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SEASONAL VARIABILITY IN TRYPSIN AND α-AMYLASE ACTIVITIES CAUSED BY THE MOLTING CYCLE AND FEEDING HABITS OF JUVENILE PINK SHRIMP *FARFANTEPENAEUS DUORARUM* (BURKENROAD, 1939)

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ABSTRACT

In view of the relationship between shifts in diet composition and the activity of digestive enzymes in penaeid shrimp, the present study focused on the analysis of digestive trypsin and α-amylase activities of wild *Farfantepenaeus duorarum* (Burkenroad, 1939) juveniles and their changes in phenotypic expression, during the molt cycle as endogenous factor and their changes due to different feeding regimes (exogenous factor) in relation with δ^{13}C and δ^{15}N isotopic signature as an index of food assimilation induced by the seasonal availability of food items in the nursery area. Wild juveniles of *F. duorarum* were captured from April 2007 to February 2008, in the Celestun coastal lagoon, Yucatan, Mexico. Samplings were carried out considering all quarters of the lunar cycle and in each of the recognized seasons for this region: dry, rainy, and the Nortes (North Wind). Copepods and amphipods were the main source of food for juveniles of *F. duorarum*. Values of δ^{13}C in the muscular tissue were near $-20‰$ hence the feeding regime of *F. duorarum* in the lagoon was composed by material of marine origin. Isotopic signature differences were found between the three annual seasons. It is an opportunist generalist organism that is located in the 4th trophic level. The digestive enzymatic activities of both trypsin and α-amylase in fresh hepatopancreas tissue showed an interaction between season and molt stages ($p < 0.05$). Activity of the trypsin was highest during the Nortes at molt stage C (140 mU mg^{-1} HP) and activity of α-amylase was higher in the Nortes at stage B_2 (674 mU mg^{-1} HP). The amylase/trypsin ratio also showed significant interaction between season and molt stages ($p < 0.05$), with higher values in premolt stages during the rainy and Nortes seasons. Isoforms of these digestive enzymes differed in expression according to the molt stage and also to the season with expression generally being greater at stage C.

KEY WORDS: coastal lagoons, digestive enzymes, *Farfantepenaeus duorarum*, feeding habits, pink shrimp

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INTRODUCTION

The distribution of postlarvae and juvenile penaeid shrimp has been associated with estuarine vegetation due to lower predation from juvenile fishes as well as to food availability, and *Farfantepenaeus duorarum* (Burkenroad, 1939) is not the exception (Sanchez, 1997). Juvenile shrimp recruited into tropical nursery grounds consume various food sources depending on their seasonal availability, location and the rate at which such materials reach the bottom, that changes during the year (Pech et al., 2007). The feeding habits of shrimp juveniles will match those of an omnivorous or a carnivorous regimen. Gaxiola et al. (2005) showed that assimilated food produces a rapid growth rate around 1 mg day^{-1} in *Litopenaeus vannamei* (Boone, 1931).

In decapod crustaceans a close relationship between molt cycle and the lunar phases has been shown (Dall et al., 1990). There is a synchrony between molt cycle and moon cycle observed in *L. vannamei* raised in earthen ponds, with 50% of shrimp population in postmolt stages during waning moon; a molting peak in new moon phase occurred after 5 days of low and high tide (Molina-Poveda et al., 2002).

Another relevant aspect related to shrimp growth is the digestive enzymatic capacity to breakdown nutrients, store energy reserves, and assimilate food items from organisms of both planktonic and benthic origin, available at different shrimp life stages. The study of such enzymatic activity is essential to establish the functioning of the shrimp digestive system in relation to food requirements (Le Moullac et al., 1996), to the origin of the components of the diet (Chong

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et al., 2001; Buillon et al., 2002a) and ontogeny (Gamboa-Delgado et al., 2003).

Digestive enzymes are substrate specific. In carbohydrate digestion of both larvae and postlarvae of *Litopenaeus schmitti* (Burkenroad, 1936), α-amylase and α-glucosidase maintained a linear correlation in their specific enzymatic activity (Arena et al., 2003). In protein digestion, trypsin is active in nutrient assimilation throughout the shrimp life cycle (Cara et al., 2004; Sainz et al., 2004) and its regulation is linked with available resources (Sanchez-Paz et al., 2007). However, the physiological conditions at different stages of the molt cycle influence significantly the digestive enzymatic activity of penaeid shrimp (Klein et al., 1996; Sanchez-Paz et al., 2003). Just prior to ecdysis, decapod crustaceans cease their feeding activity causing a general reduction in their metabolic level (Dall et al., 1990). At this critical stage, the digestive enzymatic activity is almost shut down. As shown by Casillas-Hernandez et al. (2002) enzymatic activity in the hepatopancreas during premolt and postmolt stages of *Litopenaeus stylirostris* (Stimpson, 1871) was negligible. However, there is little data about digestive enzymatic process, particularly on individuals undergoing critical molt cycle stages.

The pink shrimp *F. duorarum* is known for its omnivorous feeding behavior in the juvenile phase although it shifts to carnivory as adult. Even if data on the stomach contents of this native species are available (Schwamborn and Criales, 2000), their digestive capacity in the wild did not serve to identify the juveniles feeding habits up to now. It is hypothesized that digestive enzymes activities will be modified as a result of transcription or translation according to seasonal food availability and molt stages under lunar cycle. In view of the relation between shift in diet composition (Perez-Farfante, 1970) and activity of the digestive enzymes in penaeid shrimp (Gamboa-Delgado et al., 2003; Sanchez-Paz et al., 2003), the present study focused on the analysis of trypsin and α-amylase activities and changes in phenotypic expression during the molt cycle (endogenous factor) of *F. duorarum* juveniles. Variations were also examined with feeding regimes or changes in isotopic signature (δ^{13}C and δ^{15}N) as an index of food assimilation induced by season availability of food items in the nursery area (Buillon et al., 2002a; Macia, 2004).

Material and Methods

The tropical coastal lagoon of Celestun belongs to the biosphere reserve "Ria de Celestun" in Yucatan, which extends along the Gulf of Mexico at 20°45′N and 90°25′W (Pech et al., 2007). Herrera-Silveira et al. (1998) described this lagoon as a karstic site in which the input of freshwater from groundwater discharges varies according to the rain regime (Fig. 1). According to the hydrological conditions of the lagoon, Herrera-Silveira (1993) recognized three zones: seaward, middle and inner, and climatic regime of the region determines three seasons: dry (March-May), rainy (June-October) and the Nortes (November-February).

Samplings were made in the middle zone of the lagoon (Fig. 1) during night hours (between 19 and 23 hours), when decapods are most active and least vulnerable to potential predators (Dall et al., 1990; Simões et al., 2010)

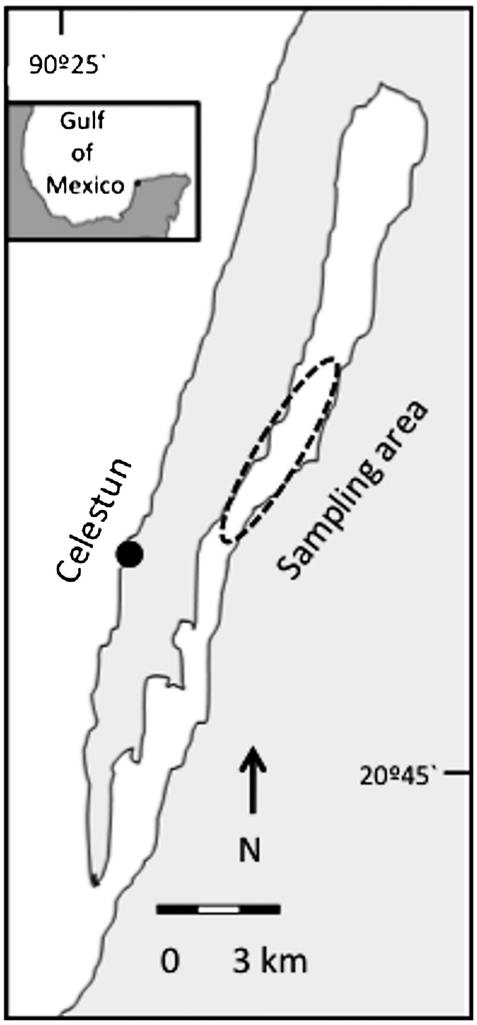

Fig. 1. Geographic position of Celestun lagoon and sampling area.

and because we observed, from samples taken in preliminary catches, that decapods were associated with seagrass and the stomach fullness was always higher during the night than during the day. A total of 1189 juvenile *F. duorarum* (mean cephalothoracic length (CL) of 16.4 ± 2.5 mm and weight of 4.07 ± 1.7 g) were collected from April 2007 to February 2008 during the dry (N = 399), rainy (N = 362), and Nortes (N = 428) seasons. The total catch was preserved for different analysis and molt stage of each specimen was assessed through the observation of the setal development of the uropod (Dall et al., 1990). Within each sampling period, organisms were collected weekly in each phase of the lunar cycle, using a local fishing gear formed by a conical net with a mesh size of 1/2 inch, attached to a triangle made with sticks of mangrove about 2 m long. Each vertex of the triangle is tied to a rope of approximately 4 m long allowing the fishing gear to be towed by foot for five minutes, covering 270 m^2 each time. Temperature, salinity, and dissolved oxygen concentrations were measured with a YSI-556 multi-parameters (YSI Incorporated, 1700/1725 Brannum Lane, Yellow Springs, OH 45387 USA). Sediment was obtained with a van Veen dredge. Two samples of 100 cm^3 of the surface material of the lagoon bottom were

collected. One of the samples was placed in a 150 mL plastic bottle and 0.6 mg L^{-1} $MgCl_2$ diluted in the water obtained from the lagoon was added. After one hour, a solution of 10% formalin neutralized with sodium tetraborate was added to preserve the meiobenthic samples. The other sample was placed on ice until preparation for isotope analysis.

Circular surface plankton tows were taken at each sampling station with a 0.5 m diameter net with 500 μm mesh, equipped with a flow meter to measure the volume of water sampled. Samples were divided in two parts, one preserved on ice without any chemical treatment until preparation for isotope analysis. The other was preserved in 10% buffered formalin solution and used for species identification.

Stomach Content Analysis

The digestive tract was dissected from individuals while in the field. The foregut of 124 specimens was extracted and kept in a 0.6 mg $MgCl_2$ L^{-1} solution and preserved in 10% formalin solution for further stomach contents analysis. Prey items from each stomach were examined under the microscope and identified according to prey types found in plankton and sediment samples collected at the same time. The Index of Importance (IIMP) of each prey was obtained following Garcia-Rodriguez et al. (2011):

$$\mathrm{IIMP}_i = \frac{1}{\mathrm{U}} \sum_{j=1}^{u} \frac{\mathrm{x}_{ij}}{\mathrm{X}_j}.$$

Where x_{ij} is the number of individuals of taxon i in stomach j, X_j is the total number of individual stomach j across all taxa and U is the number of stomachs in the sample.

The number of food components found in each stomach was also used to calculate the Trophic Level (TL) following Christensen and Pauly (1992):

$$\mathrm{TL} = 1 + \sum_{j=1}^{n} \mathrm{DC}_{ij} \cdot \mathrm{TL}_j.$$

Where the diet composition (DC_{ij}) is the proportion of prey j in the diet of individual i, TL_j is the trophic level of prey j, and n is the number of groups in the system.

Stable Isotope ^{13}C and ^{15}N Analysis

Isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ was performed according to Coplen et al. (2006) on muscle of *F. duorarum* (N = 24), estuarine bottom sediment (N = 4), plankton $\geqslant$ 500 μm (N = 2) and the seagrass *Halodule wrightii* (N = 1). The samples were prepared as follows. 1) Only muscle tissue of *F. duorarum* in intermolt stage C was used to avoid water contents variations. The exoskeleton was removed and the muscular tissue washed with distilled water, then dried for 24 hours in an oven at 60°C. 2) Sediment: 10 g of the frozen preserved sample were screened through a 270 μm mesh to remove inorganic detritus. Four grams of wet sediment were obtained. Ten more grams of the sample, without sifting, remained in a 0.5N HCl solution for one hour to remove carbonates. Both samples were washed twice with distilled water then placed in an oven to be dried at 60°C for 24 hours. 3) Plankton and *H. wrightii*: The material collected was treated with 0.5M HCl solution for one hour. Then it was washed twice with distilled water and left to dry in an oven at 60°C for 24 hours and cooled in a dessicator. Subsequently, each sample of muscle, sediment, plankton, and *H. wrightii*, was crushed into a fine powder in a mortar previously washed with water and Ingrain, rinsed with distilled water and acetone, and dried for 24 hours at 60°C. Samples were stored in 2 ml Eppendorf tubes previously labeled and kept in a foil bag. Elemental analysis was carried out in the Laboratory of Soil Science, and $\delta^{13}C$ and $\delta^{15}N$ values were obtained in the Mass Spectrometry Laboratory, both at the Institute of Geology (UNAM), using a Dumas combustion elemental analyzer coupled to a Delta Plus Mass Spectrophotometer XL, which has an accuracy of 0.2‰.

Analysis of Enzymatic Activity

Immediately after capture, 89 hepatopancreas (HP) dissected from individuals in different molting stages (five HP of each molt stage) were individually kept in small vials initially preserved in liquid nitrogen, and later maintained in the laboratory at −80°C until analysis. Each HP was homogenized in 500 μL of distilled water, with a tissue homogenizer, centrifuged at 14 000 rpm at 4°C during 20 minutes and the supernatant was removed for further enzyme analysis.

Trypsin activity was determined according to Gieger and Fritz (1988), with 100 mM BAPNA (benzoyl-arginin-paranitro-anilide, Sigma B7632) as substrate in TRIS 0.1 M pH 8 buffer at 4°C. The hydrolysis rate of substrate was measured as absorbance increment using a spectrophotometer (Spectronic model 21D) at 405 nm during two minutes, with the extinction coefficient $\varepsilon_{405} = 1.02$ L $mol^{-1}cm^{-1}$. A unit was defined as 1 mM of p-nitroanilidine released in one minute.

The α-amylase activity was measured according to a modification of Bernfeld's method (1955), using 1.5% glycogen (Fluka, 50573) as substrate diluted in a 2.5 mM $MnCl_2$, 10 mM NaCl, 10 mM phosphate buffer, at pH 7. Enzymatic activity was expressed as milligrams of maltose liberated per min at 37°C, according to van Wormhoudt (1980).

The specific isozymes of trypsin and chymotrypsin were determined by electrophoresis polyacrylamide gel with sodium dodecil-sulfate (SDS-PAGE) (Garcia-Carreño et al., 1993). The α-amylase isozymes were determined by a method previously described by Arena et al. (2003). The HPs of individuals in each molting stage were pooled and homogenized in 500 μl of Tris-phosphoric acid buffer (0.06 mol L^{-1}, pH 7) and centrifuged at 14 000 rpm (4°C, 20 min). Conventional 10% vertical polyacrylamide gel electrophoresis was used with Tris-glycine as the running buffer and separation was carried out for 4 h at a constant voltage of 250 V. Gels were then incubated in 3% boric acid for 10 minutes. Subsequently, gels were placed in a 1% starch solution-with buffer phosphate (pH 6) and incubated for 30 min at 37°C.

Activity in gels was revealed by removal of the starch solution from agar and adding lugol diluted in ultra-pure water (1 : 5 proportion). They were maintained in this state until bands became visible, at which point lugol was removed;

Table 1. Parameters monitored at the beginning of each sampling. (Moon phases: 1/4 = waning, 2/4 = new, 3/4 = crescent and 4/4 = full).

Annual season	Moon phases	Start hour	T (°C)	Salinity (ups)
Dry	1/4	19:36	29.5	33.0
Dry	2/4	20:02	25.0	18.0
Dry	3/4	20:15	29.0	29.0
Dry	4/4	19:00	29.5	28.0
	Mean =		28.2	27.0
Rain	1/4	20:10	30.3	17.6
Rain	2/4	20:45	30.9	19.6
Rain	3/4	20:30	30.0	32.8
Rain	4/4	20:00	31.7	18.0
	Mean =		30.7	22.0
N	1/4	20:22	26.2	13.1
N	2/4	19:48	27.1	20.6
N	3/4	19:38	26.1	31.3
N	4/4	19:25	27.8	33.5
	Mean =		26.8	24.6

gels fixed with a 7.5% acetic acid solution were washed with ethanol 10%.

Statistical Analysis

Differences in frequency of occurrence of each food type were evaluated using a Chi-square test. One way ANOVA and Tukey multiple range test were performed to test differences of trophic level data considering the three climatic seasons. We computed a Pearson's correlation coefficient to associate the variation of the trophic level with changes in the weight of *F. duorarum* and the expression of trypsin and α-amylase. For digestive enzymes activity a bifactorial ANOVA of 3×6 (3 seasons and 6 molt stages) of the Log_{10} transformed data was used to analyze the interactions among factors. When differences were found, a Tukey multiple test was used. In all cases a probability level (α) of 0.05 was used (Zar, 1996).

Results

The effect of season on surface water temperature was notable between the rainy season (mean 30.7°C) and the Nortes (mean 26.8°C). Salinity values were highest during dry season (27 psu) and were lowered by the input of fresh water in the rainy season (22 psu) (Table 1).

Juvenile *F. duorarum* in premolt stage D_0 comprised the majority of the captured shrimp (35.5%), followed by those in intermolt stage C (20%), and premolt stage D_1' (19.6%). Molt stages C and D_0 are the ones of the largest duration and while in these stages, shrimp display an active feeding behavior. During the last premolt (D_1'', 11.2%; D_1''', 3.1%) and early postmolt stages (A, 1.6%; B_1, 4.25%) organisms do not feed, burrow and therefore are more difficult to capture (Fig. 2).

Stomach Contents

During dry, rainy and the Nortes seasons, 17.3%, 46.6%, and 63.9% of the 124 stomachs analyzed were full. A total of 16 taxa were identified in the stomach contents and this number increased between the dry (7 taxa), rainy (11 taxa), and the Nortes (12 taxa) seasons. The percentage of the amorphous animal tissue diminished in the rainy and the Nortes seasons. According to the frequencies of occurrence and the IIMP (Table 2), Amphipoda and Copepoda were important preys of *F. duorarum* during the whole sampling period ($\chi^2 = 30.5$ and $\chi^2 = 35.3$, respectively, $\alpha = 0.05$) and were associated with all three seasons. Nematoda ($\chi^2 = 14.8$) was notable in two seasons, dry and rainy, Ostracoda ($\chi^2 = 15.6$) in dry and the Nortes and Diptera ($\chi^2 = 1.5$) in rainy season.

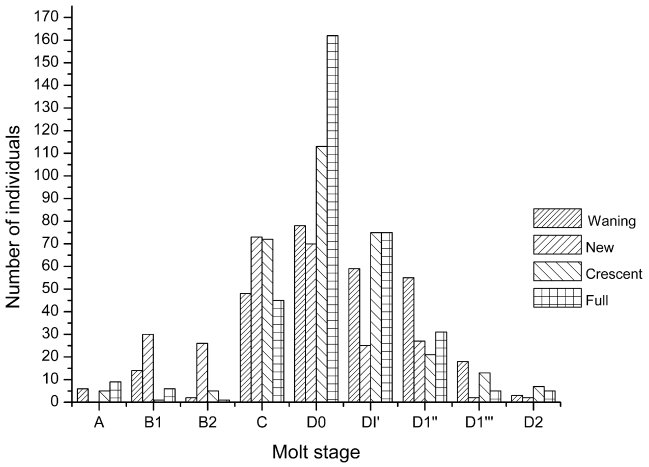

Fig. 2. Distribution of the number of juvenile *Farfantepenaeus duorarum* per molt stage and moon phase (N = 1189).

Detritus vegetal in the stomach contents represented between 7% and 8% in all three seasons. Filamentous algae, diatoms, and the seagrass *H. wrightii* could be identified. The *H. wrightii* represented 2.5% in the dry season, 27.5% in the rainy season, and 13.6% in the Nortes season (Table 2, Fig. 3). Significant differences ($p < 0.05$) were found in the trophic level of *F. duorarum* across the seasons and a carnivore diet dominated during the dry season while an omnivore diet was found in the rainy and the Nortes seasons (Fig. 4). Regression analysis indicated that the variation of the trophic level of *F. duorarum* was not related to its weight variation ($R^2 = 0.003$, $p > 0.05$), but was directly related to the variation of trypsin ($R^2 = 0.95$) and inversely to α-amylase ($R^2 = -0.67$) activities.

Stable Isotopes (δ^{13}C and δ^{15}N)

Isotopic signatures of shrimp muscle tissue showed seasonal differences. Higher values of δ^{13}C were found during dry and the Nortes seasons and were similar to those found for the *H. wrighti* samples. The δ^{13}C isotopic signature of the shrimp tissue captured in the rainy season was similar to the values obtained in the plankton samples. The lowest δ^{13}C value was observed in sediment (Table 3). The highest δ^{15}N values were found in shrimp tissue during the dry season and the lowest in the Nortes. Values from sediment, plankton and *H. wrighti* were lower than shrimp tissue values (Table 3).

Digestive Enzyme Activity

A significant interaction ($p < 0.05$) between season and molt stage was found for trypsin activity in juvenile shrimp's HPs (Fig. 5). In the dry and rainy seasons, trypsin activity did not change during the various molt stages. In contrast, in the Nortes season trypsin activity was significantly lower

Table 2. Food composition in stomach of juveniles *Farfantepenaeus duorarum* collected in annual season. Frequency of occurrence and Index of Importance (IIMP) of prey (N = 124). * (% of volume).

Food type	Ocurrence (%)			IIMP (%)	References related
	Dry	Rainy	Nortes		
Malacostraca Amphipoda	7.50	47.50	65.90	19.69	a,b
Isopoda	0.00	22.50	9.00	1.47	
Decapoda	2.50	5.00	2.20	1.30	b
Brachiopoda Anostracoda	0.00	0.00	4.50	0.25	
Cladocera	7.50	2.50	22.70	5.14	
Copepoda	12.50	50.00	77.20	29.34	a,b
Ostracoda	5.00	32.50	6.80	7.83	a
Pterygota Diptera	0.00	32.50	22.70	10.26	
Opisthobranchia Gastropoda	2.50	0.00	0.00	0.74	a
Granuloreticulosa Foraminifera	0.00	52.50	0.00	5.65	a
Acari Halacaroida	0.00	0.00	4.50	0.37	
Nematoda	7.50	2.50	29.50	9.60	a
Annelida Oligochaeta	0.00	2.50	9.00	0.82	
Polychaeta	0.00	7.50	9.00	1.02	a,b
Halodule wrightii	2.50	27.50	13.60	6.52	
Amorphous animal tissue*	79.00	55.00	64.00		
Plant detritus*	8.00	7.00	7.00		
Sand*	11.00	19.00	13.00		

a: Schwamborn and Criales, 2000 (*F. duorarum*); b: Sánchez et al., 2002 (*L. schmitti*).

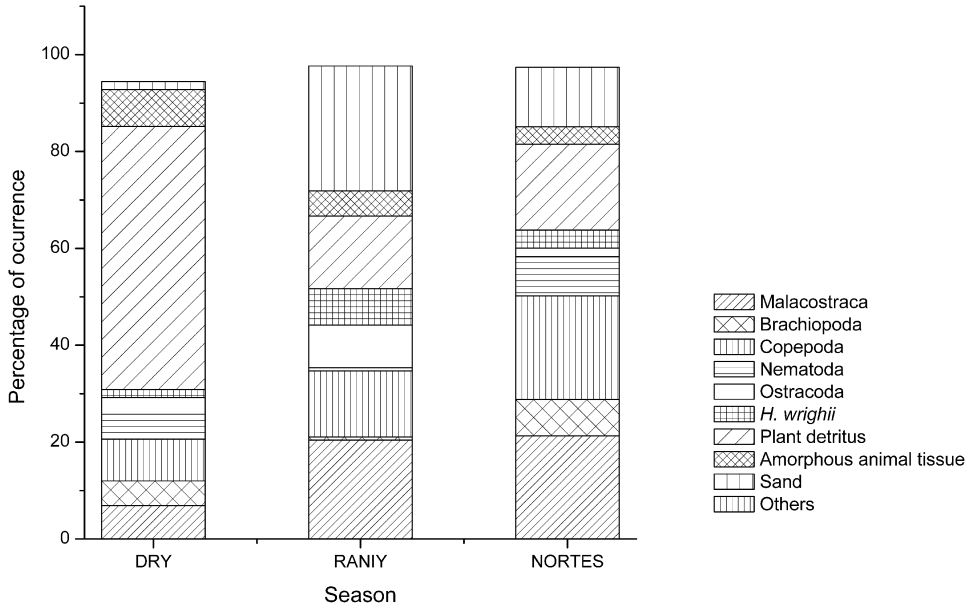

Fig. 3. Composition of the diet of *Farfantepenaeus duorarum* in dry, rainy and Nortes seasons (N = 124).

while organisms were in molt stage D_0 decreasing steadily until ecdysis ($p < 0.05$).

The α-amylase activity also exhibited a significant seasonal variation ($p < 0.05$), particularly in the dry season. During the Nortes season, the α-amylase activity reached values of 674 and 457 mU mg^{-1} HP at molt stages B_2 and C, respectively (Fig. 6).

The amylase:trypsin ratio (A/T) calculated for the three seasons and 6 molt stages had the lower value in the dry season and reached the higher values in the Nortes season, especially while organisms were in late premolt stages (Table 4). Trypsin and α-amylase analyses were not possible for molt stages A, B1 and D2 since organisms in these stages were not present in the sampling of all three seasons.

Trypsin and α-amylase Isoforms

Polyacrylamide gels revealed three trypsin isoforms during a complete molt cycle. The gel that contained trypsin from shrimp captured in the rainy season showed the main expression during a molt cycle. Three genes were identified. Molecular weight indicated a first isoform localized at 19.4 kDa, a second one at 20.7 kDa and a third one at

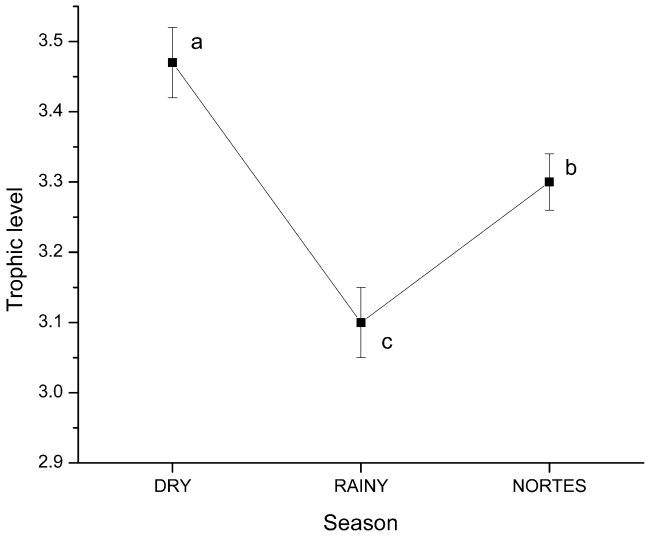

Fig. 4. Variation of trophic level calculated in *Farfantepenaeus duorarum* (N = 124).

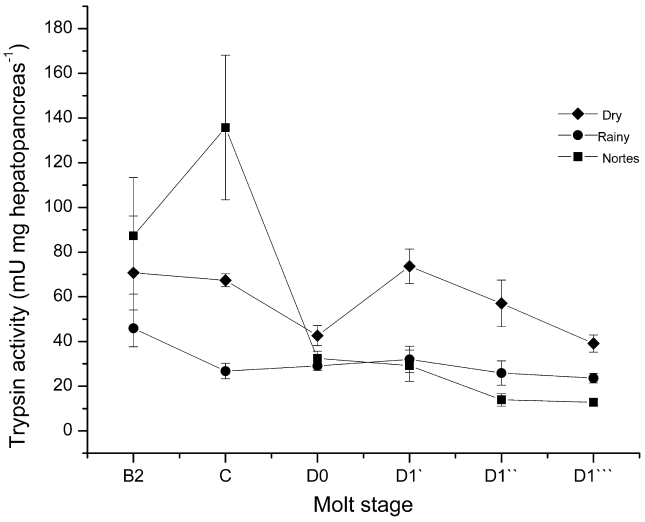

Fig. 5. Tryspin activity (mU mg^{-1} hepatopancreas) of juvenile *Farfantepenaeus duorarum* during an annual cycle. Interaction between molt stages and annual seasons (N = 89).

22 kDa. Gels analysis showed trypsin expression in the rainy season, with a unique functional gene at 19.4 KDa observed in postmolt stages A and B_1. Other stages of the molt cycle displayed all three of the identified isoforms. During the Nortes, the presence of two trypsin isoforms, at 19.4 kDa and 20.7 kDa, was observed throughout the molt cycle (Fig. 7). Another protease, chymotrypsin, was observed in bands located between 24 kDa and 36 kDa. Although its presence could be identified in the three seasons, it was more noticeable during the dry season (Fig. 7).

There is a seasonal change in the expression of α-amylase. This enzyme is a complex of two systems that are differentially expressed. If system I can be expressed by three alleles, then system II possessed 5 or 6 alleles. System I was represented by only one allele in juvenile shrimp sampled in each season. System II was expressed by 3-4 alleles in the dry season and 5-6 alleles in the rainy season throughout the molt cycle. In the Nortes season, system II was expressed by fewer alleles, except in premolt stage D_1' with 6 alleles; postmolt stage A was not present (Fig. 8).

Discussion

The results obtained in this study show that in the wild juveniles of *F. duorarum* displayed biochemical adaptations for the digestion and assimilation of food. The environmental conditions of Celestun are strongly affected by the seasonal cycle dominated by wind, temperature, and rainfall regimes, which in turn modified the nutrient input that is correlated with changes in benthic diversity (Pech et al., 2007). During the dry season, Celestun lagoon displays low primary and secondary production (Tapia-Gonzalez et al., 2008) which is reflected in the stomach contents of sampled *F. duorarum* at this time of the year. Low prey occurrence and low stomach fullness coincided with low HP's trypsin and α-amylase activities as well as high isozyme expression for both enzymes. As the lagoon productivity increases in the rainy season (Tapia-Gonzalez et al., 2008), the stomach fullness also

Table 3. Stable isotope (δ^{13}C and δ^{15}N), C and N values of juvenile *Farfantepenaeus duorarum*, sediment, plankton and *Halodule wrightii* from Celestun lagoon (N = 31). * ($\geqslant$ 0.27 mm).

	$d^{15}N_{AIR}$ (‰)	$d^{13}C_{VPDB}$ (‰)	%N	%C
Season				
Dry	14.21 ± 1.99	−21.64 ± 1.17	14.92 ± 0.14	44.88 ± 0.4
Rainy	12.75 ± 2.05	−17.68 ± 1.58	9.69 ± 0.32	43.87 ± 0.99
Nortes	11.86 ± 1.16	−23.06 ± 0.73	14.32 ± 0.14	45.10 ± 0.39
Productivity				
Sediment *	8.86	−8.04	0.33	13.7
Dry				
Rainy	9.04	−7.58	0.37	13.57
Nortes	8.51	−7.22	0.30	13.28
Plankton *				
Rainy	8.22	−10.63	0.85	16.26
Nortes	7.68	−18.43	5.06	34.44
Halodule wrightii	5.58	−20.28	1.8	36.2

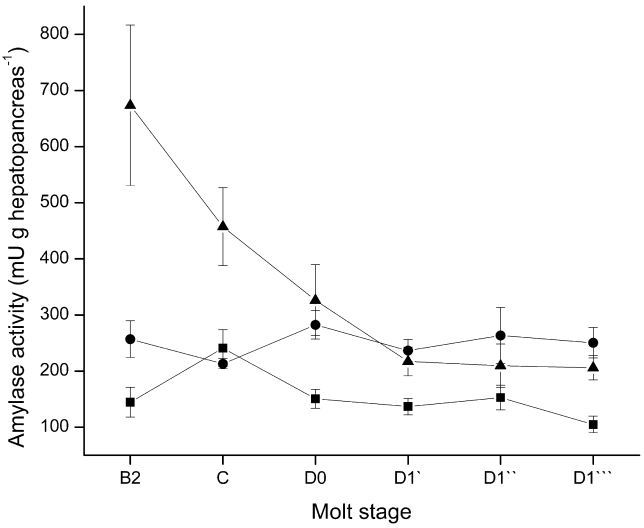

Fig. 6. α-amylase activity (mU mg^{-1} hepatopancreas) of *Farfantepenaeus duorarum* sampled during the three main seasons. Interaction between molt stages and annual seasons (N = 89).

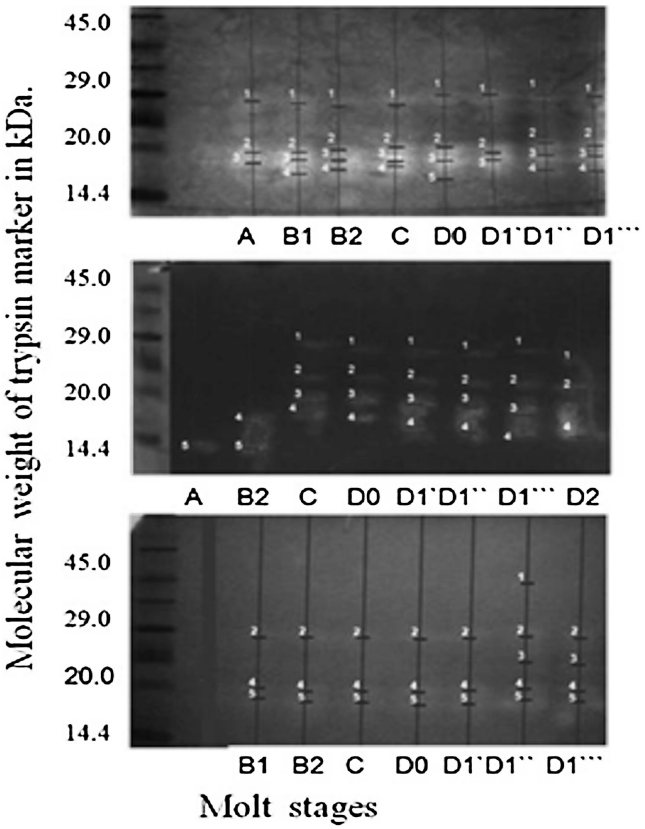

Fig. 7. Trypsin isoforms of *Farfantepenaeus duorarum* during the three determined sampling period from April 2007 to February 2008. MWM: Molecular weight of marker that identify trypsin in 19.4, 20.7 and 22.0 kDa (N = 89).

increased, but digestive enzymes activities remained low. A peak of activity appeared during the Nortes that fitted with the lowest isozyme expression of the digestive enzymes. At this time of the year, organisms showed a peak of activity for both enzymes in post and intermolt stages when feeding intensity was high.

The occurrence of prey in the benthic community of the Celestun lagoon changed according to seasons (Pech et al., 2007) and shrimp stomach contents reflected this trend. High crustacean abundance was observed during the Nortes and dry seasons both in the benthic community and in the stomach contents of juvenile shrimp. Copepods and amphipods, together with ostracodes and nematodes, dominated the shrimp's stomach contents (Table 2) and were important to define their trophic level. The main prey types upon which *F. duorarum* feeds, mainly copepods and amphipods, allowed classifying this species as carnivorous as Schwamborn et al. (2000) and Sanchez et al. (2002) also reported. *Farfantepenaeus duorarum* had a diet composed by planktonic organisms of marine origin, such as other penaeids (Dall et al., 1990) rather than prey picked out from detritus (Primavera, 1996; Macia, 2004). The feeding habits of this species changed with prey availability, controlled by abiotic factors (temperature and light intensity) that modify both the ecosystem productivity and the digestive enzymes activities in shrimp's HPs (Alpuche et al., 2005). As in *Parapenaeus longirostris* (Lucas, 1846), the molt cycle, which is controlled by lunar phases, affected the feeding behaviour of juveniles of *F. duorarum* (de Coursey, 1983). The analysis of weekly sampling allowed observe the molt stages distribution along the lunar cycle with a predominance of intermolt stages C, which has the longest time span (Fig. 2). Organisms in molt stages close to ecdysis had empty stomachs since shrimp are unable to use mouth structures formed by chitin, hence restraining the possibilities of food intake while the exoskeleton has not hardened up (Le Moullac et al., 1996; Vega-Villasante et al., 2000; Molina-Poveda, 2002). When in late premolt D_1''' and postmolt A and B, we observed a drop in enzymatic activity. During the phase of tegument stability in intermolt C (Sanchez-Paz et al., 2007), trypsin (Fig. 5) and α-amylase (Fig. 6) activities increased especially during the Nortes. Seasonal patterns modified the physicochemical parameters of the lagoon environment and the availability of prey so shrimps switched their feeding habits to maximize ingestion. During the rainy season, the lowest values of the enzyme activity can be related to a sudden stressful habitat (high

Table 4. Ratio amylase/trypsin for the identification of the level of herbivory in different molt stages of *Farfantepenaeus duorarum* (N = 178). (Mean ± Standard Error).

Annual season	B_2	C	D_0	D_1'	D_1''	D_1'''
Dry	2.3 ± 1.6	3.6 ± 0.5	3.6 ± 0.4	1.9 ± 0.2	2.8 ± 0.2	2.6 ± 0.2
Rain	6.1 ± 1.0	8.9 ± 1.8	9.8 ± 0.7	8.3 ± 1.4	12.0 ± 3.0	11.0 ± 2.0
Nortes	7.9 ± 2.5	4.3 ± 1.4	9.9 ± 1.1	8.6 ± 1.6	19.0 ± 5.0	16.0 ± 2.0

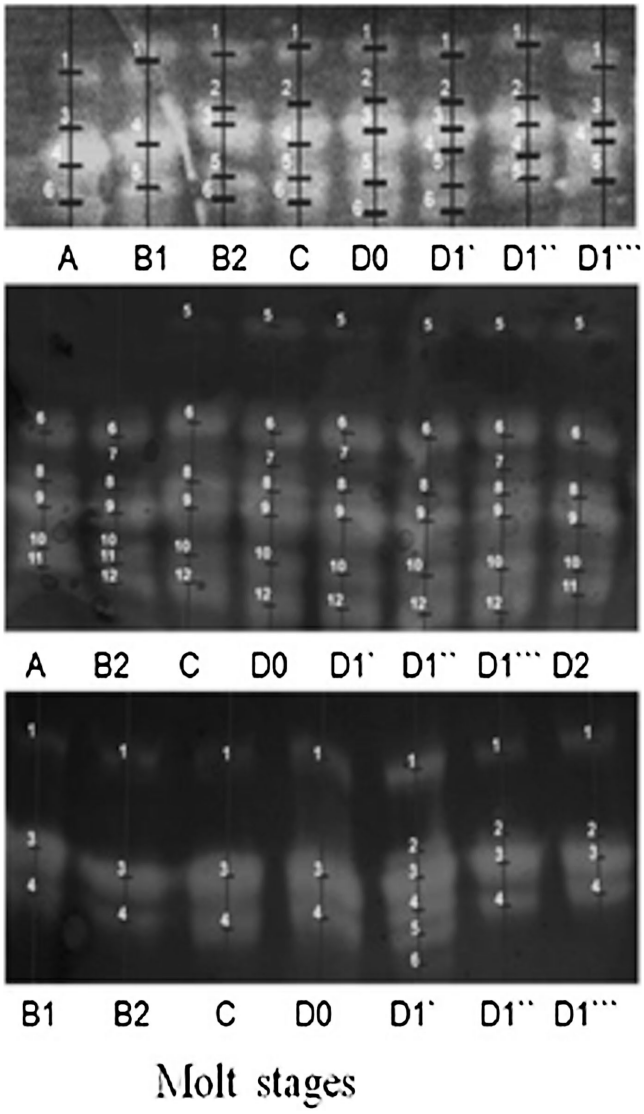

Fig. 8. α-amylase isoforms of *Farfantepenaeus duorarum* during the three determined sampling period from April 2007 to February 2008 (N = 89).

water temperature) that led to a scanty mobility of shrimp. Highest mean temperature (30.7°C) measured during this season can have an impact on trypsin activity even if its maximum between 40-70°C (Le Moullac et al., 1994; Sainz et al., 2004) but salinity was below the optimum for the species (22 psu). Trypsin activity reached a maximum during Nortes season, with a 148% increase compared to the value obtained during the dry season (Fig. 5). Changes in the environment are mirrored by variations in enzyme activity that enhanced survival in such a habitat. When a higher load of nutrients was present in the lagoon (Pech et al., 2007), the highest α-amylase activity was measured. Trypsin and α-amylase were complementary (Lovett and Felder, 1990; van Wormhoudt et al., 1998) and accounted for 60% of the digestive process. Variation in trypsin activity was similar to variations in trophic level (Fig. 9) suggesting that *F. duorarum* consumes protein as the main energy resource in the dry season (Cuzon et al., 1980; Barclay et al., 1983). Drop in trypsin activity observed in the rainy season could be a strategy to avoid the energetic use of protein, obtaining energy from glucose that is the primary source of energy used by the hepatopancreas during food scarcity events (Sanchez-Paz et al., 2007). The lowest expression of α-amylase activity during dry season could be the result of environmental stress. During the rainy season begins the removal of nutrients deposited in the estuarine soil, reactivating the food chain (Pech et al., 2007). While consuming protein sources, we observed that *F. duorarum* increased the consumption of carbohydrates reaching a peak during the Nortes. The presence of active enzymes digesting vegetal products indicates that *F. duorarum* is not a strict carnivore but is able to metabolize energy from various trophic sources, including vegetal detritus (Brethes et al., 1994).

As described for *L. stylirostris* (Wabete, 2006), shrimp reduced food intake at low temperatures due to a decrease in the metabolic activity that takes place in the cold season. α-amylase is expected to decrease in a lack-of-food event and accordingly, low values (120 mU mg^{-1}) were found during the dry season while trypsin activity showed an opposite trend.

A/P or A/T ratios give an indication on the level of herbivory or carnivory at each time of the year (Rodriguez et al., 1994; Gaxiola et al., 2009). This ratio was used to classify *Homarus americanus* Milne Edwards, 1837 as more herbivorous than penaeids (Jones et al., 1997). It was possible to identify the sequence of carnivore-herbivore shifts during the molt cycle both in the rainy and the Nortes seasons, and annual changes in the values of $\delta^{15}N$ allowed to classify *F. duorarum* as a carnivore in the dry season (trophic level 5), as an opportunistic generalist during rainy season and the Nortes (trophic level 4), which is the trophic level also given by Christensen and Pauly (1992) (Fig. 4). During these seasons there was an increase in the vegetal material available for shrimp intake (Table 3) observed as the increase in A/P ratio. In intermolt stage D_0 cells of HP produce hemocyanin and its increase at premolt stages prefigured a decrease in trypsin rather than an increase in amylase activity. The expression of enzymatic activity of *F. duorarum* examined by polyacrylamide gels (Figs. 7, 8) showed three bands next to 20 kDa in the specific case of trypsin that can be referred to the forms a, b and c, in contrast to five isoforms reported for *L. vannamei* (Klein et al., 1996). The presence of chymotrypsin was evidenced by the band located between 24 and 29 kDa. This enzyme is needed to complete the hydrolysis processes of a variety of materials usually consumed by crustaceans (Lovett and Felder, 1990; Brethes et al., 1994; Le Moullac et al., 1996).

Band patterns of α-amylase were similar to those reported by van Wormhoudt et al. (2003), showing system I formed by three alleles and system II formed by five. Low values of α-amylase expression, with five isozymes, were found in the Nortes season. At this time, a low value of the A/P ratio (2.8 ± 0.8) was also found. On the other hand, high values of α-amylase activity, with a high A/P ratio (10.7 ± 0.7), were found during the Nortes when gastric repletion reached a maximum and IIMP values showed the importance of copepods and amphipods in the diet, while vegetal detritus was present in low percentages. Variation of $\delta^{13}C$

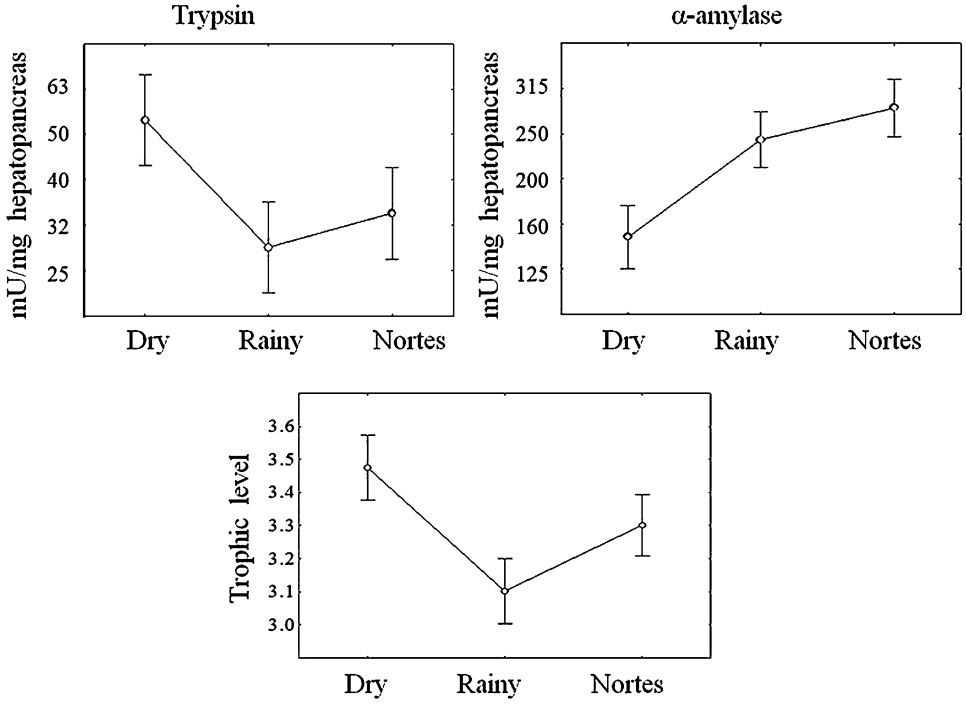

Fig. 9. Seasonal variation of trypsin (N = 89), α-amylase (N = 89) and trophic level (N = 124) of *Farfantepenaeus duorarum*.

values from $-18‰$ (in the rainy season, with an observed carnivore diet), $-21‰$ (in the dry season and omnivory) to $-24‰$ (Nortes) were observed in shrimp muscle. The shift towards herbivory can also be inferred based on the stomach contents analysis that showed greater values of occurrences of *Halodule* with a δ^{13}C value at $-23‰$. Other phytobenthic materials were found in the stomachs but δ^{13}C values for sediment and plankton were $-7‰$ and $-18‰$ respectively. *Farfantepenaeus duorarum* was usually associated with *H. wrightii*, in the Nortes when winds produce water currents that facilitate the presence of shrimp in the lagoon enhancing the importance of sea grass habitats during this time of the year. Marine or continental influences (Herrera Silveira et al., 1999; Pech et al., 2007) had an impact on the taxa available and the isotopic signatures of shrimp tissue that indicate the origin of food sources at each time of the year. In general, mangrove-derived organic matter was not the principal source of the food used by *F. duorarum* (Bouillon et al., 2002). The understanding of the dynamics of the shrimp's habitat would contribute to a better protection of this resource in the future.

The results obtained in the present study showed that juvenile *F. duorarum* display different biochemical adaptations for food digestion and assimilation in the wild. There is a synergistic effect of molt stages and seasonal variations of food availability on trypsin and α-amylase activities measured in juvenile *F. duorarum*. Phenotypic expression was a consequence of the variation of the feeding habits related to molting.

The accumulation of organic reserves to meet the energy demand required at each stage of the molting cycle of juvenile *F. duorarum* correlated well with the expression of hepatopancreatic enzymes such as trypsin and α-amylase. Both enzymes displayed a significant variability throughout an annual period of observations in this study. However, their activity was apparently triggered by the nature of the food available in the shrimp's nursery area. Higher activity of trypsin was observed in the dry season, compared to rainy season, when values of gastric repletion reached a minimum but the occurrence of vegetal material in the stomachs reached its maximum (2%) in coincidence with the highest value of the index of trophic level and δ^{15}N (14‰). At this time of the year, shrimps can be ranked in the fifth trophic level. δ^{13}C value of $-20.6‰$ suggested that juvenile *F. duorarum* grazed all the year on marine zooplankton eating Copepoda and Amphipoda associated with nursery where *H. wrightii* was present.

In the rainy season, the isotopic signature of δ^{13}C indicated that shrimp consumed materials of littoral or land origin, consistent with the drag of organic matter due to the rain. During Nortes, the isotopic signature of nitrogen was low enough (11‰) to position juveniles of *F. duorarum* in the fourth trophic level, as a general opportunistic feeder. At this time of the year, δ^{13}C values were close to the value for *H. wrightii*. This plant served as a refuge habitat as currents in the lagoon increased due to high northerly winds.

The limited composition of food resources for *F. duorarum* in the dry season, contrasted with an abundance noticed during the Nortes. However, the trypsin activity made it clear that protein utilization is the main nutrient during the three seasons. α-amylase was expressed in digestive process as part of a compensatory mechanism with carbohydrates intake during the three seasons, although vegetal material was scarce in the diet of *F. duorarum*. A modulation of enzymatic expression in *F. duorarum* was subjected to the type and abundance of food components available along the year but

physiological changes associated with ecdysis acted upon enzymes expression as well.

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