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Digestive enzyme activity and food ingesta in juvenile shrimp *Litopenaeus vannamei* (Boone, 1931) as a function of body weight

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Abstract

A study was conducted to evaluate variations of digestive enzyme activities in *Litopenaeus vannamei* (Boone) reared in commercial ponds under semi-intensive conditions. Shrimp were collected at each body weight increase of 2 g. As the shrimp grew (2–12 g), significant increases in the activities of lipase and chymotrypsin were observed. The total protease activity decreased from 6 g onwards. Trypsin activity showed a peak at 6 g and amylase activity increased two-fold after 2 g. Additionally, the stomach contents were analysed microscopically for shrimp between 2 and 10 g. Plant matter contributed above 30% of the total stomach content in 6-, 8- and 10-g shrimp. Detritus represented 58% and 62% of the total stomach content in 2- and 4-g shrimp, respectively, decreasing to 33–43% at greater shrimp weights. Artificial feed showed a maximum contribution of 20% in 6-g shrimp. The present results show changes in the enzyme activity after the shrimp reach 6 g in body weight, evidenced by a decrease in total protease and an increase in lipase and amylase activities. The amylase/protease ratio was 2.6 in 2-g shrimp and increased steadily to 9.6 in 12-g shrimp. These findings suggest an adaptation of the enzymatic activity to diets with lower protein content as body weight increases, and may be related to the variation of the different items found in the stomach.

Keywords: shrimp, *Litopenaeus vannamei*, digestive enzymes, stomach content, semi-intensive culture

Introduction

Recent investigations on digestive processes in penaeid shrimp have focused on evaluating the ability of organisms to hydrolyse, absorb and assimilate the principal dietary nutrients (Guzman, Gaxiola, Rosas & Torre-Blanco 2001). In these investigations, the study of digestive enzymes is an essential step toward understanding the mechanism of digestion and a better knowledge of nutritional needs (Le Moullac, Klein, Sellos & Van Wormhoudt 1997). The interrelationships between the data derived from these analytical techniques and growth may assist in defining the overall diet quality for both younger and older crustaceans (Lee & Lawrence 1985).

Crustacean penaeids adapt quite well to changes in diet composition by the induction of digestive enzymes synthesized and secreted in the hepatopancreas (Le Moullac *et al.* 1997). These digestive enzymes hydrolyse a variety of substrates, and various factors are involved in their regulation. Among them are diet (Le Moullac *et al.* 1997; Guzman *et al.* 2001), ontogenic changes (Lovett & Felder 1990), body size (Lee & Lawrence 1985), circadian rhythms (Molina, Cadena & Orellana 2000), moulting stage (Molina *et al.* 2000) and even a stimulant effect from the pond water has been reported (Moss, Divakaran & Kim 2001). The larval and postlarval stages of penaeid shrimp go through a series of metamorphic changes that affect enzymatic activity (Lovett & Felder 1990). Nevertheless, changes in digestive enzyme activities are also observed in juveniles and

adults. It appears that these changes are related to growth and feed digestibility (Lee & Lawrence 1985). Changes in the digestive enzyme activity may indicate physiological responses to different nutritional conditions (Le Moullac *et al.* 1997), and it has been hypothesized that the enzyme activity is high for those substrates that are more common in the diet (Moss *et al.* 2001).

In semi-intensively managed culture ponds, a substantial contribution to the nutrition of shrimp comes from the naturally occurring biota (Hunter, Pruder & Wyban 1987; Nunes, Gesteira & Goddard 1997; Focken, Groth, Coloso & Becker 1998). Therefore, once the nutritional value of natural food has been considered, feed formulas and feeding schemes should be optimized to satisfy the nutritional requirements (Focken *et al.* 1998). In this context, a better understanding of feeding preferences and the use of feed by shrimp is essential to optimize the use of nutrients and to reduce environmental pollution that originates from metabolite excretion and from uneaten artificial feed. A net waste of nutrients due to excessive feeding also represents an economic loss for the aquaculturist, as feed is the main variable cost and can represent up to 60% of the total costs in penaeid shrimp culture (Akiyama, Dominy & Lawrence 1992). The aim of this study was to measure the digestive enzyme activity of *Litopenaeus vannamei* at different body weights, reared in a commercial pond. Additionally, observations on stomach content are reported.

Materials and methods

Sampling

Shrimp were collected from an 8-ha culture pond in a shrimp farm located in Palmar (Guayas Province, Ecuador), and from a 9-ha culture pond near one of the branches of the Guayas river. The 8-ha pond was close to the shore, operated semi-intensively (9–12 shrimp m^{-2}) and had an array of feeding trays used to distribute the feed. A slow water exchange was carried out in each spring tide. The water temperature was 25–27 °C and salinity was 33–35 g L^{-1}. In the 9-ha pond, the stocking density was 10–15 shrimp m^{-2} and feed was distributed by spreading from a boat. Water exchange was practised only to compensate for losses by evaporation and percolation. The water temperature was 25–28 °C and salinity was 16–19 g L^{-1}. Both shrimp farms used a pelleted feed (22% protein) that contained antibiotics [Litofloxina™ (Farmavet, Guayaquil, Equador) in the former and Citrinal™ (Chemical Pharm, Guayaquil, Ecuador) in the latter]. The second shrimp farm was selected because of the suspension of artificial feeding in the former. Shrimp of 6, 8 and 10 g were collected to complement the stomach content examination. The sampling period was between the wet season from December 2000 to mid-May 2001. Fifty shrimp were sampled during a culture cycle every 15 days to obtain individuals with increasing weight gains close to 2 g. Shrimp in the early premoult stage (D0) were collected between 14:00 and 16:00 hours, as *L. vannamei* in this moulting stage and day period have been shown to have a higher ingestion rate and enzyme activity (Molina *et al.* 2000). The sampled shrimp were killed by immersion in a seawater–ice mix and their hepatopancreas and stomach were immediately excised. Both organs were stored at − 20 °C until assay.

Enzymatic analysis

To assess enzyme activity, shrimp of six different weights were sampled (Table 1). The hepatopan-

Table 1 Protein concentration and digestive enzyme ratios in the hepatopancreas of *Litopenaeus vannamei* at different body weights

	Shrimp groups by weight gain					
Variable	**1**	**2**	**3**	**4**	**5**	**6**
Body weight (g)	1.9 ± 0.54a	3.6 ± 0.61b	6.4 ± 0.58c	8.28 ± 0.45d	9.9 ± 0.73e	11.9 ± 0.72f
Protein*	236 ± 44a	392 ± 88b	301 ± 94c	323 ± 85c	302 ± 90c	314 ± 66c
Amylase/protease	2.6a	4.2a	4.1a	6.7b	8.1b	9.6c
Trypsin/lipase	15.0a	5.5b	5.7b	3.6b	2.7b	3.7b
Lipase/amylase	45a	36a	48a	67b	58b	50ab
Amylase/trypsin (10^{-3})	4.2a	7.1ab	9.2b	8.7b	9.6b	8.0ab
Amylase/chymotrypsin (10^{-3})	1.4a	1.1a	5.0b	0.3c	0.2c	0.1c

*Expressed as mg g^{-1} hepatopancreas.
Different letters in the same row indicate significant statistical differences ($P < 0.05$).

creases were weighed and homogenized in 1.5 mL of deionized water. Homogenates were centrifuged for 5 min at 13 000 rpm and 4 °C. The supernatant free of the lipid layer was separated and divided into subsamples for each enzyme activity test. These samples were stored in 1-mL Eppendorf tubes at − 20 °C until analysis. Dilutions of the homogenate were made in respective buffers and tested in duplicate. Enzyme activity units were expressed as units per milligram of soluble protein ($U\,mg^{-1}$). Amylase activity was determined using the method of Rick & Stegbauer (1984). A calibration curve was prepared using maltose (Kanto Chemical, Tokyo, Japan). The samples and curve standards were read at 550 nm in a spectrophotometer (Jenway, Essex, UK). One unit of amylase activity was defined as the number of micromoles of maltose released per minute per milligram of protein. Protease activity was estimated according to Garcia-Carreño (1992) using azocasein (Sigma A2765, Sigma Chemical, St Louis, USA) as the substrate. Lipase activity was assayed according to Versaw, Cuppett, Winters & Williams (1989) using β-naphthyl caprylate (Sigma, N8875) as the substrate. Units of protease and lipase activities were considered as a 0.001 increase in absorbance units per minute at 440 and 540 nm respectively. Trypsin and chymotrypsin activities were determined kinetically (Tseng, Grendell & Rothmen 1982; Geiger 1988) using *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA, Sigma, B4875) and *N*-succinyl-ala-ala-pro-phen *p*-nitroanilide (SAPPNA, Sigma, S7388), respectively, as substrates. Both reactions were registered in a spectro-photometer every 0.9 s during 3 min at 407 and 405 nm respectively. The reaction and incubation temperatures were held at 25 °C. One unit of trypsin and chymotrypsin activities corresponded to 1 μmol of 4-nitroaniline released per minute per milligram of protein. Calculations were based in an extinction coefficient $\varepsilon_{405} = 10.2\ L\,mmol^{-1}\,cm^{-1}$ (Geiger 1988; Geiger & Fritz 1988). The total soluble protein was measured using the Bio-Rad test kit (Bio Rad Laboratories, Hercules, CA, USA) based on the method described by Bradford (1976) using bovine serum albumin (Sigma, A7030) as a standard. The amylase/protease ratio was used to characterize digestive capabilities (Lovett & Felder 1990). The amylase/trypsin, amylase/chymotrypsin, lipase/amylase and trypsin/lipase ratios were also calculated as indicators of predominant activities.

Stomach content evaluation

Stomachs were dissected according to the method described by Focken *et al.* (1998) and their contents were extracted from the proventriculus (anterior chamber). Food present in the pyloric chamber (posterior chamber) was not considered because it is previously ground by the ossicles in the proventriculus and it is unrecognizable (Chong & Sasekumar 1981). The stomach contents from individual shrimp were transferred to individual Eppendorf tubes. Wet and dry weights (3 h, 60 °C) were determined. Dry samples were resuspended in a saline solution (5%) and sub-samples (0.05 mL) were placed in a Neubauer chamber and observed microscopically under 10 × and 20 × magnification. The different items in the stomach content were identified and classified into the following categories: prey (whole or parts), artificial feed, plant matter (microalgae, filamentous algae and macrophytes), detritus (organic/bacterial aggregate and semi-digested matter) and minerals. The ingestion of each item (dry weight basis) was calculated as the proportion of each item multiplied by the total weight of the stomach content. To compensate for the individual deviations from the mean body weight in each of the five samplings, the dry weight of each component was multiplied by the mean weight and divided by the individual body weight (Focken *et al.* 1998).

Statistical analysis

All the data were tested for normal distribution and homogeneity of variances using Anderson–Darling and Bartlett tests respectively. Data from the different digestive enzyme activities for each weight group were compared with each other using one-way analysis of variance. In the case of significant differences, multiple comparisons were carried out using a Fisher least significant difference test. When required, data were transformed in order to obtain normal distributions before the analysis of variance. Data that lacked homoscedasticity were analysed using a Kruskal–Wallis one-way analysis of variance on ranks test (Zar 1999) and multiple comparisons were carried out using the Dunn procedure. The Pearson correlation analysis was applied to identify significant correlations between the different enzyme activities. The statistical softwares Data Desk and Statistica were used and the level of significance employed was 0.05.

Results

Enzyme assays

The means for each size group sampled and hepatopancreas protein concentrations are shown in Table 1. All the enzymes activities assayed showed

significant differences in the different weights (Fig. 1a–e). Amylase activity was significantly lower ($P < 0.05$) in 2-g shrimp compared with larger shrimp; there were no significant differences between 4-, 6-, 10- and 12-g shrimp (Fig. 1a). Lipase activity had a significant increase between 2-, 4-, 6- and 8-g shrimp ($P < 0.05$) (Fig. 1b). The activity stayed at the same high level in 8-, 10- and 12-g shrimp. Trypsin activity was variable during the study period. No significant differences ($P > 0.05$) were detected between 2, 4, 8 and 12 g (Fig. 1c). Only chymotrypsin (Fig. 1d) and lipase activities showed a significant positive correlation with weight gain (Table 2). The total protease activity showed an overall decreasing tendency throughout the study period. Significant differences were determined ($P < 0.05$) between 6-g shrimp and larger shrimp (Fig. 1e). Soluble protein in hepatopancreas homogenates showed a significant increase ($P < 0.05$) as shrimp gained biomass. Figure 1f shows the values of shrimp body weight regressed against soluble protein. The variability of soluble protein associated with the linear model was 95% (r^2). Significant differences in soluble protein were detected in all weights, except between 4 and 6 g. Amylase and lipase activities showed a positive correlation ($r = 0.829$). Protease and chymotrypsin activities were negatively correlated ($r = -0.834$). The rest of the enzymatic activities were not significantly correlated with each other (Table 2).

Stomach content evaluation

A total of 228 stomachs were dissected during the study period and only two of these were empty. The

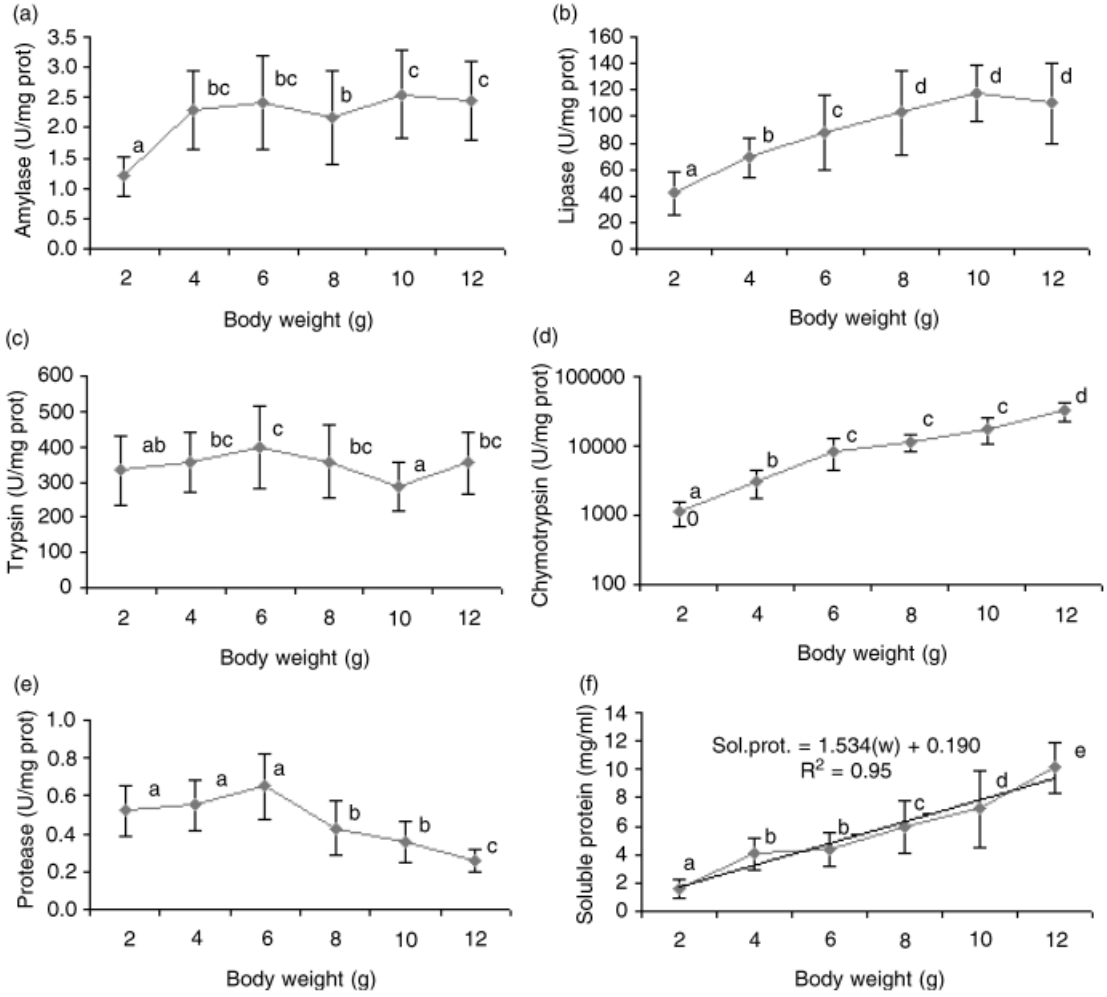

Figure 1 Specific activity of (a) amylase, (b) lipase, (c) trypsin, (d) chymotrypsin, (e) protease and (f) soluble protein content in *Litopenaeus vannamei* at different body weights. Bars and literals indicate standard deviations and significant differences ($P < 0.05$) respectively. Chymotrypsin activity is expressed in a logarithmic scale.

Table 2 Correlation matrix of mean body weight, digestive enzyme activities and soluble protein in *Litopenaeus vannamei* reared in semi-intensive conditions

	Body weight	Amylase	Trypsin	Chymotrypsin	Protease	Lipase	Protein
Body weight	1	0.716	−0.165	0.941*	−0.77	0.949*	0.962*
		$P = 0.109$	$P = 0.755$	$P = 0.005$	$P = 0.073$	$P = 0.003$	$P = 0.002$
Amylase		1	0.095	0.577	−0.278	0.829*	0.714
			$P = 0.857$	$P = 0.231$	$P = 0.593$	$P = 0.041$	$P = 0.11$
Trypsin			1	−0.161	0.589	−0.194	−0.153
				$P = 0.76$	$P = 0.218$	$P = 0.712$	$P = 0.772$
Chymotrypsin				1	−0.834*	0.797	0.968*
					$P = 0.038$	$P = 0.057$	$P = 0.001$
Protease					1	−0.64	−0.819*
						$P = 0.17$	$P = 0.046$
Lipase						1	0.876*
							$P = 0.022$
Protein							1

*Significant correlation at $P < 0.05$.

wet weight stomach content for 2-g shrimp was equivalent to 0.1% (2.7 mg) of their body weight and it increased to 0.4% (38 mg) in 10-g shrimp (Fig. 2). The stomach content weight was very variable in each of the five weight groups. Natural food represented 91% of the shrimp diet throughout the study period. The means for the different elements found in the stomach content in all the samples were 46% detritus, 28% plant matter, 12% prey, 9% feed and 5% minerals. Plant matter found in 2- and 4-g shrimp differed quantitatively and qualitatively compared with that observed in 6-, 8- and 10-g shrimp because they were collected at different shrimp farms. The percentage of plant matter in 2- and 4-g shrimp was 23% and 10% respectively (Fig. 3a and b). Fifteen percent of the stomachs from 2- and 4-g shrimp had filamentous algae, 2% diatoms and 10% fragments of macrophyte plants. In contrast, in 6-g shrimp and larger, the plant matter contribution to the total stomach content was higher than 30% (Fig. 3c and e). In the stomachs of the latter shrimp, only a small amount of filamentous algae was observed (2%) and a higher percentage of fragments of vascular plants (occurring in 18%, 65% and 74% of the stomachs in shrimp of 6-, 8- and 10-g shrimp respectively) and diatoms was determined (occurring in 40%, 15% and 29% of the stomachs in 6-, 8- and 10-g shrimp respectively). Diatoms in the stomach content were mainly represented by the genera *Cocconeis*, *Cymbella*, *Navicula*, *Amphora* and *Gyrosigma*. The prey percentage was higher in 4-g shrimp and represented 20% of the stomach content (Fig. 3b). Prey found in 6-, 8- and 10-g shrimp represented

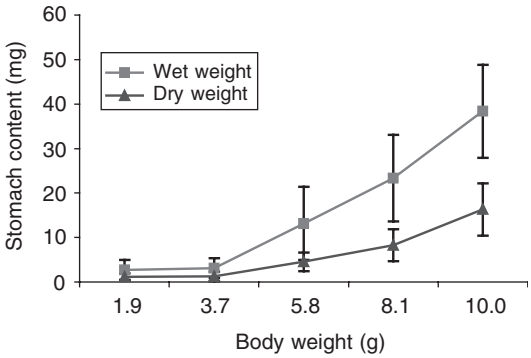

Figure 2 Amount of stomach content present in *Litopenaeus vannamei* at different body weights. Bars indicate standard deviations.

13%, 10% and 8% of the total stomach content respectively. The main prey found were harpacticoid copepods, nematodes, foraminifers, exoskeleton fragments and appendages from insects and crustaceans. Feed had a variable contribution to the total stomach content in shrimp at various weights. A minimum contribution of 2% was observed in 4-g shrimp (Fig. 3b) and a maximum of 20% in 6-g shrimp (Fig. 3c). The proportion of feed in the stomachs showed a decreasing trend in shrimp over 6 g. Detritus was an important item of the ingested material as it contributed a major percentage (> 33%) to the total stomach content at all body weights (Fig. 3a–e). Detrital material was present in 99.1% of the dissected stomachs. Contributions of 60% and 64% of the total stomach content were found in 2- and 4-g shrimp respectively. An average of 5% minerals was observed

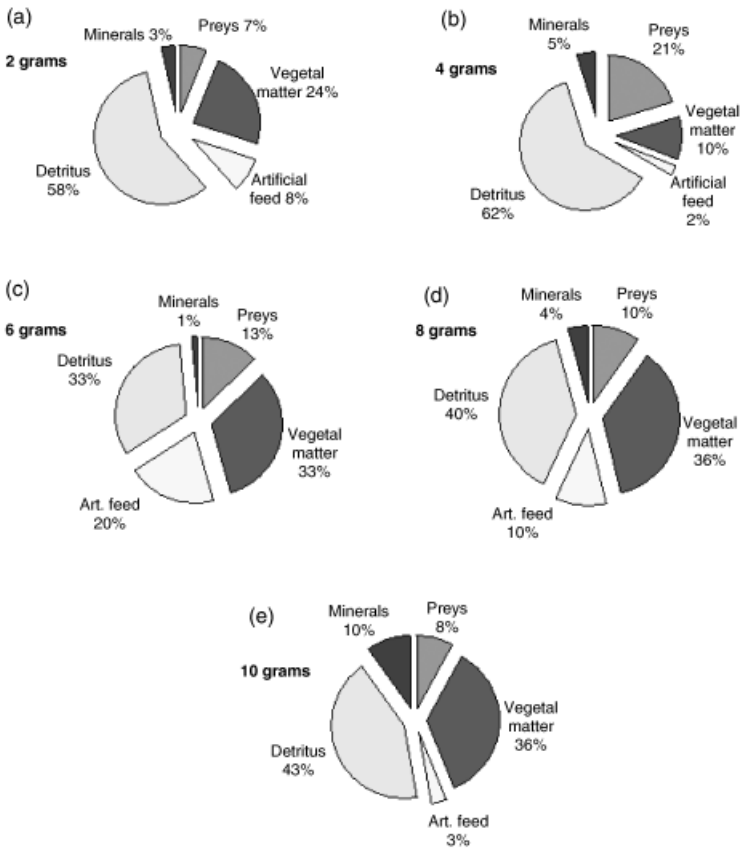

Figure 3 Percentage contribution of each feeding item to the stomach content of *Litopenaeus vannamei* at different body weights.

in the stomach content throughout the study period. The maximum mineral percentage was 10% in 10-g shrimp (Fig. 3e). Mineral material consisted in silt and fine- and medium-size sand grains.

Discussion

Enzymatical activity

Our study shows an increase in the specific activity of some digestive enzymes during development, namely, amylase, lipase and chymotrypsin. The amylase activity pattern may be related to the items found in the stomach content, where the amount of plant matter increased from less than 10% of the total stomach content in 4-g shrimp to more than 30% in larger shrimp, thus inducing amylase synthesis. In the same way, the increase in lipase activity can be because of a higher amount of compound diet observed in the stomachs of 6- and 8-g shrimp. Indeed, compound diets are formulated with a lipid level close to 10%, whereas detritus includes less than 2% lipid (Hunter *et al.* 1987). It has been hypothesized that the digestive enzyme activity is high for those substrates that are most common in the diet (Cox 1981). The specific activity of total protease decreased with growth. Lee, Smith & Lawrence (1984) consider that *L. vannamei* experiences a change in its dietary regime between 10 and 20 g. The authors based their conclusion on the observed differences in the response of protease enzymes to protein quality. They also concluded that these differences may indicate that small shrimp (4 g) have a better ability to utilize protein than larger shrimp (10 and 20 g). In another study with *L. vannamei* ranging in sizes from 1 to 14 g, Hunter *et al.* (1987) observed an increase in the C:N ratio of the ingested material, and their conclusion was that this observation may imply a selection for diets with less protein in the latter life stages. The increase in the ratio of amylase to proteases observed in our study led to the same conclusion. A digestive adaptation to new food preferences may be occurring in this period. Le Moullac (1995) reported that the amylase/protease ratio in *L. vannamei* juveniles increased by four orders of magnitude from 2 to 12 g, while the amylase/trypsin and amylase/chymotrypsin

ratios increased slightly. Similar responses were observed in the present study as the amylase/protease ratio was 2.6 in 2-g shrimp and later increased steadily to 9.6 in 12-g shrimp (Table 1). In the same way, the amylase/trypsin ratio increased slightly but significantly. Nonetheless, the amylase/chymotrypsin ratio was different, since this ratio decreased significantly as body weight increased.

Even though there is a reported correlation between trypsin and chymotrypsin activities in vertebrates (Lhoste, Fiszlewics, Gueugneau & Corring 1994), a number of studies in crustaceans have led to opposite results. Le Moullac *et al.* (1997) failed to detect this correlation in *L. vannamei* juveniles, but in another study Guzman *et al.* (2001) reported a significant correlation in these activities in *Litopenaeus setiferus* (Linnaeus, 1767) postlarvae. A lack of correlation between trypsin and chymotrypsin activities ($r = -0.16$) was observed in the present study. Lipase and amylase activities showed a positive correlation ($r = 0.82$), indicating an increase in their secretion correlated to body weight gain (Table 2).

Stomach content

Shrimp exert a constant feeding activity on the substrate in order to keep minute amounts of organic matter from reaching the proventriculus (Cuzon, Rosas, Gaxiola, Taboada & Van Wormhoudt 2000). The diet of shrimp grown in extensive and semi-intensive culture systems is almost or totally composed of natural food because the pond bottoms of these systems are organically rich and offer a variety of naturally occurring food sources (Nunes *et al.* 1997; Focken *et al.* 1998). In the present study, the different items found in the stomach contents and their percentages correspond to the omnivorous–herbivorous feeding habits described for the sub-genus *Litopenaeus* (Hunter & Feller 1987). Fragments of macrophyte plants had a contribution above 65% to plant matter present in the stomach contents of 8- and 10-g shrimp. Aquatic macrophytes were not observed in the pond, but there were shrubs and herbaceous plants growing on the levees. Therefore, fragments of plants in the stomachs of shrimp may have come from these plants. The benthic fauna in the pond bottoms can be diverse and consists of several potential prey species for the shrimp (Nunes *et al.* 1997; Focken *et al.* 1998). In the present study, the main prey found in the stomach content showed a tendency to decrease when shrimp reached a weight of 4 g. Previous studies indicate that the availability of prey organisms is related to the stocking density of the consumer organisms and is also related to the population dynamics inherent to each individual species (Nunes *et al.* 1997).

Even in semi-intensive systems, feed makes a low contribution to the shrimp diets. In the present study, the contribution of feed to stomach contents reached a maximum of 20% in 6-g shrimp. This may be related to the observation of Molina & Piña (1999), who noted that the consumption of feed increases each week until it stabilizes between 8 and 11 g. After attaining this weight, a decrease in consumption was registered, but the shrimp growth rate kept increasing. It appears that in this phase of culture, a change in the feeding preference of *L. vannamei* occurs, in which growth rate is mainly supported by the nutrients found in the different items of the natural productivity. Focken *et al.* (1998) state that the low percentage of feed in the shrimp diet may have an important nutritional contribution because of higher digestibility when contrasted with plant tissues and organic material with high fibre contents. In the present study, it was observed that the high amounts of detritus in the stomach contents (Fig. 2a–e) were similar to those reported for other penaeid species such as *Farfantepenaeus subtilis* (Pérez Farfante, 1967) (Nunes *et al.* 1997). Hunter *et al.* (1987) determined that the biochemical composition of detritus is 14.8% protein, 1.6% lipid and 1.1% carbohydrate on a dry weight basis. The protein:energy ratio was higher for detritus than any other component of the biota. Bacteria associated with the detrital mass may serve as a source of food in that the bacterial protein, released upon cell lysis, may be utilized by the shrimp (Hood & Meyers 1974). Nunes, Goddard & Gesteira (1996) consider that the presence of mineral material in the stomach content may be related to accidental consumption when other benthic material has been ingested. In the present study, stomachs with a high percentage of sand grains showed a null or very low amount of diatoms. Therefore, it is possible to assume an additional grinding effect (besides the stomach compression) by the sand grains on the silicified cell walls.

In conclusion, the results of this study suggest an adaptation of enzymatic activity to diets with a lower protein content as the shrimp body weight increases. The change in stomach content composition may also support a different food preference occurring as shrimp grow. More research in this area is necessary to assess the activity of these and other digestive

enzymes in shrimp with higher body weights than those evaluated in the present study. The estimation of trends in digestive enzyme activities and the evaluation of food preferences may contribute to the improvement of both feeding schemes and diet formulations for shrimp in specific grow-out stages.

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