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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 Peneid shrimp have focused on evaluating the ability of organisms to hydrolyze, 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 our understanding of the mechanism of digestion and the better knowledge of nutritional needs (Le Moullac, Klein, Sellos & Van Wormhoudt 1997). The interrelationships between data derived from these analytical techniques and growth, may assist in defining overall diet quality for both young and older crustaceans (Lee & Lawrence 1985).

Crustacean penaeids adapt quite well to changes in diet composition by induction of digestive enzymes synthesized and secreted in the hepatopancreas (Le Moullac *et al.* 1997). These digestive enzymes are able to hydrolyze 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), molting 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 the enzymatic activity (Lovett & Felder 1990). Nevertheless, changes in the digestive enzyme activities are also found in juveniles and adults. It appears that these changes are related to growth and feed digestibility (Lee & Lawrence 1985). Changes in 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 optimised to satisfy the nutritional requirements (Focken *et al.* 1998). In this context, a better comprehension of the feeding preferences and the use of feed by the shrimp are essential in order to optimise the use of nutrients and to reduce the environmental pollution that originates from metabolite excretion and from the artificial feed not consumed. A net waste of nutrients due to an excessive feeding, represents also

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 determine the digestive enzyme activity of *Litopenaeus vannamei* (Boone) at different body weights, reared in a commercial pond. Additionally, observations on stomach content are reported.

Materials and Methods

Sampling

Shrimps were collected from an 8-Ha culture pond in a shrimp farm located in Palmar, Guayas, Ecuador and from a 9-Ha culture pond near the Guayas river. The 8-Ha pond was close to the shore, operated semi-intensively (9-12 shrimps m^{-2}) and feeding trays were used to distribute the feed. A slow water exchange was done in each spring tide. Water temperature was 25-27 ºC and salinity 33-35 g L^{-1}. The 9-Ha pond was located near to one of the branches of the Guayas river. The stocking density was 10-15 shrimps m^{-2} and feed was distributed by spreading from a boat. Water exchange was practised only to compensate loses by evaporation and percolation. Water temperature was 25-28 ºC and salinity 16-19 g L^{-1}. Both shrimp farms used an artificial feed with 22% protein which was formulated with antibiotics (litoflaxine™ in the former and citrinal™ in the latter). The second shrimp farm was selected due to the suspension of artificial feeding in the former, shrimps in 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 in order to obtain individuals with increasing weight gains close to 2 g. Shrimps in early premolt stage (Do) were collected between 14:00 and 16:00 hours as in these molting stage and day period a higher ingestion rate and enzyme activity has been determined in *L. vannamei* (Molina *et al.* 2000). Selected shrimps were sacrificed by immersion in a seawater-ice mix and their hepatopancreas and stomach were immediately excised. Both organs were stored at –20 ºC until assayed.

Enzymatic analysis

In order to assess the enzyme activity, shrimps in 6 different weights (Table 1) were sampled. The hepatopancreas were weighted and homogenized in 1.5 ml deionized water. Homogenates were centrifuged for 5 min at 13,000 rpm and 4 ºC. The supernatant free of the lipid layer was separated and divided for each enzyme activity test. These samples were stored in 1-mL Eppendorf tubes at –20 ºC until analysis. Dilutions of the homogenate were made in respective buffers and tested in duplicate. Enzyme activity was expressed as units per mg soluble protein (U mg^{-1}). Amylase activity was determined by the method of Rick & Stegbauer (1984). A calibration curve was prepared using maltose (Kanto Chemical, Japan). The samples and curve standards were read at 550 nm in a spectrophotometer (Jenway, UK). One unit of amylase activity was defined as number of micromoles of maltose released per min per mg of protein. Protease activity was assayed according to García-Carreño (1992) using azocasein (A2765, Sigma, USA) as substrate. Lipase activity was assayed according to Versaw, Cuppett, Winters & Williams (1989) using β-naphthyl caprylate (N887

5, Sigma, USA) as 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-alfa-benzoyl-DL-arginine p-nitroanilide (BAPNA, B4875, Sigma, USA) and N-succinyl-ala-ala-pro-phen p-nitroanilide (SAPPNA, S7388, Sigma, USA) respectively, as substrates. Both reactions were registered in a spectrophotometer each 0.9 sec during 3 min at 407 and 405 nm, respectively. Reaction and incubation temperatures were held at 25 ºC. One unit of trypsin and chymotrypsin activities corresponded to 1 micromol of 4-nitroaniline

released per min per mg protein. Calculations were based in an extinction coefficient ε_{405} = 10.2 L $mmol^{-1}$ cm^{-1} (Geiger 1988; Geiger & Fritz 1988). Total soluble protein was measured using the Bio-Rad® test kit (Bio Rad Laboratories, USA) based on the method described by Bradford (1976) using bovine serum albumin (A7030, Sigma, USA) as a standard. All enzymatic units were divided by the soluble protein. 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.

Table 1. Digestive enzyme activity in the hepatopancreas of *Litopenaeus vannamei* at different weights.

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

Different literals indicate significant statistical differences ($P < 0.05$)

Stomach content evaluation

Stomachs were dissected according to the method described by Focken *et al.* (1998) and its content was extracted from the proventriculus (anterior chamber). Food present in the pyloric chamber (posterior chamber) was not considered because is previously triturated by the ossicles in the proventriculus and it is unrecognisable (Chong & Sasekumar 1981). The stomach content was transferred to individual Eppendorf tubes. Wet and dry weight (3 h, 60 ºC) 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 in 10 and 20X magnification. The different items in the stomach content were identified and classified into the following categories: preys (whole or parts), artificial feed, vegetal 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 by Anderson-Darling and Bartlett tests, respectively. Data from the different digestive enzyme activities for each weight group were compared with each other by one way analysis of variance. In case of significant differences, multiple comparisons were done by a Fisher least significant difference test. When required, data were transformed in order to obtain normal distributions before the analysis of variance.

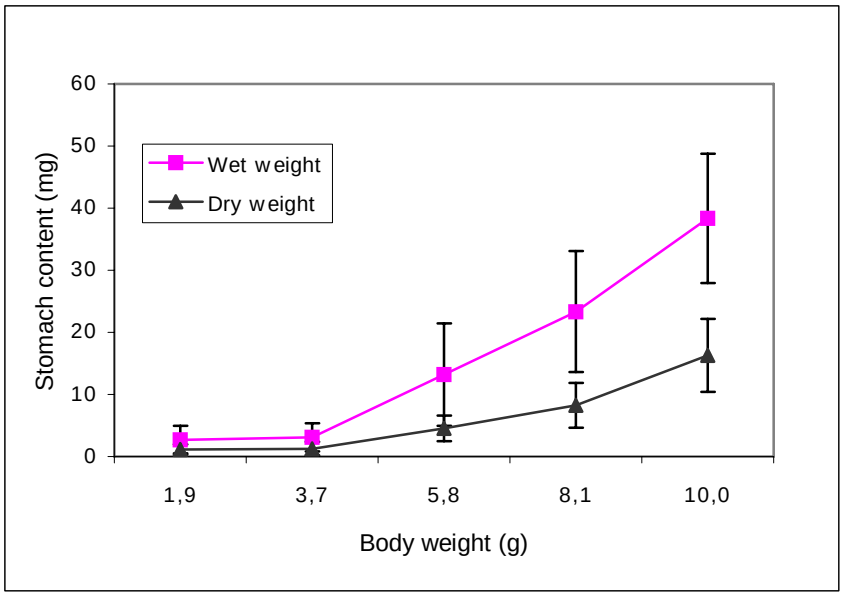
Data which lacked homocedasticity were analysed by a Kruskal-Wallis one-way analysis of variance on ranks test (Zar 1999) and multiple comparisons were done by the Dunn procedure. Pearson correlation analysis were applied to identify significant correlations between the different enzyme activities. The statistical software Data Desk and Statistica were used and the level of significance employed was 0.05.

Results

Enzyme assays

Means for each size group sampled and hepatopancreas protein concentrations are shown in Table 1. The stomach content was very low, less than 2 mg in 2 g shrimp, but increased up to 40 mg wet weight in 12 g shrimp (Fig 1).

Fig. 1. Amount of stomach content present in *L.vannamei* at different body weights. Bars indicate standard deviations.



All the enzymes activities assayed showed significant differences in any of the different weights (Fig. 2a-e). Amylase activity was significantly lower ($P < 0.05$) in 2 g

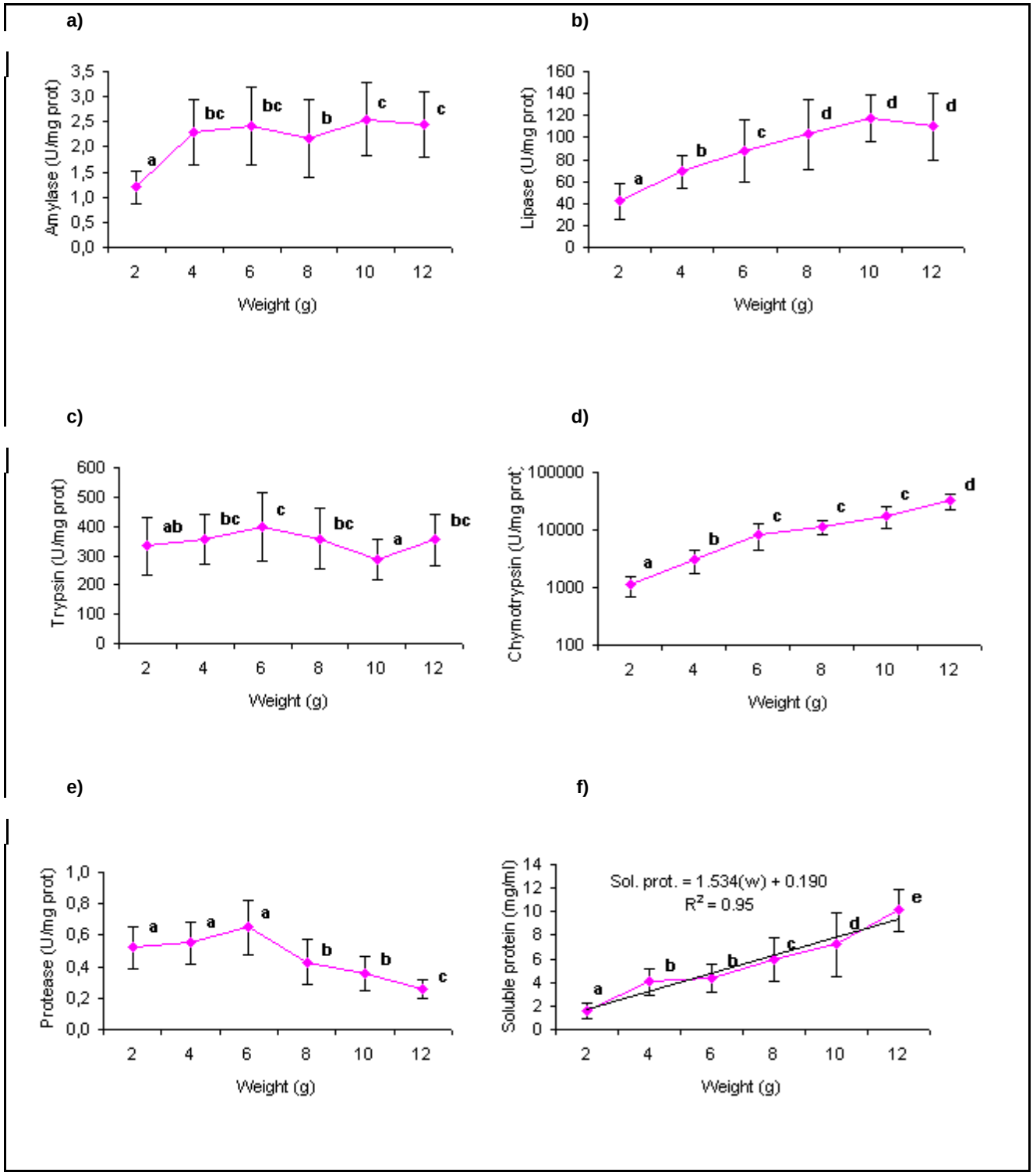
shrimp as compared with bigger weights, there was no significant difference between 4, 6, 10 and 12 g (Fig. 2a). Lipase activity had a significantly increased in between 2, 4, and 6 and 8 g shrimp ($P < 0.05$) (Fig 2b). The activity stayed at the same high level in 8, 10 and 12 g shrimp. Trypsin activity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

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

	Body weight	Amylase	Trypsin	Chymotrypsin	Protease	Lipase	Sol. 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
Sol. protein							1

*Significant correlation at P < 0.05

Fig 2. Digestive enzyme activity of (a) amylase, (b) lipase (c) trypsin, (d) Chymotrypsine (e) protease and (f) soluble protein in *L. 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.

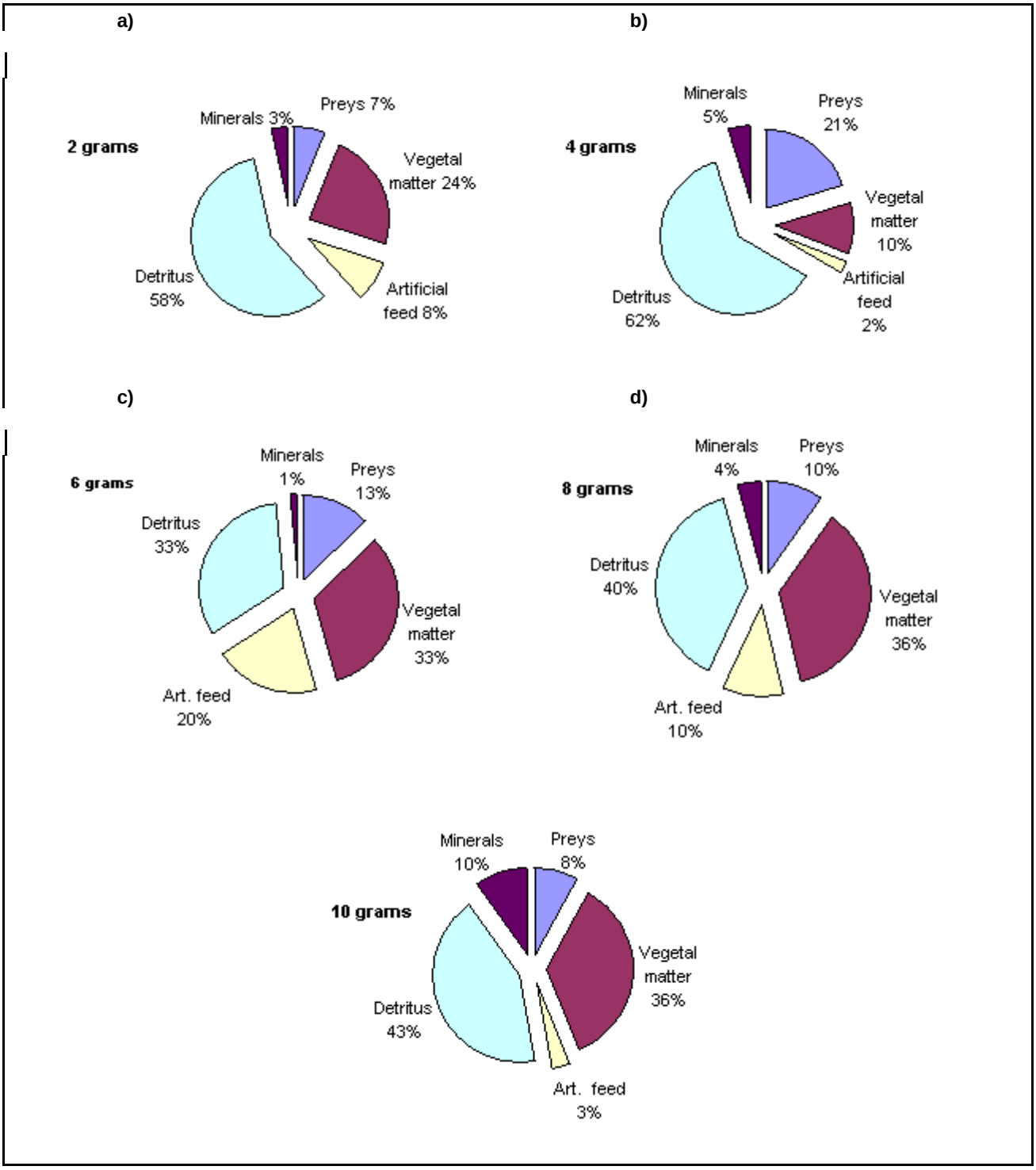


Stomach content evaluation

A total of 228 stomachs were dissected during the study period and only 2 of these were empty. Wet weight stomach content for 2 g shrimps 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. 1). The stomach content weight was very variable in each of 5 weight groups. Natural food represented 91 % of the shrimp diet throughout the study period. The mean for the different elements found in the stomach content in all the samples were: 46% detritus, 28% vegetal matter, 12% preys, 9% artificial feed and 5% minerals. Vegetal matter found in 2 and 4 g shrimp differed quantitative and qualitatively compared to the observed in the body weights 6, 8 and 10 g because they were collected in different shrimp farms. The percentage of vegetal matter in 2 and 4 g shrimp was 23 and 10% respectively (Fig 3a-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 bigger, the vegetal matter contribution to the total stomach content was higher than 30% (Fig. 3c-e). In the stomachs of the latter shrimps a minimal 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 shrimps of 6, 8 and 10 g respectively) and diatoms was determined (occurring in 40, 15 and 29% of the stomachs in shrimps of 6, 8 and 10 g 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). Preys found in 6, 8 and 10 g shrimps represented 13, 10 and 8% of total stomach content, respectively. The main preys found were harpacticoid copepods, nematodes, foraminifers, exoskeletons fragments and appendages from insects and crustaceans. Artificial feed had a

variable contribution to the total stomach content in different shrimp weights. A minimum contribution of 2% was observed in 4 g (Fig 3b) shrimp and a maximum of 20% in 6 g shrimp (Fig 3c). The proportion of artificial feed in the stomachs showed a decreasing trend in shrimps bigger than 6 g. Detritus was an important item of the ingested material as it conformed a major percentage (> 33%) to the total stomach content in 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 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.

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



Discussion

Enzymatical activity

Our study shows an increase in specific activity of some digestive enzymes during development, amylase and lipase. This pattern may be related to the stomach content: the increased ingestion of vegetal matter -10% of total stomach content in 4 g shrimp to more than 30% in bigger shrimp- may have induced amylase synthesis. In the same way, the increase in lipase activity can be due to a higher ingestion of compound diet observed in 6 and 8 g shrimp. Indeed, compound diet includes around 10% lipid, when detritus includes less than 2% lipid (Hunter *et al*., 1987). It has been hypothesized that digestive enzyme activity is high for those substrates that are most common in the diet (Cox 1981). Specific activity of total protease decreased with growth. Lee, Smith & Lawrence (1984) consider that *L. vannamei* experiments a change in its dietary regime when it reaches a weight between 10 to 20 g. The authors based their conclusion on the observed differences in the response of the protease enzymes to the protein quality. They also concluded that these differences may indicate that small shrimp (4 g) have a better ability to utilize the protein than bigger shrimp (10 and 20 g). In another study with *L. vannamei* ranging in body 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 amylase/proteases (Table 1) 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 amylase/protease ratio in *L. vannamei* juveniles increased in 4 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 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 proportion had a significant decrease 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 has led to opposite results. Le Moullac *et al*. (1997) failed to detect this correlation in *L. vannamei* juveniles but in other study, Guzman *et al*. (2001) determined a significant correlation in these activities in *L. setiferus* 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 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 food sources naturally occurring (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). Vegetal matter had a contribution above

30% to total stomach content in 6, 8 and 10 g shrimp. Fragments of macrophyte plants were present in 65 and 74% of the stomachs of shrimp in 8 and 10 g respectively. 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 shrimps 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, copepods, exoskeleton fragments, nematodes and foraminifers represented the main items within the prey category. 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, artificial feed presents a low contribution to the shrimp diet. In the present study, the contribution of artificial feed to the stomach content reached at the maximum 20% in 6 g shrimp. This may be related to the observation by Molina & Piña (1999), who noted that the consumption of artificial feed increases each week until it stabilizes between 8 and 11 g. After that weight, a decrease in consumption was registered but the shrimp growth rate kept increasing. It appears that in this phase of the culture occurs a change in the feeding preference of *L. vannamei* in which the growth rate is now mainly supported by the nutrients found in the different items of the natural productivity. Focken *et al.* (1998) state that the low percentage of artificial feed in the shrimp diet may have an important nutritional contribution due to higher digestibility when is contrasted with vegetal tissues and organic material with high fibre content. In the present study, it was observed that the high amounts of detritus in the stomach contents (Fig. 2a-e) is

similar to those reported for other penaeids species as *Farfantepenaeus subtilis* (Pérez Farfante) (Nunes *et al*. 1997). Hunter *et al.* (1987) determined that the biochemical composition of detritus is 14.8% protein, 1.6% lipids and 1.1% carbohydrates on a dry weight basis. The protein:energy ratio was highest for the detritus than any other element in the biota. Bacteria associated to 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 an accidental consumption when other benthic material has been ingested. In the present study, it was observed that 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 triturating 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 the enzymatic activity to diets with lower protein content as body weight increases. The change in stomach content composition may also support a different food preference occurring as shrimps grow. More research in this area is necessary in order to asses the activity of this and other digestive enzymes in shrimps 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 improve both, feeding schemes and diet formulations for shrimp in specific grow-out stages.

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