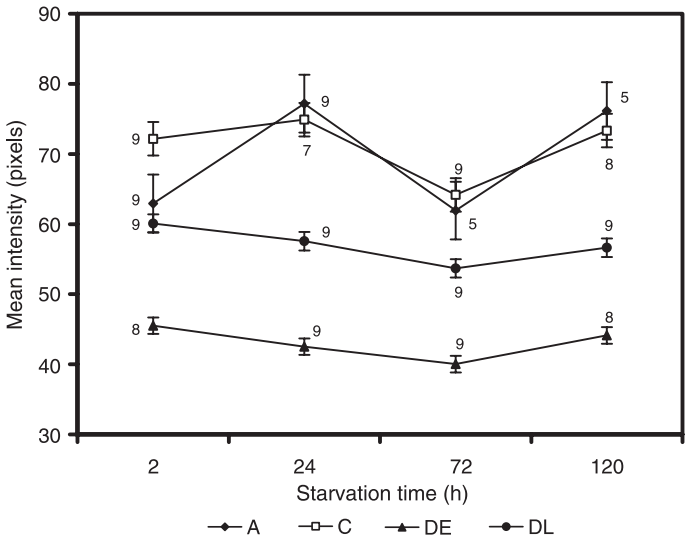

Fig. 3. Statistical analysis of the steady-state levels of actin mRNA in *P. vannamei* midgut gland during fasting. β-actin mRNA concentration is expressed as mean intensities of 131 samples, obtained from individual whole midgut gland homogenates. For post hoc analysis, Tukey honest significant difference (HSD) test was used. Values are means ± 95% confidence intervals. Sample size for each mean is indicated by a number above or below bars.

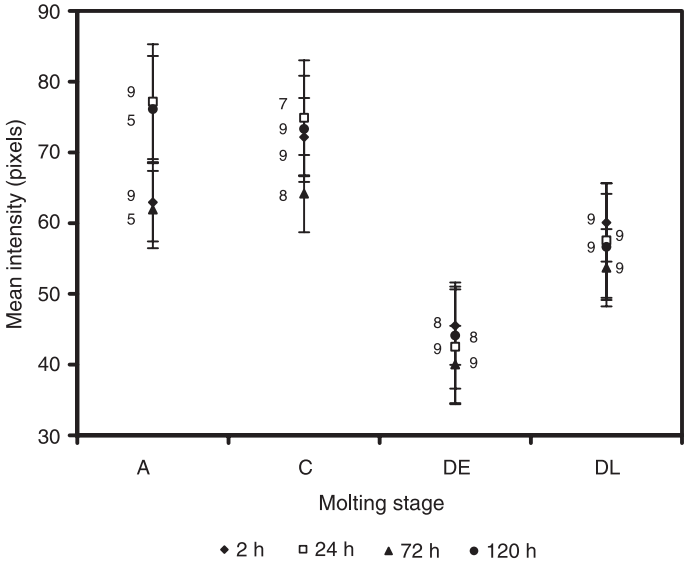

Fig. 4. Actin mRNA concentration during molting. RNA samples were obtained from individual whole midgut gland homogenates. For post hoc analysis, Tukey honest significant difference (HSD) test was used. Values are means ± 95% confidence intervals. Sample size for each mean is indicated by a number above or below bars.

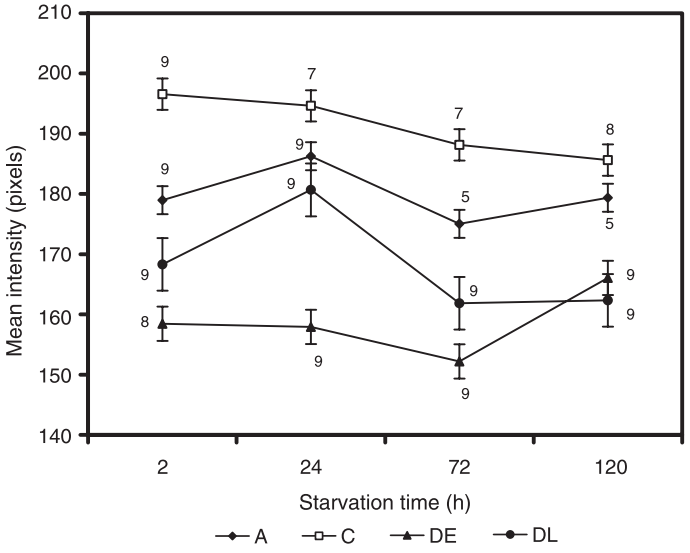

Fig. 5. Statistical analysis of the steady-state content of 16S rRNA in *P. vannamei* midgut gland during starving at different molt stages. 16S rRNA concentration is expressed as mean intensities of 130 samples obtained from individual whole midgut gland homogenates. For post hoc analysis, Tukey honest significant difference (HSD) test was used. Values are means ± 95% confidence intervals. Sample size for each mean is indicated by a number above or below bars.

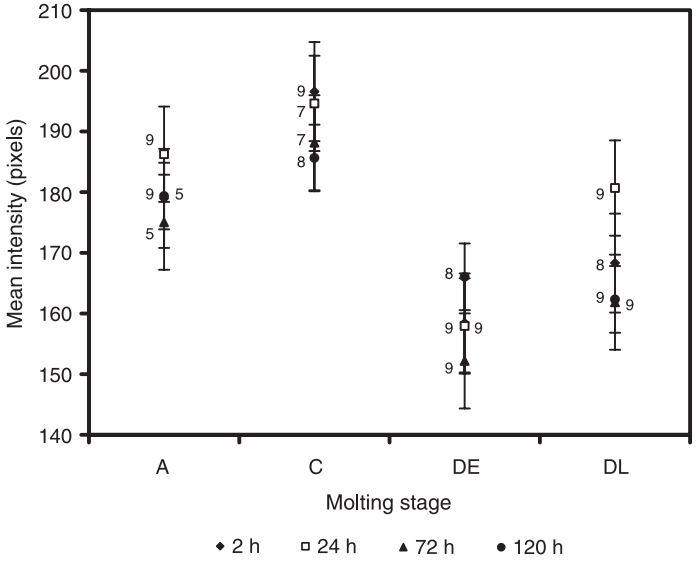

Fig. 6. 16S rRNA concentration during molting. RNA samples were obtained from individual whole midgut gland homogenates. For post hoc analysis, Tukey honest significant difference (HSD) test was used. Values are means ± 95% confidence intervals. Sample size for each mean is indicated by a number above or below bars.

of starvation, the highest concentration of 16S rRNA was observed during intermolt, and the lowest concentration at early premolt (24% difference), but the difference diminished to 10.5% ($p < 0.05$) between intermolt and late premolt after 120 h starvation.

Trypsin mRNA levels, as measured by dot blot hybridization, varied through the starvation period, increasing during the first 24 h, except during late premolt (Fig. 7). A significant sharp decrease in trypsin mRNA levels started after 24 h of starvation, bottomed out at 72 h, and then increased slightly. However, the final transcript abundance was always significantly lower than those between 0 and 24 h starvation (Fig. 7).

Trypsin mRNA levels in the intermolt and postmolt stages were close, as during early and late premolt. The importance of this finding will be discussed later. It is also noteworthy that the concentrations of trypsin mRNA during intermolt and early premolt stages were not significantly different, nor were they during postmolt and late premolt.

It is remarkable that trypsin mRNA levels tended to be different than that of β-actin or 16S rRNA throughout the molting stages (Fig. 8). During early premolt both β-actin and 16S levels decreased significantly, while trypsin mRNA levels increased. Three-way ANOVA showed that there was no interaction among genes, starvation periods, or molting stages (data not shown). However, it is interesting that trypsin mRNA levels were noticeably different than those of actin and 16S. In fact, trypsin, actin, and 16S show similar patterns through starvation period, with a striking increment in trypsin mRNA after 24 h of starvation, and a sharp fall after 24–72 h (Fig. 9). Similarly, when comparing trypsin mRNA levels with actin and 16S through molting stages, completely different patterns were observed. As seen in Fig. 10, while control genes showed similar patterns, trypsin mRNA differed: actin and 16S levels decreased all through postmolt (A) to early

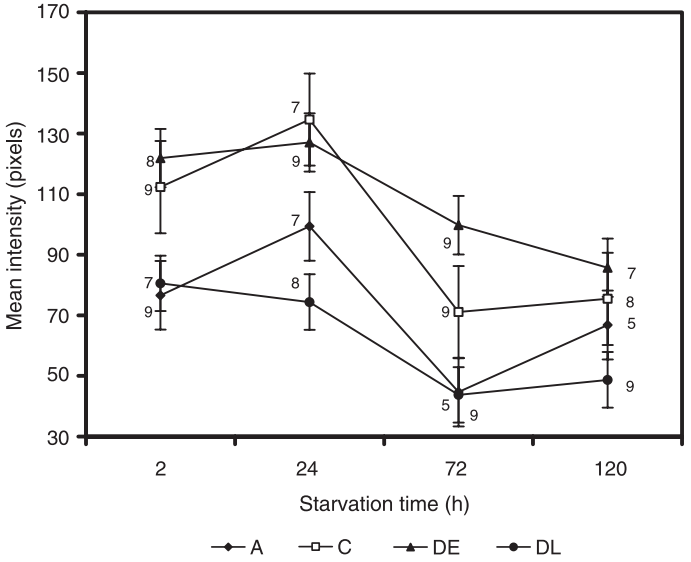

Fig. 7. Trypsin mRNA concentration in midgut gland during starving and molting. Trypsin mRNA concentration is expressed as mean intensities of 126 organisms obtained from individual whole midgut gland homogenates. For post hoc analysis, Tukey honest significant difference (HSD) test was used. Values are means ± 95% confidence intervals. Sample size for each mean is indicated by a number above or below bars.

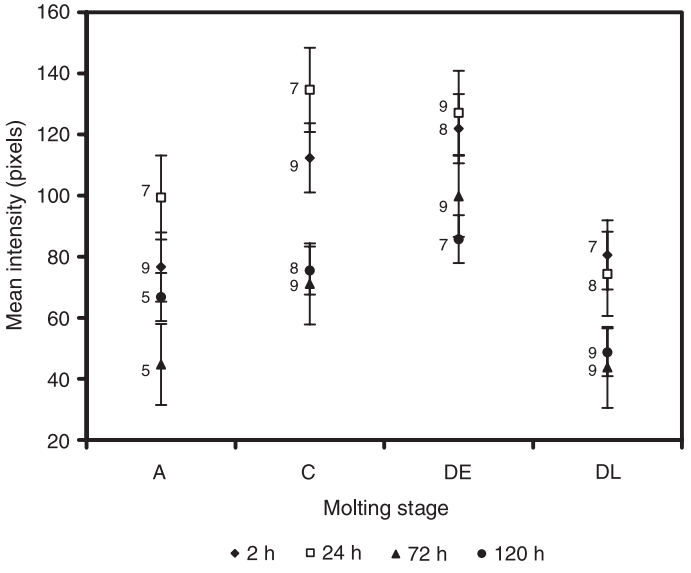

Fig. 8. Trypsin mRNA concentration during molting. RNA samples were obtained from individual whole midgut gland homogenates. For post hoc analysis, Tukey honest significant difference (HSD) test was used. Values are means ± 95% confidence intervals. Sample size for each mean is indicated by a number above or below bars.

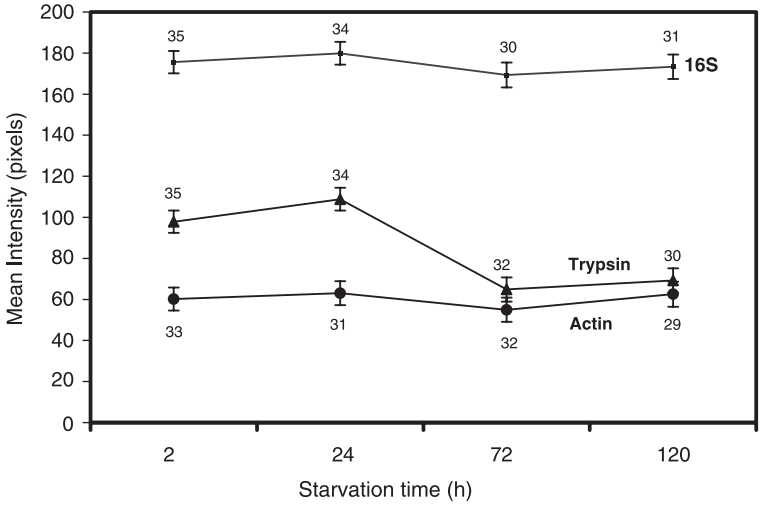

Fig. 9. Trypsin and actin mRNA, and 16S rRNA levels during different starvation periods. RNA samples were obtained from individual whole midgut gland homogenates. RNA levels were quantified by densitometry of dot intensity. Error bars indicate 95% confidence intervals about mean values for each starvation period. Sample size for each mean is indicated by a number above or below bars.

premolt (DE), while trypsin mRNA levels increased notably; after that, actin mRNA and 16S rRNA increased slightly until late premolt. Trypsin, in contrast, displayed an abrupt decrease in mRNA levels.

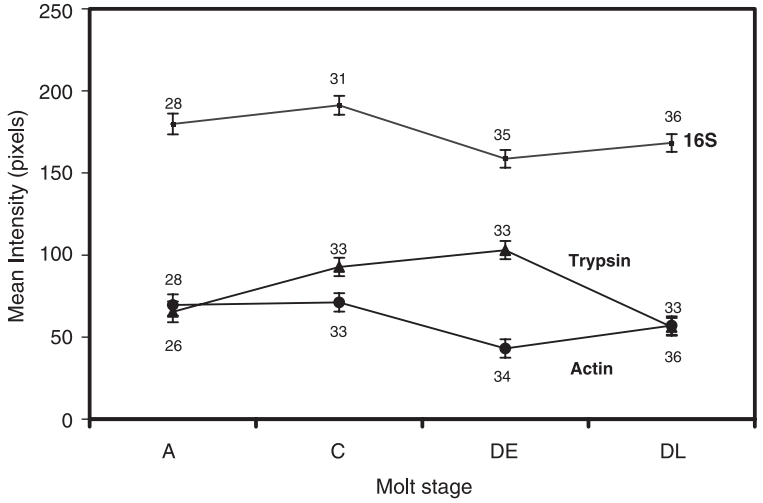

Fig. 10. Trypsin and actin mRNA, and 16S rRNA levels in molting stages of *Penaeus vannamei*. RNA samples were obtained from individual whole midgut gland homogenates. RNA levels were quantified by densitometry of dot intensity. Error bars indicate 95% confidence intervals about mean values for each starvation period. Sample size for each mean is indicated by a number above or below bars.

4. Discussion

The midgut gland in decapods is a vital organ involved in diverse metabolic activities, one of which is the synthesis and secretion of digestive enzymes, and absorption of nutrients. It is also involved in excretion, molting, and lipid and carbohydrate metabolism. Most of these functions are controlled neuroendocrinally (Gibson and Barker, 1979). However, the digestive gland is a site of lipid storage, which appears to be used mainly to obtain energy during starvation prior to molting (Barclay et al., 1983). This explains the midgut gland weight loss of 27% after 120 h of starvation, as a consequence of use and mobilization of reserves. Similar results were obtained in *P. japonicus* (Cuzon et al., 1980), in which a decline of about 50% in hepatosomatic index was observed in specimens starved during 28 days. The same effect was described in the shrimp *Metapenaeus ensis* (Cuzon et al., 1980), where the hepatosomatic index after 4 days of starvation was less than 50% of the corresponding value in daily fed shrimp. In starved *P. japonicus*, the use of reserves follows the sequence: carbohydrates, lipids, and protein (Cuzon et al., 1980). It has been suggested that the lack of variation in the concentration of protein in the hepatopancreas of *M. ensis* during starvation might be the result of the metabolism of protein, the major constituent of the organ, so that a decrease in protein concentration would be accompanied by a decrease in the organ weight, and therefore, a change in protein concentration might not be observed (Leung et al., 1990). Consequently, the type of biochemical components metabolized preferentially in starved decapods appears to be linked to the extent of starvation and might also be species specific, and possibly related to the feeding habits of the animal (Leung et al., 1990).

It is accepted that the transcription rate of housekeeping genes, or those expressed in a constitutive manner, such as β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cytochrome *c* oxidase (COX), is constant. However, several authors have reported that β-actin gene transcription can be altered significantly under conditions of alimentary stress. Food-deprived rats exhibited reduced rate of β-actin mRNA transcription than nourished rats (Yamada et al., 1997), whereas pathological conditions, such as food deprivation, appear to alter the expression of this gene (Graf et al., 2000). Some reports showed that the relative concentrations of β-actin mRNA in 12- and 24-month-old Fischer rats were diminished by 23% and 37%, respectively, when compared with 6-month-old animals (Moshier et al., 1993). Our current data show that β-actin mRNA expression might vary as a consequence of molting. This observation is consistent with results described in a study of the regulation of muscle gene expression in the American lobster (*Homarus americanus*), during a molt cycle (El Haj et al., 1992), in which an elevated expression of this gene was found in claw and leg muscle during premolt and postmolt. However, varying patterns of expression were found at specific stages of premolt and postmolt. In contrast with these results, we found the lowest actin transcript concentration during premolt. Two likely explanations for this are: (a) β-actin gene expression during the molt cycle follows a species-specific pattern, or (b) differences in activities of ecdysteroid hormone receptors depend on the organism. It has been suggested that differential regulation of protein synthesis is related to ecdysteroid receptor activity (El Haj et al., 1994). There is evidence of a 2-fold increase of protein synthesis in midgut gland and dermis of the land crab (*Gecarcinus lateralis*) in response to the application of

20-hydroxyecdysone (20-HE) hormone (Paulson and Skinner, 1991). In contrast, the crayfish (*Orconectes virili*) hepatopancreas gene expression was not stimulated by treatment with 20-HE (Gorell and Gilbert, 1971), even though this steroid hormone has been reported to be a stimulant in the synthesis of mRNA and protein (Skinner, 1965).

In the present study, we focused on alterations in trypsin mRNA levels in starved molting organisms. Because of the lack of information concerning expression of β-actin in *P. vannamei*, we assumed that it could be used as an internal control, since β-actin genes generally are considered constitutive (Selvey et al., 2001; Schmittgen and Zakrajsek, 2000). However, when results proved us wrong, we decided to evaluate 16S rRNA, by extrapolating to vertebrates, since we found no report on regulation of this gene in arthropods.

When compared with RNA levels of β-actin and trypsin, 16S rRNA in all cases was more abundant. This is explained by either (a) rRNA constitutes the largest fraction of RNA in the cell (up to approximately 80%), or (b) animal mitochondria are essential energy-producing organelles. It is well known that the number of mitochondria in different organs depends on its function, so in an organ with high metabolic activity, such as liver or midgut gland, the number of this organelles is elevated. Thus, we can explain that the signal intensity of 16S rRNA blots was so high.

Evaluation of 16S rRNA during the time course of starvation provided some interesting observations. The 16S rRNA levels varied due to starvation and molting. Kramer and Singleton (1992) reported that the marine bacteria *Vibrio* spp., retained between 10% and 26% of the original 16S rRNA content after 15 days of starvation. However, no report of eucariots was found. An explanation for such fluctuation is that 16S rRNA is regulated hormonally. This regulation might not act directly, but through some intermediate factor affecting nucleo-mitochondrial communication. Mitochondria biogenesis requires expression and duplication of the mitochondrial DNA (mtDNA) genome, and relies heavily on the nuclear genome, which provides all protein components required for this process and also those involved in their replication (Ruiz de Mena et al., 2000). We assume that 16S rRNA levels dropped as a consequence of the midgut gland weight decrease due to starvation, which might lower the metabolic activity or the number of cells in the organ, similarly to the effect of alimentary stress on cell density has been reported by Lhoste et al. (1993), who fed pigs with 7% protein inducing a marked reduction in the number of pancreatic cells.

The present work is the first report of variation in 16S rRNA levels through starvation and molt in a crustacean. Undoubtedly, the causes for this variation need further investigation and its use as an internal control need be achieved carefully. That 16S rRNA changed less dramatically than β-actin mRNA makes it more suitable as internal control, mainly because finding a gene that expresses in a strictly constitutive way has proven difficult (Siebert, 1999). Schmittgen and Zakrajsek (2000) suggested that genes used as internal controls need to be validated properly when designing quantitative gene expression studies.

This study did not intend to evaluate the amount of protein (β-actin and trypsin), simply because quantification of active trypsin in shrimp midgut gland is complicated by the fact that the enzyme is synthesized as trypsinogen, which in turn could activate spontaneously during tissue homogenization, so that the amount evaluated of trypsin could not be

distinguished from the enzyme that is activated and secreted for food digestion by natural causes. Accordingly, protein or activity quantification might not necessarily reflect the amount of mRNA translated into protein. However, although mRNA gives some idea about the ''potential'' for synthesis of a particular gene product, there is no direct relationship between mRNA and the amount of protein eventually produced. In fact, some studies suggest that certain amount of mRNA is never translated into protein because the translational efficiency of eukaryotic mRNAs varies considerably, depending on sequence characteristics (for review, see Kozak, 1994; Gallie, 1996; Pain, 1996). In this way, it is necessary to underline the fact that the observed changes in mRNA concentration might or might not result in physiologically relevant changes in enzyme concentration.

The effects of starvation and molting on trypsin mRNA are notable. The increase in trypsin mRNA abundance after 24 h of starvation and its subsequent decline was demonstrated. More outstanding is the finding of changes in the steady-state content of trypsin mRNA through molting. The highest level of trypsin transcript was found in shrimp at intermolt and early premolt, while the lowest concentration was found during early premolt and postmolt. These results are consistent with the series of events taking place throughout molting in penaeids. At postmolt, when the exoskeleton is soft and limp, the organism is incapable to intake food. Feeding does not begin until the organism is well into stage B, in which the exoskeleton is still soft but rigid enough to support the weight of the animal and handle food. During intermolt, when the exoskeleton reaches maximum rigidity, the organism feeds actively. Prior to molting, feeding declines during early and late premolt as a preparation for ecdysis, in which the old cuticle is shed. In this way, the higher concentration of trypsin mRNA during intermolt and early premolt, and the lowering of the transcript level during late premolt and postmolt stages, are responses of the organism to feeding habits constrained strongly by molt cycle.

It is notable that the trypsin mRNA content drops sharply after 24 h of starvation, with a slight rise occurring near the end of the starvation period. This might be a response to the acclimatization strategy in which organisms were fed at certain times each day, in a way that they prepare themselves for food digestion. Even more, the decrease of transcript concentration after 24 h of starvation could be a strategy to reduce enzyme synthesis, and consequently, the energy required for protein synthesis, thereby, preventing autolysis. Our results suggest a mechanism of trypsin synthesis regulation during transcription by external (starvation) and internal (molt cycle) factors. Additionally, the fact that trypsin mRNA reaches its lowest level after 72 h of starvation might be an indicator of trypsin mRNA turnover rate, which could prove to be an effective strategy for handling the effects of medium- to long-term starvation.

The different patterns of trypsin and actin mRNA and 16S rRNA levels are notable. Even when RNA levels of the three genes fluctuate equally during starvation (Fig. 9), trypsin shows an abrupt increase in mRNA level after 24 h of starvation, while those changes are more gradual in actin and 16S. On the contrary, when considering the effect of molting stage on mRNA concentrations, the different patterns of trypsin and control genes are obvious (Fig. 10). This might be a strategy to deal with starvation periods due to molting process. Differences in mRNA levels of trypsin, and actin and 16S during starvation and molting stages indicate that this is not due to general transcription changes but a strategy to deal with the harmful effect of starvation.

It must be remarked that statistical demonstration of the effects of starvation on trypsin mRNA concentration would not be possible due to the highly variable individual behavior exhibited by the obtained samples. Only through the use of a large number of samples and replicates could a significant correlation between starvation and expression concentrations of this gene become perceptible.

In the study of the biology of any organism, adaptation must be a dominant topic. This concept was defined as the modification of characteristics of the organisms that facilitates an enhanced ability to survive and reproduce in a particular environment (Hochachka and Somero, 1984). White shrimp possess biochemical adaptations to feeding habits that depend strongly on the physiology of the molt cycle thereby avoiding self-damage by diminishing the amount of enzyme-producing transcripts that can affect cellular and tissue organization.

In conclusion, we believe that the white shrimp possesses adaptation mechanisms to cope with short- and medium-term changes. When dealing with starvation, a response might be regulated during transcription. For many functions, control at the first step of gene expression is paramount (Calkhoven and Ab, 1996). Furthermore, the fast increase and decrease in trypsin mRNA indicates that, besides regulation during transcription, there is a form of post-transcriptional regulation established by stability of mRNA. Future studies on this subject might provide clues as to the regulation features enabling this organism to deal with starvation.

Acknowledgements

Authors thank the financial support from the Consejo Nacional de Ciencia y Tecnología (CONACyT, Mexico): grant 28257-B given to FLGC and a scholarship to ASP, the financial support from CIBNOR for project funds (PAC 16), Tec. Ma. Navarrete del Toro for technical assistance, CIBNOR editing staff, and Drs. Julio Cordova and Carmen Blázquez for their appreciable help with statistical analysis. **[SS]**

References

Barclay, M.C., Dall, W., Smith, D.M., 1983. Changes in lipid and protein during starvation and the moulting cycle in the tiger prawn, *Penaeus esculentus* Haswell. J. Exp. Mar. Biol. Ecol. 68, 229–244.

Calkhoven, C.F., Ab, G., 1996. Multiple steps in the regulation of transcription-factor level and activity. Biochem. J. 317, 329–342.

Chan, S.-M., Rankin, S.M., Keeley, L.L., 1988. Characterization of the molt stages in *Penaeus vannamei*: setogenesis and hemolymph levels of total protein, ecdysteroids and glucose. Biol. Bull. 175, 185–192.

Chung, J.S., Dircksen, H., Webster, S.G., 1999. A remarkable, precisely timed release of hyperglycemic hormone from endocrine cells in the gut is associated with ecdysis in the crab *Carcinus maenas*. Proc. Natl. Acad. Sci. U. S. A. 23, 13103–13107.

Cuzon, G., Cahu, C., Aldrin, J.F., Messager, J.L., 1980. Starvation effect on metabolism of *Penaeus japonicus*. Proc. World Maric. Soc. 11, 410–423.

Dall, W., Hill, B.J., Rothlisberg, P.C., Sharples, D.J., 1990. Molting and growth. In: Blaxter, J.H.S., Southward, A.J. (Eds.), The Biology of the Penaeidae. Advances in Marine Biology, vol. 27. Academic Press, London, pp. 213–250.

Eisen, A.Z., Jeffrey, J.J., 1969. An extractable collagenase from crustacean hepatopancreas. Biochim. Biophys. Acta 191, 517–526.

El Haj, A.J., Harrison, P., Whiteley, N.M., 1992. Regulation of muscle gene expression in Crustacea over the moult cycle. In: El Haj, A.J. (Ed.), Molecular Biology of the Muscle. Soc. Exp. Biol. Sem. Series, vol. 46. Company of Biologists, Cambridge, UK, pp. 151–165.

El Haj, A.J., Harrison, P., Chang, E.S., 1994. Localization of ecdysteroid receptor immunoreactivity in eyestalk and muscle tissue of the American lobster, *Homarus americanus*. J. Exp. Zool. 270, 343–349.

Furie, B., Furie, B., 1988. The molecular basis for blood coagulation. Cell 53, 505–518.

Galgani, M.L., Benyamin, Y., Ceccaldi, H.J., 1985a. Identification of digestive proteinases of *Penaeus kerathurus* (Forskal): a comparison with *Penaeus japonicus*. Comp. Biochem. Physiol. 78B, 355–361.

Galgani, F., Benyamin, Y., Van Wormhoudt, A., 1985b. Purification, properties and immunoassay of trypsin from *Penaeus japonicus*. Comp. Biochem. Physiol. 81B, 447–452.

Gallie, D.R., 1996. Translational control of cellular and viral mRNAs. Plant Mol. Biol. 32, 145–158.

García-Carreño, F.L., Carrillo, O., Navarrete del Toro, M.A., 1998. Control of digestive functions in shrimp: I. An inhibitor of trypsin activity in the hepatopancreas. Proc. Fourth Int. Crust. Cong., Amsterdam, Netherlands.

Gibson, R., Barker, P.L., 1979. The decapod hepatopancreas. Oceanogr. Mar. Biol. Annu. Rev. 17, 285–346.

Gorell, T.A., Gilbert, L.I., 1971. Protein and RNA synthesis in the premolt crayfish *Orconectes virilis*. Z. Vgl. Physiol. 73, 345–356.

Graf, R., Valeri, F., Gassmann, R., Hailemariam, S., Frick, T.W., Bimmler, D., 2000. Adaptative response of the rat pancreas to dietary substrates: parallel regulation of trypsinogen and pancreatic secretory trypsin inhibitor. Pancreas 21, 181–190.

Hochachka, P.W., Somero, G.N., 1984. Biochemical adaptation: basic mechanisms and strategies. Biochemical Adaptation. Princeton Univ. Press, New Jersey, pp. 537.

Kato, G.J., 1999. Human genetic diseases of proteolysis. Human Mutat. 13, 87–98.

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.

Klein, B., Sellos, D., Van Wormhoudt, A., 1998. Genomic organization and polymorphism of a crustacean trypsin multi-gene family. Gene 216, 123–129.

Kozak, M., 1994. Determinants of translational fidelity and efficiency in vertebrate mRNAs. Biochimie 9, 815–821.

Kramer, J.G., Singleton, F.L., 1992. Variations in rRNA content of marine *Vibrio* spp., during starvation–survival and recovery. Appl. Environ. Microbiol. 58, 201–207.

Lee, P.G., Smith, L.L., Lawrence, A.L., 1984. Digestive protease of *Penaeus vannamei* Boone: relationship between enzyme activity, size and diet. Aquaculture 42, 225–239.

Leung, K.M., Chen, H.L., Chu, K.H., 1990. Effects of starvation on biochemical composition and digestive enzyme activities in the hepatopancreas of the shrimp *Metapenaeus ensis*. In: Hirano, R., Hanyu, I. (Eds.), The Second Asian Fisheries Forum. Asian Fish. Soc., Manila, Philippines, pp. 445–448.

Lhoste, E.F., Fiszlewicz, M., Gueugneau, A.M., Wicker-Planckart, C., Puigserver, A., Corring, T., 1993. Effects of dietary proteins on some pancreatic mRNAs encoding digestive enzymes in the pig. J. Nutr. Biochem 4, 143–152.

Lu, B., Gerard, N., Kolakowski, L., Finco, O., Carrol, M., Gerard, C., 1996. Neural endopeptidase modulates septic shock. Ann. N. Y. Acad. Sci. 780, 156–163.

Moshier, J.A., Cornell, T., Majumdar, A.P., 1993. Expression of protease genes in the gastric mucosa during aging. Exp. Gerontol. 28, 249–258.

Neurath, H., 1984. Evolution of proteolytic enzymes. Science 224, 350–357.

Neurath, H., 1986. The versatility of proteolytic enzymes. J. Cell. Biochem. 32, 35–49.

Neurath, H., 1989. Proteolytic processing and physiological regulation. TIBS 7, 217–268.

Noriega, F.G., Shah, D.K., Wells, M.A., 1997. Juvenile hormone controls early trypsin gene transcription in the midgut of *Aedes aegypti*. Insect Mol. Biol. 1, 63–66.

Noriega, F.G., Wells, M.A., 1999. A molecular view of trypsin synthesis in the midgut of *Aedes aegypti*. J. Insect Physiol. 45, 613–620.

Pain, V., 1996. Initiation of protein synthesis in eukaryotic cells. Eur. J. Biochem. 236, 747–771.

Paulson, C.R., Skinner, D.M., 1991. Effects of 20-hydroxyecdysone on protein synthesis in tissues of the land crab *Gecarcinus lateralis*. J. Exp. Zool. 257, 70–79.

Pearson, W.R., Lipman, D.J., 1988. Improved tools for biological sequence analysis. Proc. Natl. Acad. Sci. U. S. A. 85, 2444–2448.

Ruiz de Mena, I., Lefai, E., Garesse, R., Kaguni, L.S., 2000. Regulation of mitochondrial single-stranded DNA-binding protein gene expressión links nuclear and mitochondrial DNA replication in *Drosophila*. J. Biol. Chem. 275, 13628–13636.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Schmittgen, T.D., Zakrajsek, B.A., 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J. Biochem. Biophys. Methods 46, 69–81.

Selvey, S., Thompson, E.W., Matthaei, K., Lea, R.A., Irving, M.G., Griffiths, L.R., 2001. Beta actin—an unsuitable internal control for RT-PCR. Mol. Cell. Probes 5, 307–311.

Siebert, P.D., 1999. Quantitative RT-PCR. In: Kochanowski, B., Reischi, U. (Eds.), Methods in Molecular Medicine, vol. 26. Humana Press, NJ, USA.

Skinner, D.M., 1965. Amino acid incorporation into protein during the molt cycle of the land crab *Gecarcinus lateralis*. J. Exp. Biol. 160, 225–233.

Tan, S.H., Degnan, B.M., Lehnert, S., 2000. The *Penaeus monodon* chitinase I gene is differentially expressed in the hepatopancreas during the molt cycle. Mar. Biotechnol. 2, 126–135.

van Wormhoudt, A., 1973. Variations des proteases, des amylases et des protéines solubles au cours de dévelopement larvaire chez *Palemon serratus*. Mar. Biol. 19, 245–248.

van Wormhoudt, A., Favrel, P., 1988. Electrophoretic characterization of *Palaemon elegans* (Crustacea Decapoda) aamylase system: study of amylase polymorphism during the intermoult cycle. Comp. Biochem. Physiol. 89B, 201–207.

Vogelstein, B., Gillespie, D., 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. U. S. A. 76, 615–619.

Whitaker, J.R., 1994. Principles of Enzymology for the Food Sciences, 2nd ed. Marcel Dekker, New York.

Yamada, H., Chen, D., Monstein, H.J., Hakanson, R., 1997. Effect of fasting on the expression of gastrin, cholecystokinin, and somatostatin genes and of various housekeeping genes in the pancreas and upper digestive tract of rats. Biochem. Biophys. Res. Commun. 231, 835–838.

Zimmerman, M., Mumford, R.A., Steiner, D.F., 1980. Precursor processing in the biosynthesis of proteins. Ann. N. Y. Acad. Sci. 343, 156–163.