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Research Article

Effect of protein level in diet and feeding schedule on the digestive enzymatic activity of *Macrobrachium tenellum* juveniles

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ABSTRACT. This study assessed the effect of protein level in diet and feeding schedule on the digestive enzymatic activity of *Macrobrachium tenellum* juveniles. Freshwater prawn juveniles (0.31 ± 0.004 g and 32.48 ± 0.27 mm) were fed diets with 20, 25, 30, 35 and 40% crude protein (CP) (p/p). In a second experiment, feeding schedules were assessed at 08:00, 14:00 and 20:00 h with feed at 30% CP with juveniles of 0.22 ± 0.002 g and 29.36 ± 0.28 mm. In both experiments, five organisms were sampled after 60 days of treatment in a time-lapse of one hour (08:00 to 09:00 h) per experimental tank and treatment. The enzymatic extract was obtained from the complete organisms to determine specific general protease, trypsin, chymotrypsin, amylase and lipase activities. Greater general protease, trypsin and lipase activities were observed in feed at 40% CP. The feeding schedules with greater general protease, trypsin, lipase and amylase activities were found at 14:00 and 20:00 h. Chymotrypsin did not show differences among treatments at CP levels or feeding schedules. Protein level in diets for *M. tenellum* modified basal digestive enzymatic activity and increased proteolytic and lipolytic activities, while it decreased amylolytic activity as the inclusion of CP level increased in the diet; likewise, the activity was modified by feeding schedule, showing a direct correlation between the increase in digestive enzymatic activity and food offering at the end of the day.

Keywords: *Macrobrachium tenellum*; prawn; feeding schedule; digestive enzymes; digestive physiology

INTRODUCTION

Among the most important commercial *Macrobrachium* species, the one that stands out in the Mexican Pacific coasts is the fresh-water prawn *M. tenellum* (Ponce-Palafox *et al.*, 2002; Espinosa-Chaurand *et al.*, 2011). Given its high aquaculture potential (Vega-Villasante *et al.*, 2015), its tolerance to environmental conditions as well as to its low aggressiveness toward its congeners, and its high density in the environment (Ponce-Palafox *et al.*, 2002). Currently, cultivation techniques for its farming are in the developmental stage based on the knowledge that has been generated on the species through biological, ecological, physiological and nutritional research. Within the last ones, both the type of food and feeding strategy should be considered as they represent factors of vital importance in providing the necessary energy for its adequate growth and development, which is reflected in the growth in farmed animals (Jayachandran, 2001). In the successful cultivation of prawn, management and feeding are the most important factors because food constitutes from 40 to 60% of the production costs (Vega-Villasante *et al.*, 2011, 2014), and protein is the most essential and expensive ingredient (Fernández-Giménez, 2013).

The previous information shows the great importance of studying the physiological digestive process because it modulates consumption and better use of food; specifically, the digestive enzymatic activi-

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ty is responsible for crucial activity in the digestive processes (Molina *et al.*, 2000). The digestive enzymes in crustaceans can hydrolyze a great variety of essential substrates as nutrients that are regulated by factors, such as life stage, molt cycle, body size (Cadena-Llomitoa, 2000; Brito *et al.*, 2001; Guzmán *et al.*, 2001), protein source and level in the diet, feeding amount and frequency, and the circadian cycle (Molina *et al.*, 2000).

In several crustacean species, the effect of food quality (different protein sources, biofloc use, food supplements or additives) (Perera *et al.*, 2012; Xu & Pan, 2012; Anand *et al.*, 2013; Fernández-Giménez, 2013; Gallardo *et al.*, 2013) and feeding frequency (Casillas-Hernández *et al.*, 2006; Carrillo-Farnés *et al.*, 2007; Avenido & Serrano, 2012; Sacristán *et al.*, 2013) on digestive enzymatic activity have been studied. Particularly, the protein level in the diet has been mentioned to affect the digestive enzymatic activity in crustaceans, increasing or decreasing the presence of enzyme units in the hepatopancreas (Lee & Lawrence, 1985; Chen & Lin, 1989; Sridhar *et al.*, 1995; Galindo *et al.*, 2002). Likewise, Casillas-Hernández *et al.* (2006) confirmed synchrony between shrimp feeding activity and enzymatic variation, which could be exploited to improve the production parameters by adjusting feeding schedules according to enzymatic variation.

Therefore, this research aimed to assess the effects of protein levels in the diets and feeding schedule on the digestive enzymatic activity of *M. tenellum* juveniles, contributing to the knowledge on its digestive biology and physiology and for aquaculture management improvement.

MATERIALS AND METHODS

Organisms, experimental conditions, and sampling

The fresh-water prawn *Macrobrachium tenellum* juveniles were collected in a natural affluent zone of Puerto Vallarta, Jalisco, Mexico (20°42'19"N, 105°13'16"W; 10 m.a.s.l.) and transferred to the Experimental Aquaculture Laboratory of Centro Universitario de la Costa, Universidad de Guadalajara. The prawns were acclimated to laboratory conditions and made up a stock until further use.

Two 60-day independent bioassays were performed to assess the response of *M. tenellum* to protein levels in the diet and feeding schedules, particularly on digestive enzymatic activity. The first one consisted on feeding *M. tenellum* juveniles with five experimental diets (20, 25, 30, 35 and 40% CP; Table 1) using 225 prawns randomly distributed (initial weight and size of 0.31 ± 0.004 g and 32.48 ± 0.27 mm, respectively) in 15 experimental 70-L tanks (15 ind per tank), and fed once a day (14:00 h). In the second bioassay, 135 *M. tenellum* juveniles (weight 0.22 ± 0.002 g and size 29.36 ± 0.28 mm) distributed randomly in nine experimental 70-L tanks (15 ind per tanks) were fed a diet with 30% CP (Table 1), and the treatments corresponded to three different feeding schedules, 08:00, 14:00 and 20:00 h. In both experiments, treatments were assessed in triplicate, and the experimental tanks were maintained with clear water by cascade filters and under controlled oxygen conditions (5.00 ± 0.26 mg L^{-1}), temperature (28.0 ± 1.5ºC), pH (7.8 ± 0.4) and photoperiod (12 h light: 12 h darkness); prawn feeding was once a day with 10% of live weight to assure satiation.

Once the 60-day bioassay (at day 60) concluded, five individuals in the intermolt stage were randomly sampled in a time-lapse of one hour (08:00 to 09:00 h) per experimental tank and treatment. Sample taking in each case was carried out after the juveniles fasted and rested for 24 h. The sampled individuals were sacrificed with cold water at 4°C, and then frozen at -20°C for storage until biochemical analysis at the Northwest Biological Research Center, Comparative Physiology Laboratory, La Paz, BCS, Mexico.

Analysis of digestive enzymatic activity

Whole organisms were rinsed with distilled water, dried with absorbent paper and integrated to a homogenized pool with cold distilled water in a proportion 1:3, using a tissue homogenizer (Potter PRO 250®; Pro Scientific. Oxford, CT, USA). The resulting macerated tissue was clarified by double centrifugation (15,000 *g*; Ø rotor 9.5 cm) at 4ºC for 10 min, eliminating the lipid fraction, which was extracted by absorption with a micropipette for its disposal when it was found as supernatant. The clarified was recovered and preserved at -20ºC, considered as enzyme extract (EE) to which soluble protein and amylase, lipase, general protease, trypsin and chymotrypsin were determined by triplicate.

The micro-method Bradford (1976) was used to determine the amount of protein in enzyme extract using bovine albumin serum as standard. General protease activity was determined according to Vega-Villasante *et al.* (1995) and modified as follows: mixing 20 µL of EE, 230 µL of buffer Tris-HCl (50 mM, pH 8) and starting the reaction adding 500 µL of azocasein (0.5% in Tris-HCl 50 mM, pH 8) as substrate. The mixture was incubated in a water bath at 37°C for 60 min. The reaction was stopped with 500 µL TCA 20%, and the mixture was clarified by centrifugation (15,000 *g*, 25°C, 5 min). Absorbance was measured at 440 nm.

Table 1. Ingredients and proximal composition of experimental feed (g per 100 g of dry weight). [a]g per 200 g mineral premix: KCl, 28.57; $MgSO_4.7H_2O$, 28.57; $ZnSO_4.7H_2O$, 5.14; $MnCl_2.4H_2O$, 1.34; $CuSO_4.5H_2O$, 0.29; Kl, 0.29; $CoCl_2.2H_2O$, 0.14; Na_2HPO_4, 135.43, [b]g per 900 g vitamin premix: vitamin A acetate, 100,000 UI; vitamin D_3, 850 UI; di-alpha-tocopheryl acetate 2,000 UI; menadione, 2; thiamine-HCl, 0.5; rivoflavin (B_2), 3; pyridoxine HCl (B_6), 1; DL-Ca-pantothenate, 5; nicotinic acid, 5; biotin, 0.05; inositol, 5; Vitamin B_{12}, 0.002; folic acid, 0.18. *Proximate analysis of tested diet. Analytical method: [c]AOAC, 2012 (micro-Kjendahl method), [d]AOAC, 2012 (Soxhlet method), [e]AOAC, 2012 (Weende method), [f]AOAC, 2012 (oven at 550°C), [g]nitrogen free extract = 100 - (% crude protein + % total lipids + % crude fiber + % ashes).

	Protein level				
	20%	25%	30%	35%	40%
Inclusion of ingredients by experimental feed (g per 100 g)					
Whole wheat flour	38.00	38.00	38.00	38.00	35.09
Corn starch	27.18	19.82	12.47	5.11	0.00
Soy pasta	10.00	10.00	10.00	10.00	10.00
Fish meal	9.11	16.82	24.58	32.31	40.67
Cod liver oil	5.71	5.33	4.96	4.58	4.24
Squid meal	5.00	5.00	5.00	5.00	5.00
Mineral crustacean premix[a]	2.50	2.50	2.50	2.50	2.50
Calcium carbonate	1.00	1.00	1.00	1.00	1.00
Soy lecithin	1.00	1.00	1.00	1.00	1.00
Vitamin crustacean premix[b]	0.30	0.30	0.30	0.30	0.30
Vitamin C	0.10	0.10	0.10	0.10	0.10
Choline chloride	0.10	0.10	0.10	0.10	0.10
Proximal composition in dry weight* (g per 100 g in dry weight)					
Crude protein[c] (N×6.25)	20.79 ± 0.27	26.41 ± 0.04	32.01 ± 0.16	37.63 ± 0.19	42.55 ± 0.23
Total lipids[d]	7.89 ± 0.12	7.86 ± 0.13	8.15 ± 0.07	7.99 ± 0.04	8.47 ± 0.10
Crude fiber[e]	0.67 ± 0.06	0.43 ± 0.03	0.47 ± 0.05	0.36 ± 0.06	0.34 ± 0.06
Ash[f]	6.55 ± 0.04	7.84 ± 0.05	9.04 ± 0.02	10.52 ± 0.02	11.73 ± 0.01
Nitrogen-free extract[g]	64.11	57.46	50.32	43.50	36.91
Energy (kcal g^{-1})	4.36 ± 0.03	4.42 ± 0.01	4.55 ± 0.02	4.42 ± 0.03	4.34 ± 0.03

The control was prepared similarly, but the EE was added after the TCA solution. The general protease activity was expressed as the number of protease units per mg of soluble protein. One protease unit was defined as the amount of enzyme required for increasing 0.01 absorbance units at 440 nm per minute.

The chymotrypsin activity was determined according to Asgeirsson & Bjamason (1991): 10 µL of EE, 890 µL of Buffer (Tris 44.4 mM, $CaCl_2$ 5.5 mM, pH 8) were mixed, and the reaction started with 100 µL de BTEE (5 mM in DMSO) as substrate. The mixture was incubated at room temperature (25°C) for 10 min. The reaction stopped by boiling it for 15 min. Once the reaction mixture cooled down (25°C), absorbance was read at 256 nm. The control was prepared similarly with denaturalized enzyme extract. The chymotrypsin activity was expressed as the number of units of chymotrypsin per mg of soluble protein. One unit of chymotrypsin was defined as the amount of enzyme required for increasing 0.01 absorbance units at 410 nm per minute.

The trypsin activity was determined by mixing 20 µL of EE and 1,250 µL of substrate solution (100 mM of Na-Benzoyl-DL- Arginine- p-Nitroanilide in 1 mL of DMSO, graduated at 100 mL with buffer Tris-HCl 50 mM, $CaCl_2$ 10 mM, pH 8.2). The mixture was incubated at room temperature (25°C) for 10 min, and 250 µL of acetic acid at 30% to stop the reaction, was added. Absorbance was read at 410 nm; the control was prepared similarly with EE denatured (5 min in boiling water bath). Trypsin activity was expressed as the number of trypsin units per mg of soluble protein. One trypsin unit was defined as the amount of enzyme required for increasing 0.01 absorbance units at 410 nm per minute.

Amylase activity was determined by the method of Vega-Villasante *et al.* (1993): 500 µL of buffer Tris-HCl (50 mM, pH 8), 5 µL of EE were mixed, and the reaction started with 500 µL of soluble starch (1% in Tris-HCl, 50 mM, pH 8) as substrate. The mixture was incubated at room temperature (25°C) for 10 min, and 200 µL of sodium carbonate (2 N) and 1,500 µL of dinitrosalycilic acid (DNS) reagent to stop the reaction,

were added. The mixture was placed in a water bath to boil for 15 min; then, volume was adjusted at 10 mL with distilled water, and absorbance was read at 550 nm. The control was prepared similarly, but EE was added after the DNS reagent. The amylase activity was expressed as the number of units of amylase per mg of soluble protein. One amylase unit is defined as the amount of enzyme required for increasing 0.01 absorbance units at 550 nm per minute.

The lipase activity was determined by the method of Nolasco *et al.* (2018): The incubation mixture (200 µL total volume) of the micro method procedure was as follows: 10 µL sodium taurocholate (NaTau) (100 mM) emulsifier reagent in a 96-well microplate, plus 50 µL Tris-HCl buffer (200 mM) and 10 µL lipase extract (LE) (it can be diluted 1:10) were added. Then, to start the reaction 10 µL BNC (20 mM), substrate reagent was added. The microplate was agitated by "8" movements on the table, and incubate for nine minutes at room temperature (25ºC). After incubation, the color reaction was produced by the addition of 10 µL FBBB (20 mM) color developer, followed by an additional 1-min incubation, to get a 10 min total incubation. The reaction was stopped with 110 µL TCA-SDS stopper-clarification reagent (trichloroacetic acid (TCA) 2 and 12.5% sodium dodecyl sulfate (SDS)) and then read for absorbance at 540 nm, after 1-min shaking at 1,500 rpm. Lipase activity was expressed as the number of lipase units per mg of soluble protein. One lipase unit is defined as the amount of enzyme required for increasing 0.01 of absorbance units at 540 nm per minute.

Statistical analyses

Data generated on the digestive enzymatic activity by the effect of feeding schedule and different protein levels in diet were analyzed by one-way analysis of variance (ANOVA). All the analyses were performed after the Kolmogorov-Smirnov normality ($\alpha = 0.05$) and Bartlett's homogeneity of variances ($\alpha = 0.05$) tests. Significant differences among treatments were determined by Tukey's multiple comparisons method ($\alpha = 0.05$). All the tests were performed by Sigmastat v.3.1 statistical software.

RESULTS

The enzymatic activities of the general protease, trypsin, chymotrypsin, amylase and lipase in *Macrobrachium tenellum* juveniles (final weight of 0.70 ± 0.11 g and final size of 38.79 ± 1.79 mm) fed five protein levels are shown (Fig. 1). The highest digestive protease level was observed in the organisms fed with 40% of CP in the diet. A significantly higher concentration of trypsin was observed in the treatment with 40% of CP in the diet ($P < 0.05$) while no difference was observed in chymotrypsin among treatments ($P > 0.05$). The amylolytic activity was significantly higher when organisms were fed with the diet containing 20% of CP, a lower protein concentration used in the experimental diets, followed by one with 25% of CP ($P < 0.05$). Lipolytic activity was significantly higher under the treatments of 35 and 40% of CP ($P < 0.05$).

The activities of the general protease, trypsin, chymotrypsin, amylase and lipase activities of *M. tenellum* juveniles (final weight of 0.56 ± 0.10 g and final size of 36.79 ± 2.71 mm) fed in different time schedules (08:00, 14:00, 20:00 h) are shown (Fig. 2). The general proteolytic activity significantly increased at 14:00 and 20:00 h compared to that expressed in the organisms fed at 08:00 h ($P < 0.05$) with no statistical differences between these two-time schedules ($P > 0.05$). In the case of the digestive trypsin activity, the concentration was statistically more significant at 14:00 h, followed by its activity at 20:00 h ($P < 0.05$). The digestive chymotrypsin activity did not show statistical differences between the three feeding schedules assessed ($P > 0.05$). The lipolytic activity was significantly higher at 20:00 h, followed by the activity at 14:00 h ($P < 0.05$). The amylolytic digestive activity was significantly higher at 14:00 and 20:00 h concerning the activity at 8:00 h ($P < 0.05$).

DISCUSSION

In general, this research found an increase in the digestive enzymatic activity as the inclusion of protein level increased, which agreed with that reported by Lee *et al.* (1984), Le Moullac (1995), and Gamboa-Delgado (2001), who demonstrated that in *Penaeus vannamei* an adaptation of digestive enzymes occurred according to the organism size, protein sources and level in the diet. Naik & Murthy (2012) indicated that the relative activity of digestive enzymes could be correlated to the nature and composition of the food consumed by the organisms. In contrast, for *P. setiferus,* Lee & Lawrence (1985) and Lovett & Felder (1990) mentioned that enzymatic activity varied inversely to protein level in the diet; this last behavior only agreed with that found in the amylase expression in our study.

Sagar *et al.* (2009) did not find a relationship between protein level and enzymatic activity of proteases, lipases and amylases in the organisms subjected to two crude protein levels (27 and 32%) while assessing the digestive enzymatic activity of *Macrobrachium rosenbergii* under these two diets. This result agrees with the activity observed in our study on chymotrypsin, where independently of the protein level

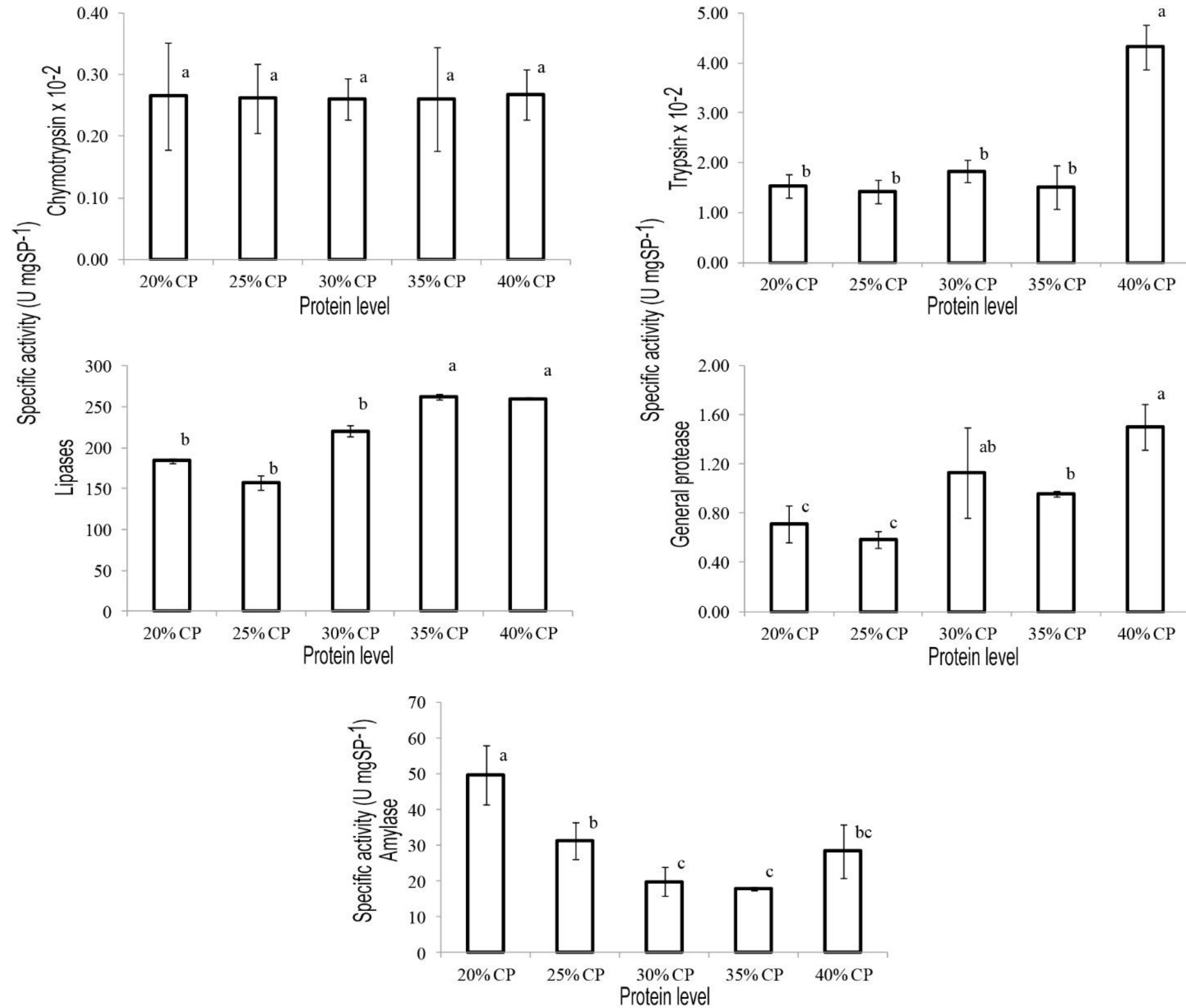

Figure 1. Comparison of general proteases, trypsin, chymotrypsin, amylases and lipases (specific activity, U mg of soluble protein^{-1}) of *Macrobrachium tenellum* juveniles fed five protein concentrations. Vertical lines in each bar indicate the standard deviation. Different letters on the bars indicate statistical differences ($P < 0.05$).

of the diet, a uniform concentration was maintained in *M. tenellum* individuals. On the other hand, Naik & Murthy (2012) found a higher protease activity in *M. rosenbergii* with diets related to high values of essential fatty acids.

Galindo *et al.* (2002) reported that the highest proteolytic activities in *Litopenaeus schmitti* were obtained with the diets of higher protein content, which was also observed in *M. tenellum* juveniles in our study. On the contrary, Perera *et al.* (2005) did not find differences in protease levels while testing two protein levels in the diet (25 and 35% of CP) with *Panulirus argus.* Besides, they mentioned that a difference was found depending on the type of ingredient used, observing that the digestive protease activity increased with the inclusion of squid flour in the diets compared to those without it.

A similar case was reported by Le Moullac *et al.* (1994) for the chymotrypsin activity in *Penaeus (=Litopenaeus) vannamei* larvae, and Perera *et al.* (2012) for trypsin activity in *P. argus* lobster, finding a higher activity of these enzymes when they added squid flour in the diets, concluding that feed type and quality influenced the digestive enzymatic activity (Isiordia-Pérez *et al.*, 2006; Naik & Murthy, 2012). On the other hand, Sridhar *et al.* (1995) observed no significant differences in trypsin activity in *Penaeus indicus* while using the three diets at different protein levels (30, 34 and 36% CP), which differed with our research on *M.*

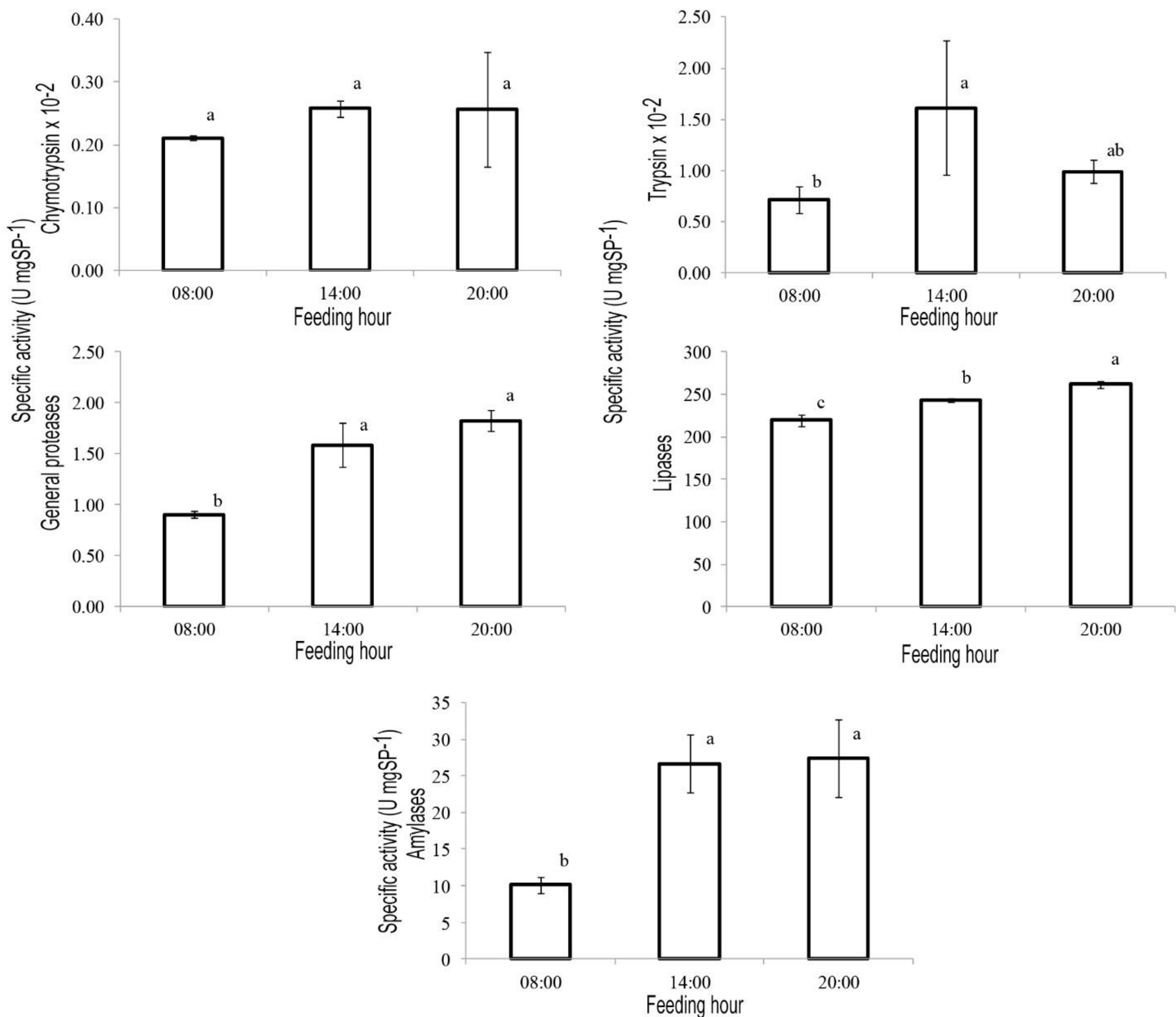

Figure 2. Comparison of general proteases, trypsin, chymotrypsin, amylases and lipases (specific activity, U mg protein^{-1}) of *Macrobrachium tenellum* juveniles in feeding schedules. Vertical lines in each bar indicate the standard deviation. Different letters on the bars indicate statistical differences ($P < 0.05$).

tenellum, where a difference was found in trypsin activity while increasing protein level to 40%.

In general, the digestive enzymatic behavior in *M. tenellum* was affected by the protein level in the diet, increasing proteases and lipases and decreasing amylases as the protein level of the diet increased. One cause of the inverse behavior between proteases and amylases could be related to the inclusion of different levels of other nutritive substrates in the experimental diets, while increasing protein concentration, a decrease in the level of nitrogen-free extract occurred (mainly starch provided by vegetal ingredients used in feed formulation that are generally used to adjust protein concentration in diets). Notably in this study, corn starch inclusion was reduced as protein level in diets increased, while the use of lipids could have increased to satisfy the energy demand of the organism due to the relationship mentioned between carbohydrates and proteins present in the experimental diets, it resulted in the increase of lipolytic activity as protein increased in the diets.

The hepatoprancreas is the main lipid reservoir; the composition and availability of the fatty acids present will depend on the combination between the diets offered, compound biosynthesis, and temperature (Ahmed *et al.*, 2015). To summarize, substrate specificity is one of the valid criteria to distinguish the type of enzymes present in an organism (Forjan *et al.*, 2000; Bornscheuer, 2002).

Substrate hydrolysis not only depends on the digestive enzymatic capacity of the organism to convert food in useful substances for its nutrition (Cruz-Suárez *et al.*, 2002), but also on the substrate quantity present and the feasibility of being hydrolyzed (its digestibility) besides the fact that each enzyme has a characteristic substrate concentration to be saturated (Whitaker, 1996; Nelson & Cox, 2009).

Although, undoubtedly, the presence and activity of digestive enzymes in crustaceans are influenced by many internal and external factors (Casillas-Hernández *et al.*, 2006; Fernández-Giménez, 2013), synchrony between feeding activity and digestive enzymatic variation could be confirmed when the digestive enzymatic activity was analyzed to see if it was affected by zootechnical management practices (Casillas-Hernández *et al.*, 2006). Thus, the possibility of performing an exogenous stimulation exists through different feeding schedules despite having a biphasic circadian rhythm in the enzymatic activity (Nolasco-Soria & Vega-Villasante, 2000).

By knowing the organisms' circadian rhythms of the digestive enzymatic activities, their feeding hour and frequency could be established (González *et al.*, 1995; Nolasco *et al.*, 1997; Nolasco-Soria & Vega-Villasante, 2000; Casillas-Hernández *et al.*, 2006), which would avoid excessive nutrient losses by lixiviation (Cuzon *et al.*, 1982; Casillas-Hernández *et al.*, 2006) and would help management programs for incorporating food to the digestive tract close to the time schedule of its maximum digestive capacity. Moreover, it would also increase hydrolysis speed and favor complete digestion of nutrients for their absorption and metabolism (Nolasco *et al.*, 1997; Casillas-Hernández *et al.*, 2006), and in consequence, improve organism growth (Cuzon *et al.*, 1982; Nolasco *et al.*, 1997; Casillas-Hernández *et al.*, 2006), pond conditions and feeding costs (Nolasco-Soria & Vega-Villasante, 2000).

Unequal patterns have been found, in enzymatic activity peaks, when the organisms are fed at different time schedules, modifying them depending on feeding time (Díaz-Granda, 1997; Molina *et al.*, 2000). Our research study agrees with those of Nolasco *et al.* (1997) on *Litopenaeus californiensis,* and Molina *et al.* (2000) on *P. vannamei* in which the behavior of general proteases, trypsin, chymotrypsin, amylases and lipases showed greater activity in the afternoon at night schedules. However, it disagreed with that mentioned by Díaz-Granda (1997) in *L. schmitti* for these enzymes, as a higher activity was reported in our study in the morning schedule (10:00 h), indicating a daytime habit preferably for the feeding activity of this last species.

Digestive enzymes play an important role in digestion and maximum use of food (Van Wormhoudt, 1977; Rosas *et al.*, 2001); knowing the moment at which the organisms are more capable of consuming and digesting food will have advantages for its management. For this reason, considering adjusting feeding schedules to digestive enzyme variation could result in better practices (Casillas-Hernández *et al.*, 2006).

To conclude, the protein level in the diets of *M. tenellum* modified basal enzymatic activity, increasing proteolytic and lipolytic activities, and decreasing amylolytic activity as the inclusion of crude protein level in the diet increased. Likewise, the activity was modified as the feeding schedule changed, showing a direct relationship in the increase of the digestive enzymatic activity with food offering at the end of the day (08:00 h < 14:00 h < 20:00 h). To our knowledge, these results have been reported for the first time in *M. tenellum*, which will allow establishing management techniques and nutritional considerations at the moment of formulating a diet for the species. Moreover, they provide information to understand better the digestive physiology of these organisms and their application to establish better feeding schedules.

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