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Digestive lipase activity through development and after fasting and re-feeding in the whiteleg shrimp *Penaeus vannamei*

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ABSTRACT

Lipase activity of the midgut gland during larval and postlarval stages of *Penaeus vannamei* was assayed to determine its capability to digest lipids from feed and involvement in digesting lipids from reserves during fasting conditions. Lipase activity was detected at all larval stages, increasing from nauplii to protozoea. Lipase isoenzymes at larval stages were evaluated by SDS-PAGE using 4-methylumbelliferone butyrate as the substrate. Results showed that shrimp larvae possess a nearly complete set of lipases starting with the first larval stage. In addition, to understand the effects of fasting conditions as a stress factor on lipase activity, intermolt shrimp were fasted up to 5 days, a period corresponding to the normal time that shrimp starve during molting, in which they cannot eat. Digestive lipases were affected by fasting, increasing in activity after 24 h of treatment, suggesting that lipid is used as an energy reserve during fasting. Proteins with lipase activity were identified and characterized by zymograms; the presence of more than one lipase enzyme could be one way to hydrolyze triacylglycerides more efficiently as the first step of fat assimilation and to obtain energy from fatty acids under fasting conditions.

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1. Introduction

Whiteleg shrimp *Litopenaeus vannamei* (=*Penaeus vannamei*) is widely farmed (FAO, 2007). Efforts to reduce costs by modifications of feed formulations have generated a strong interest to understand this species' nutritional needs, such as preferences in energy sources, carbohydrates, lipids, and proteins.

The knowledge of digestion of lipids in whiteleg shrimp by digestive lipases over their life cycle is limited to a few studies, although the topic is important in commercial production and from ecological viewpoints. Penaeid species produce large numbers of eggs that hatch into nauplii beginning with a lecithotrophic stage, but becoming phytotrophic within days. Information on the natural food of larvae is scarce, but larvae of many species have been successfully reared in laboratories to prey on *Artemia*. After passing through several planktonic larval stages, shrimp metamorphose into postlarvae stages and migrate shoreward to lagoons and protected nursery areas (Brito et al., 2001). Development of digestive lipase activities in *Procambarus clarkii* (Ying et al., 2009), *Macrobrachium borelli* (González-Baró et al., 2000), *Penaeus monodon* (Fang and Lee, 1992), and *Penaeus setiferus* (Lovett and Felder, 1990) have been studied. Many other studies have shown that digestive enzymes in Crustacea vary with the developmental stage and there is little information on the causes of these variations and the conditions that regulate lipases in crustaceans.

Additional information on the physiological response of lipase variation and regulation mechanism can be obtained from the study of fasting, which takes place during molting (Dall et al., 1990). The midgut gland, besides its role in digestion, participates in molting as the major lipid storage organ, mainly storing triacylglycerides (TAG), which are the main source of energy during fasting or increased energy demands (Birnbaum, 2003). Variations in lipase activity in crustaceans have been studied during fasting in lobsters, such as *Jasus edwarsii* (Johnston et al., 2004); other studies on lipase activity showed that oligotrophic and eutrophic pond water induce changes in lipase activity in *L. vannamei* (Moss et al., 2001), as does the use of probiotics in feeds for shrimp farming (Yan-Bo, 2007), but these results are inconclusive since lipase activity is commonly confused with esterase activity when using unspecific substrates.

Lipase present in the digestive tract has been studied in lobster *Panulirus argus* (Perera et al., 2008), crab *Carcinus mediterraneus* (Slim et al., 2007), and shrimp, such as *M. borelli* (González-Baró et al., 2000), *P. monodon* (Deering et al., 1996) and *P. setiferus* (Lovett and Felder, 1990). These studies mainly covered biochemical aspects of lipases. Our objective was to determine and characterize the activity of digestive lipases at different larval stages and during fasting in *L. vannamei* as part of their digestive physiology and nutritional needs during development.

2. Materials and methods

2.1. Animals and experimental conditions

A shipment of one million nauplii was transported from a commercial hatchery to the laboratory and kept in three 1000-l plastic

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tanks maintained at 28 °C, pH 7.5 to 8.2, salinity of 33‰, 12:12 h photoperiod, and constant aeration for the experiment. During nauplii (N) stages, larvae were unfed because differentiation of the digestive tract is not complete. At the protozoea (PZ) stage, larvae were fed *Chaetoceros calcitrans* and *Chaetoceros gracilis* algae. At the mysis (M) and postlarval (PL) stages, they were fed *Artemia franciscana* to satiation. Larval stages were identified according to Martínez-Córdova (1993). Larvae pools (0.3 g wet weight) were sampled randomly at each stage when 80% of the population had reached that stage and used for assays.

A second experiment was designed to measure the lipase activity from midgut gland at different fasting times in adult specimens. Here, 350 shrimps (23.0 ± 1.0 g each) were maintained under controlled conditions (28 °C, 34‰ salinity, 7.4 mg l^{-1} dissolved oxygen). During a 7-day acclimatization period, specimens were kept in two 1000-l tanks containing 175 specimens each. Daily exchange of filtered marine water was 70% of the total volume. Specimens were fed twice daily with commercial feed containing 35% protein. Feces and uneaten feed were discarded before the next feeding. At the end of the period of acclimation, one group served as a control and the other as the fasted group. Three sample sets of both groups were assayed for lipase activity at 2, 4, 8, 12, 24, 48, 72, 96, and 120 h and an additional three sample sets were fed after fasting for 96 h and then assayed at 120 h. Three specimens of each replicate were selected by setogenesis (Chan et al., 1988) at intermolt C. In our study all C substages were considered as intermolt. Shrimps were individually weighed, and then decapitated. The midgut gland was dissected and processed in the laboratory for lipase assays.

2.2. Assay of enzyme activity

Samples (0.3 g wet weight) of whole larvae at each stage were homogenized in ice-cold distilled water. Homogenates were centrifuged at 10,000 × *g* for 30 min at 4 °C, and the supernatant, which contained the soluble lipase protein (enzyme extract), was then stored at −20 °C until further analysis. For adult extracts, individual midgut gland extracts were also assayed in three replicates. The protein concentration in the supernatant solutions was assayed by the Bradford method (Bradford, 1976) and compared with a standard, bovine serum albumin (Sigma, B-4287, St. Louis, MO).

Lipase activity was measured using β-naphthyl caprylate in DMSO as the substrate (Versaw et al., 1989). The assay mixture modified for microplate assay contained: 30 µl 100 mM sodium taurocholate (Sigma, T4009), 570 µl 50 mM Tris·HCl at pH 8, 6 µl of the enzyme extract, and 6 µl 200 mM β-naphthyl caprylate (Sigma, N8875). The reaction mixture was incubated for 30 min at 25 °C for the reaction to proceed and then 6 µl of 100 mM fast blue BB dissolved in DMSO (Sigma, 917A) was added. The reaction was stopped with 60 µl of 0.72 N trichloroacetic acid. Finally, 813 µl of 1:1 (v/v) ethyl acetate/ethanol solution was added and absorbance was recorded at 540 nm in a microplate spectrophotometer (Versamax, Molecular Device, Sunnyvale, CA). Pancreatic lipase Type II (Sigma, L3126) was used as the control. Lipase activity was expressed as U mg^{-1} lipase protein, where one lipase unit is the quantity of enzyme required to increase absorbance by 0.01 U at 540 nm/min.

A second assay for lipase activity was done on adult specimens; the release of fatty acids by lipid hydrolysis was measured titrimetrically at pH 8 and 30 °C with pH-Stat (Metrohm, 718 STAT Titrino, Riverview, FL) using tributyrin (C4:0; Sigma, 8628), emulsified in 10 ml 0.5% $CaCl_2 \cdot 2H_2O$ (w/v) and 2% (w/v) Arabic gum (Sigma, G9752) as substrate and mixed mechanically for 7 min in a commercial blender (Minning et al., 1998). The free fatty acids were titrated with 0.01 N NaOH. Lipase activity was calculated as:

$$U/L = (T/t_T - B/t_B) \times 0.1 \times 10^3,$$

where U is free fatty acids (in micromoles per min), L is liters, T and B are 0.01 N NaOH volumes (in µl); t_T and t_B are reaction time (in min) for the assay and blank, respectively.

2.3. SDS-PAGE and zymograms

Composition of proteins in the enzyme extracts (larvae and adult) was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gels according to Laemmli (1970). Enzyme extracts, 0.01 mg of protein, of each individual extract (one volume of sample with one volume of loading buffer) were loaded per lane, in a temperature controlled (4 °C) electrophoresis device. Molecular weight standards (4 µl) were included in each plate. Electrophoresis was done on a constant current of 15 mA per gel. After electrophoresis, gels were stained for protein with a solution containing 40% ethanol, 7% acetic acid, and 0.05% Coomassie brilliant blue R-250. After 24 h of staining, gels were de-stained with the same solution without Coomassie dye.

Substrate SDS-PAGE (12% polyacrylamide gels) was used to examine the composition of lipases in larvae and adult specimens using 4-methylumbelliferone butyrate (MUF-butyrate) and β-naphthyl acetate as the substrates. Enzyme extracts, 0.01 mg of protein, were neither boiled nor treated with mercaptoethanol before loading into the gel. After separation of proteins, gels were soaked for 30 min in 2.5% Triton X-100® at room temperature, washed in 50 mM Tris–HCl buffer, pH 8.0, and covered by 100 µM MUF-butyrate solution (19362, Fluka, St. Louis, MO) in the same buffer (Prim et al., 2003). Activity bands became visible in a short time after exposure to UV.

For preparing the zymograms with β-naphthyl acetate as the substrate, after separation of lipase proteins, the gel was soaked for 10 min at room temperature in a solution of 25 ml 50 mM Tris–HCl at pH 8.0, 2.5 ml acetone, and 10 mg of β-naphthyl acetate (N6875, Sigma). After incubation, the gel was rinsed with 50 mM Tris–HCl at pH 8.0, and then soaked for 10 min in a solution of 25 ml of 50 mM Tris–HCl at pH 8.0, Triton X-100®, and 25 mg of Fast red (44720, Fluka). Reddish bands indicated activity.

2.4. Statistical analysis

Results are presented as means of three samples. All data were tested for normal distribution and homogeneity of variance using the Anderson–Darling's and Bartlett's tests. Data from the different digestive enzyme activities for each stage were compared, using one-way ANOVA. Data that lacked homocedasticity (Bartlett's test) were analyzed using the Kruskal–Wallis one-way ANOVA on ranks test (Zar, 1999). Software (Statistica v. 6.0) performed the analyses. Differences were regarded as statistically significant at $P < 0.05$.

3. Results

3.1. Lipase activity during development

A clear variation in the specific activity of lipase was observed during the studied postlarvae. Lipase activity was first detected at the nauplii stage; during this stage, activity was lower than at later larval stages, which was related to the use of the yolk reserves. Highest activity appeared at stage PZ2–PZ3, when the mouth opened and feeding started. Activity significantly decreased from 3.65 ± 0.5 U mg^{-1} at stage PZ2 to 0.75 ± 0.02 U mg^{-1} at stage M3. Activity then increased to 1.85 ± 0.04 U mg^{-1} at stage PL1 (Fig. 1).

The zymogram showed activity bands at all larval stages (30–>100 kDa; Fig. 2). During the nauplii stage, the number of activity bands increased slightly, displaying six bands at stage N5, which is related to the use of yolk reserves (Zagalsky et al., 1990). The immature digestive system for feeding suggests that lipids from the yolk, at this stage, are the only source of energy. In contrast to the

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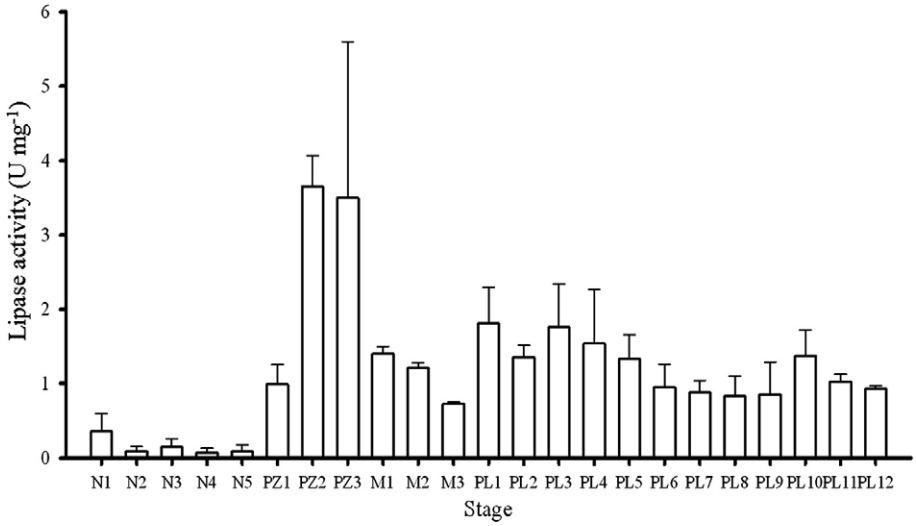

Fig. 1. Lipase activities of pooled larval samples during developmental stages of whiteleg shrimp *Penaeus vannamei*. Activity given as U mg^{-1}, the bars indicate standard error.

nauplii stage, lipase in protozoea, mysis, and postlarval stages showed distinct activity bands (48–100 kDa) and juveniles showed seven bands of lipase activity (Fig. 2).

3.2. Effect of fasting on lipases

Protein content in the midgut gland of fed versus fasted shrimp (Fig. 3) revealed a decline of protein content on fasted specimens. After 4 h of fasting, protein content decreased 23% and returned to the initial concentration after 8 h without food. The highest amount of protein was observed at 24 h for fed and fasted groups. When fasted shrimp were fed at 96 h, a recovery of protein content was evident at 120 h (14.08 ± 1.5 mg ml^{-1}).

In fasted shrimp, lipase activity, measured by β-naphthyl caprylate hydrolysis, was in the range of 1.10 ± 0.2 and 3.59 ± 0.9 U mg^{-1} in fasting specimens (Fig. 3). No significant differences were observed in the control group. In the experimental groups, after 24 h of fasting, lipase activity increased continuously, reaching a maximum activity at 120 h (3.59 ± 0.4 U mg^{-1}). In the experimental group, shrimp fed after fasting for 96 h had declining lipase activity, reaching the initial value of 1.44 ± 0.1 U mg^{-1}. Lipase activity of fasted group significantly exceeded that of fed group at almost all sampling intervals.

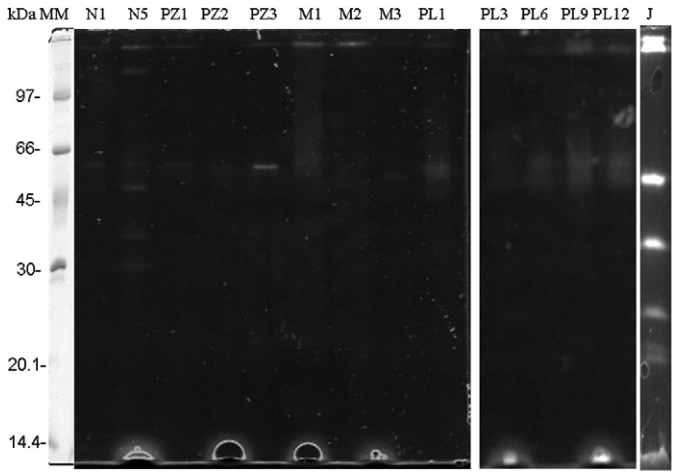

Fig. 2. Isoenzyme patterns of lipases at different developmental stages of *Penaeus vannamei*. MM = molecular marker; Stages: N = nauplii, PZ = protozoea, M = mysis, PL = postlarvae, and J = juvenile.

The effect of fasting (Fig. 4) was also observed when lipase activity was assayed with tributyrin as the substrate by the pH-stat method, showing an increase in activity at 120 h of fasting. A minimum value of 388 ± 133 U L^{-1} at 2 h was observed; it was statistically different from the maximum value of 2788 ± 46 U L^{-1} observed after 120 h of fasting. In the group that was fasted for 96 h and then fed, lipase activity decreased, showing no statistical difference from the fed group.

To find differences in the number of activity bands of fasted and fed shrimp, zymograms were prepared. Lipase zymograms using MUF-butyrate as the substrate show differences in the intensity of active bands between individual specimens (Fig. 5). The bands with lipase activity were identified in adult shrimps by MUF-butyrate substrate and β-naphthyl acetate zymogram (Fig. 6). In Fig. 6D, bands with lipase activity produced a fluorescent signal in which six bands were identified as lipases, while only five was observed on β-naphthyl acetate zymograms (Fig. 6C). The molecular mass of proteins with lipase activity was determined by R_f, where two bands of molecular mass higher than 220 and others with 151.5, 50.57, 36.5, 24.1 kDa were observed.

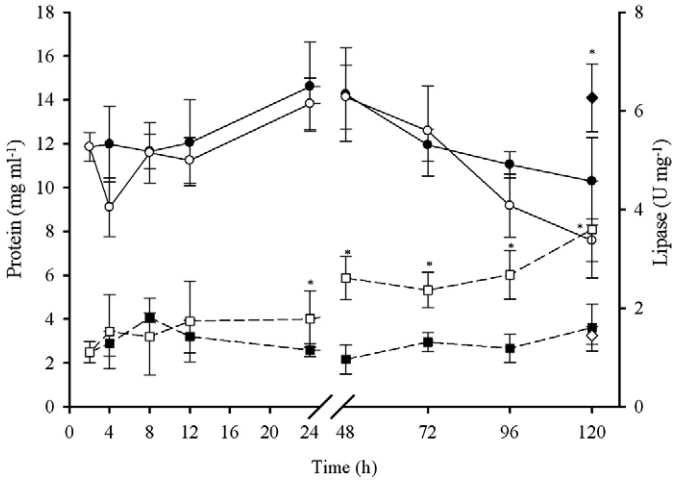

Fig. 3. Protein content and lipase activity using naphthyl caprylate as substrate of midgut gland of adult shrimps during starvation period (up to 120 h) and re-feeding (96 h). * = significant differences at ($P < 0.05$), protein content: ● = fed group, ○ = fasted group, ◆ = re-fed group; lipase activity: ■ = fed group, □ = fasted group, ◊ = re-fed group. Activity given as U mg^{-1}, the bars indicate standard error.

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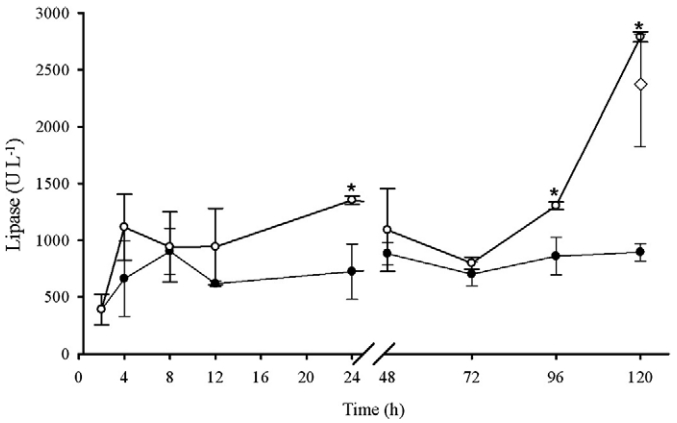

Fig. 4. Lipase activity using tributyrin as substrate of adult shrimps during starvation period (up to 120 h) and re-feeding (96 h). * = significant differences at $P<0.05$; ● = fed group, ○ = fasted group and ◊ = re-fed group. Activity given as U L^{-1}, the bars indicate standard error.

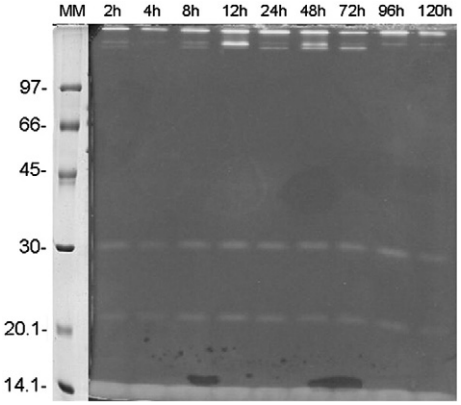

Fig. 5. Isoenzyme pattern of lipases of adult shrimps at different fasting periods. MM = molecular marker.

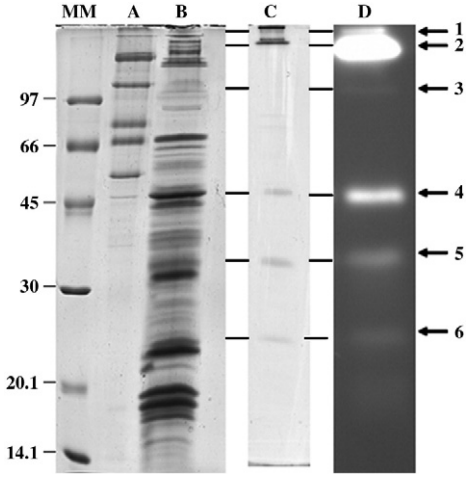

Fig. 6. Protein profile and zymograms of enzymatic extract from the midgut gland of adult shrimps. MM = molecular marker; A = high molecular marker; B = crude extract; C = zymogram of lipase using β-naphthyl acetate as substrate; D = zymogram of lipase using MUF-butyrate as substrate. Arrows indicate bands with lipase activity.

4. Discussion

4.1. Effect of development on lipases

In contrast with other malacostracan groups, which do not show nauplii stage (Scholtz, 2000), penaeid shrimps are ideal crustacean models to study sequential changes of digestive enzymes during development because all larval stages are free swimming rather than embryonated and metamorphosis to adult morphology and habits takes several weeks (González-Baró et al., 2000; Ying et al., 2009). In crustaceans, lipases have been detected (Deering et al., 1996; Gamboa-Delgado et al., 2003; Slim et al., 2007). They are required in many biochemical reactions, but differ in their primary structure, tissue specificity, and cellular compartmentalization. Lipases include digestive lipases, which facilitate extracellular cleavage of alimentary lipids (Vogt, 2002), while intracellular lipases are involved in the mobilization of triacylglycerides in adipose tissue and have a critical role in regulating fatty acid metabolism and energy supply (Viherväara and Puig, 2008). We found that all larval stages of *P. vannamei* showed a certain amount of lipolytic activity, with the lowest concentrations in the nauplii stage, which is the consequence of the poorly developed stage of the embryonic digestive system. Lipase activity, which is under the control of gene expression during the course of metamorphosis, reflects the digestion of the nutrients from yolk to supply energy to continue metamorphosis (Calado et al., 2007). The lipase zymogram confirmed that lipase activity is present at all nauplii stages, with different isoenzyme pattern among nauplii, and increasing lipase activity with development. As in vertebrates, lysosomal enzymes, proteinases and lipases are involved in yolk lipoprotein hydrolysis, with yolk containing phospholipids and neutral lipids, primarily triacylglycerol (Murray et al., 2003; Yeong Kwon et al., 2001). We found that the nauplii shrimp are physiologically prepared to metabolize endogenous yolk reserves, the main metabolic substrate for energy production, allowing larvae independence of external feeding before the mouth is open, as had been demonstrated in crabs, such as *Lithodes santolla* (Saborowski et al., 2006), *Armases angustipes* (Anger and Moreira, 2002), and *P. clarkii* (Ying et al., 2009). Several studies did not detect lipase activity in crustaceans at larval stages (Berner and Hammond, 1970; Lovett and Felder, 1990), and this can be attributed to the use of a nonspecific substrate (β-naphthyl acetate). The use of triacylglycerides and/or fluorogenic substrates overcomes this problem (Gupta et al., 2003).

The selection of phytoplankton or zooplankton as primary sources of food results in a divergent nutritional composition of larval diet. The mean value of energy content of lipids on phytoplankton is 5.7 J mg^{-1} while zooplankton is 16 J mg^{-1} (Watanabe et al., 1983). In penaeid protozoea, which are dependent on phytoplankton as food, they tend to have high metabolic rates combined with elevated energy turnover rates, e.g. high ingestion, digestive enzyme activity and feces production, compared to larger carnivorous larvae. This difference is reflected in the higher activity of digestive enzymes at the protozoea stage, mainly trypsin and chymotrypsin (Jones et al., 1993; Le Vay et al., 1993; Lemos et al., 1999) and lipase in this study. In spite of the major content of lipid found in zooplankton, lower values of lipase activity had been observed in mysis and postlarval stages. This could be related to the use of the main component of zooplankton, which is protein, because it was proposed that in crustaceans, the primary source of energy is protein (New, 1976). In addition, this indicates a modulation of lipase activity in response to dietary quality on larvae stages. Accordingly, isoenzyme patterns of lipases changed during development as shown in the lipase zymogram of larvae (Fig. 2). The significant increase in lipase activity reflects increasing digestive capacity with development that is required to meet the metabolic and structural requirements of the species.

4.2. Effect of starvation on lipases

Unsuitable food or acute starvation may constitute a particularly widespread and ecologically significant form of environmental stress. As a general response, resistance to fasting or starvation may confer adaptation to a range of marginal food conditions, including fluctuating

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availability of resources, as during molting. Resistance to fasting could be achieved by a reduction in metabolism and/or the use of stored energy compounds (carbohydrates, lipids, and proteins). The use of stored energy compounds has been documented in several species as a response to fasting and starvation conditions. For example, in some crustaceans the protein becomes the main energy source after 21 days of starvation and conserving the lipid reserves (Helland et al., 2003), while other crustaceans such as *J. edwarsii* use lipids before using protein reserves (Ritar et al., 2003).

The lipid reserves available in crustaceans for energy is small, with only 8% of body lipids stored as triacylglycerides in the midgut gland (Chandumpai et al., 1991). This could explain the lowest values of lipase activity in different animals, including shrimp, such as *P. setiferus* (Lovett and Felder, 1990). In crustaceans, neutral lipids are preferentially catabolized during fasting, while polar lipids are conserved to fulfill structural roles (Stuck et al., 1996). Catabolism of triacylglycerides achieved by lipases is scarcely studied in crustaceans, however, there is a variation of digestive enzymes, such as proteinases (trypsin and chymotrypsin) during starvation conditions in shrimp (Muhlia-Almazán and García Carreño, 2002), as in other invertebrate species, such as insects (Harshman and Schmid, 1998).

Nonspecific substrates can provide incorrect values of lipases activity or an association between lipase and esterase activities. Substrates in this study discriminated between lipase and esterase activities through fasting experimentation, suggesting that the results are very well addressed. On the other hand, zymograms were attempted to identify true lipases, allowing us to separate lipases from esterases. The measured lipolytic activities in the midgut gland of the digestive and intracellular lipases come from B cells (Loizzi and Peterson, 1971). Holocrine secretion in the midgut gland suggests that both enzymes are involved in food digestion. This topic remains to be addressed.

In whiteleg shrimp, lipase activity was significantly affected by starvation, increasing after 24 h and having the highest activity at 120 h (Figs. 3 and 4), which agrees with findings by Sánchez-Paz et al. (2007), where a decrease of 86% of the lipids in the midgut gland of whiteleg shrimp occurred during the same treatment period. This is an increase in the amount of lipolytic enzymes and a consequent decline in the triacylglyceride storage in the midgut gland because of starvation. The increase in lipase activity during fasting is opposite to the decreased amount of lipase activity that Johnston et al. (2004) reported in starving larvae of the spiny lobster *J. edwarsii*, in which specimens were starved for 44 days, as well as the activity reported by developmental growth in the same species (Perera et al., 2008). These observations suggest that lipase activity can be affected by food quality, developmental status, as well as by lack of food and the changes in lipase activity differ according to the crustacean species (Sanchez-Paz et al., 2006) and the length of time under starvation conditions.

In addition, the change in the amount of lipases where specimens were fasted and later fed offered insight in the response capacity of the species and the degree of reversibility in the changes after periods without food. In whiteleg shrimp, lipid levels in the midgut gland did not recover to values before the starvation period (Sánchez-Paz et al., 2007). Another crustacean, the isopod *Stenasellus virei* showed complete recovery of lipid levels after 180 days of starvation and 15 days of feeding, as well as the isopod *Asellus aquaticus,* that recovered all energy reserves after 28 days of starvation followed by 7 days of feeding (Hervant and Renault, 2002). Recovery of triacylglycerides concentration after fasting-feeding suggests a decrease in lipase activity since triacylglycerides are stored as a source of energy for other physiological purposes, as shown here for the whiteleg shrimp, recovering initial amount of lipase after fasting-feeding. It is clear, that the time necessary to restore energy reserves, as well as digestive capability, differs among species. Reduction of lipase activity in whiteleg shrimp to a base level suggests restoration of body reserves once triacylglycerides from food is available and accumulated for future physiological stress, as an adaptive response.

The difference in lipase activity observed during the ongoing fasting period and the variation in activity bands in the zymograms suggest an enzymatic adaptation mechanism in the midgut gland, leading it to secrete more or less enzymes for digestion of triacylglycerides from food or those stored as lipid droplets as energy reserves. This process showed that fasting is a natural stimulus that allows the shrimp to regulate rates of enzyme synthesis (Muhlia-Almazán and García Carreño, 2002).

In contrast to other studies on the effect of lack of feeding on lipase activity in crustaceans, this is the first evidence of changes in pattern of isoenzymes. Whiteleg shrimp have five bands with lipase activity, which may be present or absent, depending on the needs of the specimen during triacylglycerides hydrolysis. Although we observed changes in lipase activity (Figs. 3 and 4) and changes in the intensity of lipase bands of high molecular weight (>97 kDa), two bands with lipase activity (~22 kDa and ~32 kDa) were always present in midgut gland extracts (Fig. 5). However, when a pool of shrimps was used as the source of the crude extract to evaluate isolipases, six bands with lipase activity were identified (Fig. 6). The presence of multiple lipases in the midgut gland of the whiteleg shrimp probably provides a selective advantage for efficient hydrolysis of lipids from different sources.

5. Conclusions

In this work, we demonstrated that shrimp larval stages possess a set of digestive lipases which are stage-specific and contribute to the digestion of external nutrients. Fasting stimulates the digestive system and this affects the activity of lipases, mobilizing lipid reserves during starvation; however, the study did not discriminate among digestive lipases and intracellular lipases. There are still unanswered questions regarding lipid digestion. Among these are the role of intracellular lipases and digestive lipases and their specificity as well as their regulation during stress. Studies of starving shrimp, in combination with molecular tools, can help in understanding the mechanisms for regulating digestive enzyme activity.

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