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Research Article

Effect of dietary chitin on digestive enzyme activity, growth and survival of *Macrobrachium tenellum* juvenile prawns

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ABSTRACT. *Macrobrachium tenellum* is a freshwater prawn native from Mexico to Peru, with potential for cultivation. Currently, it is extensively exploited by local fisheries. To understand its chitin requirements, a formulated diet with chitin added (5, 10 15, 20, and 25%) was given to juveniles for 60 days. Growth, survival, and enzyme activity (trypsin, chymotrypsin, lipase, amylase, and chitinase) were measured every 15 days. Highest final weight and specific growth rate resulted from diets with 20% chitin. Poorest performance resulted from diets with 5 and 10% chitin. Chitin in the diet did not have a significant effect on survival. Statistically significant differences in enzyme activity were not found in any treatment.

Keywords: chitin, diet, nutrition, *Macrobrachium tenellum*, aquaculture.

INTRODUCTION

Within the American species of the genus *Macrobrachium* (Bate, 1868) only few are economically important. Some species have potential for cultivation: *M. carcinus* (Linnaeus, 1758), *M. americanum* (Bate, 1868), *M. digueti* (Bouvier, 1895), *M. acanthurus* (Wiegmann, 1836), *M. amazonicus* (Heller, 1962), and *M. tenellum* (Smith, 1871). *M. tenellum*, the long arm river prawn, has been considered a candidate for cultivation because it grows quickly, tolerates high densities and wide fluctuations in water conditions, and adapts well to captivity (Ponce-Palafox *et al*., 2002; Espinosa-Chaurand *et al*., 2011; García-Guerrero *et al*., 2013). This species is still common in its natural environment, which is necessary for recruitment.

Currently, there are few works focused on *M. tenellum* cultivation, particularly feeding and nutrition. Previous studies include García-Ulloa *et al*. (2004) who determined the effects of protein diets on growth, replacing fish meal with soybean meal and Espinosa-Chaurand *et al*. (2012) who determined the effect of levels of protein in diets on growth and survival of juveniles. Additional studies of feeding and nutrition are required to establish the type and balance of feed ingredients to produce good results under cultivation. Espinosa-Chaurand (2013) describes enzyme activity in this species. However, enzymes that act on chitin are not well studied yet. This information is required because *Macrobrachium* prawns consume crustacean exoskeletons, which provide ingredients for new exoskeletons after molting (Kumar *et al*., 2006; Zhang *et al*., 2014). Chitin digestive enzyme in this prawn has not been studied (García-Guerrero *et al*., 2013).

Most of the works on the effect of chitin in the diet of crustaceans have been developed with penaeid species (Chang *et al*., 1988; Shiau & Yu, 1998; Terwilliger, 1999; Chang *et al*., 2001; Casillas-Hernández *et al*., 2006; Chih-Hui & Chen-Chun, 2012). For *Macrobrachium* prawns, Kumár *et al*. (2006) compared the effect of natural chitin and purified chitin on the growth of *Macrobrachium rosenbergii* (De Man, 1879).

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Chitin in diets of prawns advance growth (Kumar *et al.*, 2006), promotes antimicrobial activity in diets (Somjit *et al*., 2005), and supports molting (Zhang *et al.*, 2014). In our study, we measured the effect of different levels of chitin in the diet for *Macrobrachium tenellum* juveniles on growth and digestive enzyme activity, with special emphasis on chitinases.

MATERIALS AND METHODS

Experimental design

A batch of *Macrobrachium tenellum* juveniles were collected in the Chacahua Lagoon (15°58'20"N, 97°32'28"W) in Oaxaca, Mexico. They were transported in a 250 L tank with permanent aeration to laboratory facilities and then, acclimated during two weeks at 25°C. During this time, they were fed with marine shrimp commercial pelletized food (40% crude protein, Camaronina 40® Agribrands Purina, Mexico).

In order to evaluate chitin effect on growth, a completely randomized experimental design of five treatments was realized. Every treatment had a different amount of chitin, included in a isoproteic-isolipidic diet. Different chitin amounts were 5, 10, 15, 20 and 25% of the food. An experimental control (no chitin) was also included. The experimental diet was prepared in agreement with Cortes-Jacinto *et al.* (2003) and Espinosa-Charaund *et al.* (2012) with the following ingredients: *Cyprinodontid* fish meal (50.05%), soybean meal in bulk (25.03%), Maseca® commercial corn flour (17.80%), and San Antonio® commercial wheat flour (7.12%) and vitamin and mineral supplements (Fervinac with selenium®, Laboratorios Brovel, Mexico). All ingredients were blended and mixed with an blender. Chitin was prepared by pulverizing dried shrimp carcasses (*Litopenaeus* sp.). The carcasses were cleaned by rinse and then, dried at sunlight. When completely dry, they were pulverized with the blender and added to the mixture. Then, the mixture was sieved through a 500 µm mesh and mixed again with the same blender. At this step, cornstarch were added as agglutinant. The resultant mixture was passed through a meat grinder in order to obtain 2 mm diameter pellets. Those pellets were dried in a convection oven at 30°C during 12 h. Table 1 shows the proximate analysis of the diet, to establish the values for gross energy, the conversion factors suggested by Anh *et al*. (2009) were applied: 4.2 for carbohydrates; 5.56 for proteins; and 9.54 for lipids.

Every treatment had six replicates and every replicate consisted on a dark non-translucid plastic container (50 L). All replicas of the same treatment were placed together at different levels. In the container at the lowest level no specimens were placed. A water pump was placed with a hose that throw water to the highest tray. Every tray had an outcome flowing to its lower next. This way, a cascade is produced and water is constantly recirculating through all containers of the same treatment. In every treatment, water temperature was maintained always at 28°C, with submersible 300 W heaters (RENA Aquatic Supply; US) installed in the lowest non-specimen tray of every treatment. Oxygen concentration was daily measured with a Hanna® HI98186 oximeter (Hanna Instrumenta Inc; Italy) and maintained always above 5 mg O_2 L^{-1}. The photoperiod was 12 h light -12 h dark. In each container, a 0.5 m^2 of plastic mesh was placed inside the water to provide shade and shelter. Ten organisms (0.2 ± 0.04 g) were placed in every container. A week before the first day of the experiment, total daily food was provided at 7% of the prawn biomass. After one week, the ration was adjusted to 10% of their body weight for all treatments. They were fed every day at 18:00 h. Survival was registered daily. The trial lasted 60 days. At days 0, 15, 30, 45, and 60, all prawns were individually weighed (±0.001 g).

Table 1. Proximate analysis of tested diet. Analytical method: [1]Differences in moist and dry weight, [2]AOAC, 2012 (micro-Kjendahl method), [3]AOAC, 2012 (Soxhlet method), [4]AOAC, 2012 (Weende method), [5]AOAC, 2012 (oven at 550°C).

	g 100 g^{-1} of dry food Average value ± SD
Humidity (%)[1]	4.783 ± 0.37
Crude protein[2]	44.137 ± 0.19
Total lipids[3]	6.517 ± 0.20
Crude fiber[4]	1.557 ± 0.09
Ash[5]	9.700 ± 0.38
Nitrogen-free extract	38.090 ± 0.77
Gross energy (kcal g^{-1})	4.741

The following parameters were obtained: average individual weight gain (g): AIWG = final weight-initial weight; gain weight per day (g day^{-1}): GWPD = (final weight-initial weight) t^{-1}); gain weight in percentage (%): GW = 100 × (final weight - initial weight)/initial weight); and specific growth rate (%): SGR = [(ln final weight - ln initial weight) t^{-1}] ×100. Also the feed conversion ratio was calculated as: FCR = supplied food (g)/weight gain (g); and feed efficiency ratio: FER = weight gain (g)/supplied food (g). SGR and FCR were calculated in agreement with Cortes-Jacinto *et al.* (2003); Gitte & Indulkar (2005) were followed for GW and GWP; Vega-Villasante *et al*. (2011) for AIWG and SR; and Hasan *et al*. (2012) for FER.

Enzyme activity

For measuring enzymatic activity, two prawns from each replica (eight by treatment) were randomly selected on days 0, 30 and 60. These were weighed as fresh weight and kept at -20°C until analysis. Only the hepatopancreas and intestine were analyzed. By the size of juvenile prawns the anterior section of head, all appendages, and the exoskeleton were discarded during dissection. The remaining tissue after dissection were individually weighed and separately homogenized with cold distilled water (4°C) in a v/w proportion of four mL water for g fresh organ tissue. Raw extracts were separated by centrifugation at 14.000 rpm for 10 min at 4°C, and clarified crude extract was kept at -40°C until analyzed for soluble proteins and enzyme activity. All measurements were made in quadruplicate for all enzymatic analysis, a control sample (blank) was also included, where the enzyme reagent was added after the reaction ceased. Protein concentrations in the enzyme raw extracts were quantified by the Bradford method (1976). In glass tubes (100 × 15 mm), 8 µL of crude extract, 792 µL distilled water, and 200 µL Bradford reagent were mixed and gently vortexed. Absorbance was measured at 595 nm. Bovine serum albumin (05470, Sigma-Aldrich, St. Louis, MO) was used as the protein standard. Lipase activity was determined as described by Versaw *et al*. (1989), using β-naphthyl caprylate as the substrate. Lipase activity was expressed as lipase units mg^{-1} protein (one lipase unit was the quantity of enzymes required for an increase of 0.01 absorbance units at 540 nm min^{-1}). Amylase activity was measured, as described by Vega-Villasante *et al*. (1993), using 1% starch in 50 mM Tris-HCl at pH 7.5, as the substrate. This activity was expressed as amylase units/mg protein (one amylase unit was defined as the quantity of enzyme to increase absorbance units by 0.01 at 550 nm min^{-1}). Trypsin activity was determined, using BAPNA as the substrate (García-Carreño & Haard, 1993), adapted to a 96-well microplate by adding 10 µL crude extract, 160 µL 60 mM Tris-HCl at pH 8.0, 10 µL 192 mM $CaCl_2$ at pH 8.0), and 10 µL 9.6 mM BAPNA dissolved in DMSO, in each well to start the reaction. Chymotrypsin activity was determined, using 9.6 mM SAAPNA dissolved in DMSO. For both enzymes, absorbance at 414 nm was recorded every 15 s for 30 min. At the end of the assay, a linear coefficient was calculated to determine the increase of absorbance per second. Enzyme activity was calculated using molar extinction coefficient of ρ-nitroaniline (8800). Chitinase activity was determined by mixing 20 µL of crude extract, 50 µL 60 mM Tris-HCl at pH 8, and 530 µL substrate (C3020, Sigma-Aldrich). The reagent mixture was shaken with a vortex at 120 rpm (at 45° angle) for incubation during 2 h. The reaction was stopped by centrifugation after 5 min at 14,000 rpm for 10 min at 4°C. The supernatant was separated immediately and absorbance was measured at 570 nm (one chitinase unit was defined as the quantity of enzymes required for an increase of 0.001 absorbance units at 570 nm min^{-1}).

Statistical analysis

Survival rate (%), average individual weight gain (g), gain weight per day (g day^{-1}), specific growth rate (%), feed conversion ratio, feed efficiency ratio and enzyme activity (U mg^{-1} Prot Sol) were determined and then compared by one way ANOVA test. Normality tests were performed (Kolmogorov-Smirnov test, $\alpha = 0.05$). Significant differences between treatment were determined by the Duncan multiple range test ($P > 0.05$). Minitab 17 statistical package (Minitab, College Park, PA) was utilized for statistical analyses.

RESULTS

During the 60 day trials, the water in all experimental units were maintained at 28.3°C, pH 7.5, and 6.8 mg O_2 L^{-1}. After 60 days, only the growth parameter of absolute weight was statistically significant (Fig. 1).

The range of each growth parameter was: GWPD (0.0048 to 0.0085 g day^{-1}); GW (147.1 to 286.5%); SGR (1.42 to 1.88%); FCR (3.86 to 2.91); and FER (2.72 to 3.90). Additionally, Table 2 shows growth (weight) per treatment.

The inclusion of 20% in one diet produced the best average individual weight gain (0.508 ± 0.101 g) and the best gain weight per day (0.0085 ± 0.0017 g day^{-1}). Treatment with 15% and 25% chitin were less successful. The best growth rate occurred by day 45, afterward declining. The least growth occurred in the 10% and 5% chitin treatment and in the control treatment (Fig. 2). There was no statistically significant difference in survival between treatments, ranging from 72.2% (treatment 5) to 83.3% (treatments 1, 2, 3, and control). For FCR, the best treatments were the 20% chitin (2.91 ± 0.39) and 25% chitin (3.50 ± 0.55), with the highest feed conversions around day 45.

For soluble protein concentrations, there were no statistically significant differences between treatments. Chitinase activity was the only enzymatic activity with statistically significant differences between treatments. Other enzymes had similar trends in the final phase of the culture (Fig. 3). Like specific growth data (see Fig. 2), chitinase activity was highest at day 45, declining thereafter to the end of the trials (Fig. 4), differing from lipase, amylase, trypsin, and chymotrypsin enzyme activity.

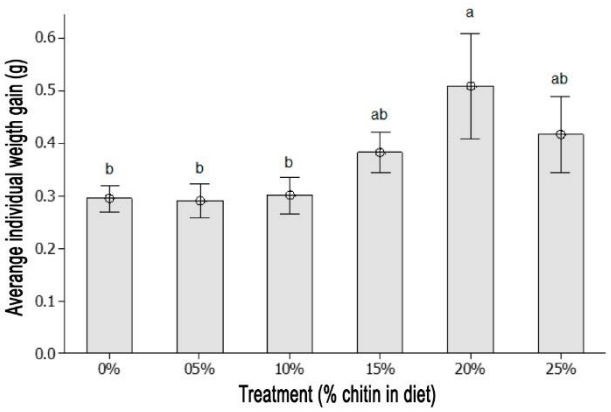

Figure 1. Average individual weight gain (g) of *Macrobrachium tenellum*: effect of chitin level in diet. Same letters represent no differences between treatments (P = 0.05). Bars represent amount of chitin in each treatment. Whisker lines are SE from the mean.

DISCUSSION

The water physical and chemical conditions were always maintained as required by *Macrobrachium tenellum* in culture, as stated in previous studies (García-Ulloa *et al*., 2008; Espinosa-Chaurand *et al*., 2011; Vega-Villasante *et al*., 2011). For this species, the best growth used to be observed at a water temperature range from 28-32°C in agreement with Espinosa-Chaurand *et al*. (2011). Schiff & Hendrickx (1997) and Vega-Villasante *et al*. (2011) report that *M. tenellum* in culture grows very well with 5.0 mg O_2 L^{-1}. For pH, Hernández *et al*. (2007) and Vega-Villasante *et al*. (2011) suggest pH from 7.5 to 8.5 for *M. tenellum* cultivation conditions.

Two trends on survival rate were observed in present work. Prawns fed chitin at 0%, 5%, 10%, and 15% had an average survival of 83.3%. Prawns fed 20% and 25% chitin had lower survival (75% and 72.2%, respectively). Lower content of chitin apparently had no direct effect on survival, what is in agreement with previous studies. Kumar *et al*. (2006) and Shiau & Yu (1998) studied chitin and shrimp head meal, respectively, on survival of *M. rosenbergii* and *Penaeus monodon* (Fabricius, 1798). Kumar *et al*. (2006) find a survival of 40-80% while Shiau & Yu (1998) reported 74-84%, without significant differences or correlation between the amount of chitin and survival. These results are similar to present work although a precise comparison cannot be executed

Previous studies with present species includes the works of Espinosa-Chaurand *et al*. (2012), who fed *M. tenellum* crude protein in diets (20, 25, 30, 35, and 40%) and maintained 95 to 100% survival, without statistical differences between treatments. In contrast, Ponce-Palafox *et al*. (2006) reported 50% survival after prawns were fed a diet containing 25% of protein. The most common cause of death during cultivating prawns is from cannibalism. Common causes for this behavior are lack of shelters or large variations in size among individuals in the same tank (Nair *et al*., 1999; García-Guerrero & Apun-Molina, 2008) observe that adding chitin to the diet reduced cannibalism in *M. rosenbergii*. It is important to state that the only chitin source was form diet, since every time one specimen moult, the exuviae was inmediatly removed from the tank. Survival of 75 and 72% in diets containing 20 and 25% chitin, respectively, had the lowest survival, despite having the highest growth. There were no significant differences in survival among treatments and it is suggested that different diets do not influence survival since chitin variation effect could be mostly on growth rate.

Present results suggest that the adding of chitin in the diet has a statistically significant effect on *M. tenellum* growth. The highest average individual weight gain (AIWG = g) was obtained with the 20% treatment. Chitin in the di*et al*so increases chitinase activity, which was evident at day 30, when the highest weight gain per day (GWPD = g day^{-1}) occurred. The GWPD are in agreement with Ceccaldi (1989) and Zhang *et al*. (2014) who state that chitinase is essential for digestion and molting. However, molt frequency were not determined in present study so its direct relation with growth could not be determined even considering that in present study, a decrease in chitinase activity occurred when growth rate decreased. As Lemos *et al*. (2000) find, different growth stages engenders different feeding behavior and different digestion tract functional structures, thus, different digestion capabilities. This contributes to differences in enzyme activity, as observed in shrimp by Chang *et al*. (1988). Casillas-Hernández *et al*. (2002) observe that enzymatic, hormonal, and environmental issues are involved with molting, causing an indirect effect on growth rate. Chitin at 15 and 25% in the diet contribute to molting, as reported by Chang (1995). In present study, treatments containing 20, 25, and 15% chitin produced better weight gain in prawns. Prawns fed diets containing 10, 5, and 0% chitin had poorer weight gain. Statistical differences (P = 0.046) suggest that small variations in the amount of chitin may not affect weight gain in a measurable way, consequently, no differences in specific growth rates, increase in weight; feed conversion, and feed conversion efficiency were found. Kumar *et al*. (2006) report a similar trend in growth rate, using diets with different chitin content for *M. rosenbergii*. They report that diets containing 5% natural chitin (22% shrimp head meal), 10% natural

Table 2. Parameters of growth in weight for each treatment of chitin added to diet. AIWG: average individual weight gain, GWPD: gain weight per day, GW: gain weight in percentage, SGR: specific growth rate, FCR: feed conversion ratio and FER: feed efficiency ratio. Values with the same letter do not show statistically significant differences (P = 0.05), using Duncan multiple range test.

Treatment	AIWG (g)	GWPD (g day^{-1})	GW (%)	SGR (%)	FCR	FER
0% chitin	0.294 ± 0.025^{b}	0.0049 ± 0.00041^{b}	151.3 ±15.1^{a}	1.48 ± 0.09^{a}	3.53 ± 0.31^{a}	0.296 ± 0.029^{a}
5% chitin	0.291 ± 0.033^{b}	0.0048 ± 0.00055^{b}	150.8 ±17.0^{a}	1.45 ± 0.11^{a}	3.77 ± 0.51^{a}	0.295 ± 0.047^{a}
10% chitin	0.301 ± 0.036^{b}	0.0050 ± 0.00060^{b}	147.1 ±17.4^{a}	1.42 ± 0.11^{a}	3.86 ± 0.40^{a}	0.272 ± 0.026^{a}
15% chitin	0.383 ± 0.038^{ab}	0.0064 ± 0.00063^{ab}	201.7 ±23.2^{a}	1.74 ± 0.12^{a}	3.53 ± 0.30^{a}	0.293 ± 0.022^{a}
20% chitin	0.508 ± 0.101^{a}	0.0085 ± 0.0017^{a}	286.5 ±72.6^{a}	1.88 ± 0.20^{a}	2.91 ± 0.39^{a}	0.390 ± 0.071^{a}
25% chitin	0.417 ± 0.073^{ab}	0.0070 ± 0.0012^{ab}	271.9 ±66.2^{a}	1.78 ± 0.22^{a}	3.50 ± 0.55^{a}	0.329 ± 0.060^{a}

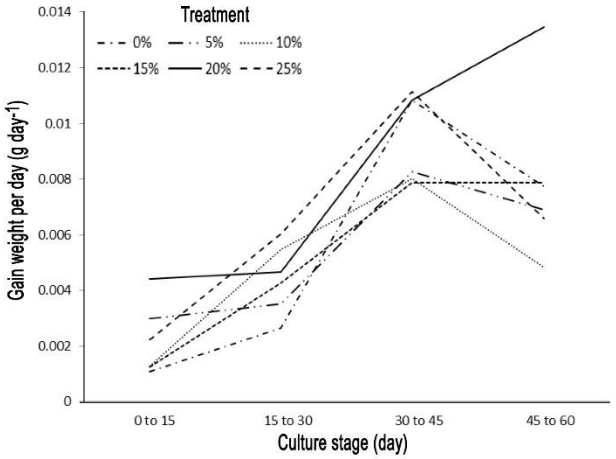

Figure 2. Gain weight per day (g day^{-1}) of *Macrobrachium tenellum*: effect of chitin amount in diet, at four intervals in trials. Chitin amount ranges from 0% to 25%.

chitin (44% shrimp head meal), and 5% purified chitin produce good weight gain and low feed conversion ratio. Shiau & Yu (1998) report a high weight gain in giant tiger shrimp (*Penaeus monodon*) fed a diet containing 22% chitin (equivalent to 5% purified chitin). They state that chitin in diets that is >22%, reduces weight gain. Fox (1993) mentions that natural chitin from shrimp head meal has high nutritional value for *P. monodon*, compared to purified chitin. In his study, there were not significant differences in growth where diets contained 0, 4, 8, 12, or 16% natural chitin. It seems that similar percentages of chitin can produce different results in different species. Prawns may take better advantage of dietary chitin compared to shrimp. Ceccaldi (1989) states that there is no difference in the use of chitin or other carbohydrates between shrimp and prawn digestive tracts; however, there are differences in tissue and biochemical activity (Cruz-Suarez, 2002). More research is required before conclusions on this topic.

In *M. tenellum*, Espinosa-Chaurand (2013) states that different concentrations of digestive enzymes in each phase of the life cycle are related to protein amount in diets and with circadian rhythm, since alterations of these variables affects proteolytic activity of chymotrypsin, trypsin, lipase, and amylase. Since we used identical protein diets, enzyme activity did not differ significantly between treatments, however, lowest activity was observed for all enzymes during the middle of the trial (day 30). Lower physiological activity was observed by Espinosa-Chaurand (2013). This depends on fitness, metabolic rate, and nutrient requirements of specific stages (Vega-Villasante *et al*., 1999). Casillas-Hernández *et al*. (2006) report a relationship between feeding and enzyme activity, caused by demands of each developmental stage and production of biomass. Alexandre *et al*. (2014) and Cordova-Murrueta *et al*. (2003) also consider variations in enzyme activity as responses to growth stage, molting, digestion, and nutritional condition. These factors, together, determine enzyme activity independent of dietary ingredients. Other factors also influence enzyme activity. Since enzyme activity was not significantly different between treatments, growth also depends on other factors, such as availability and expenditure of energy, as in the case of *Penaeus monodon* (Chen & Lin, 1989).

It was also found an inverse relationship between enzymatic activity and digestion of proteins, lipids, and carbohydrates and the amount of chitin in the diet. Kumar *et al*. (2006) observe a decrease in digestibility with an increase in dietary chitin. They used purified chitin, which counteracts hydrolytic activity of other enzymes, probably because chitin was present in excess. Chitin content at 20% increases chitinase, compared to diets with less chitin (P < 0.01). Similar to their findings, we observed significant differences in enzyme activity between different stages (P < 0.01), chitinase activity peaked at day 30 and lower at the beginning and end of the trial. We also observed that

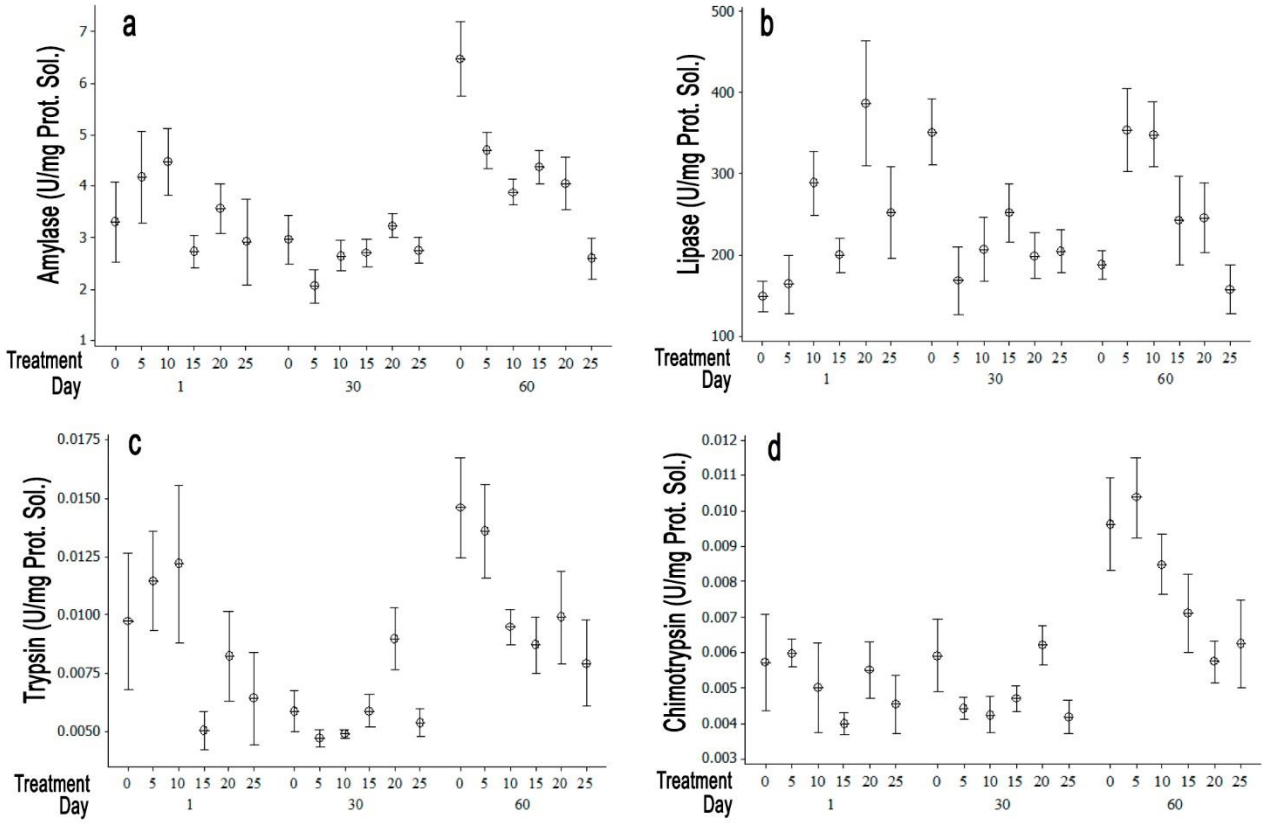

Figure 3. Effect amount of chitin in the diet on the digestive enzyme activity in *Macrobrachium tenellum*. a) Amylase, b) lipase, c) trypsin, and d) chymotrypsin. Samples taken on days 1, 30, and 60. Treatments are chitin amounts in diet (0%, 5%, 10%, 15%, 20%, and 25%). Whisker lines are SE from the mean.

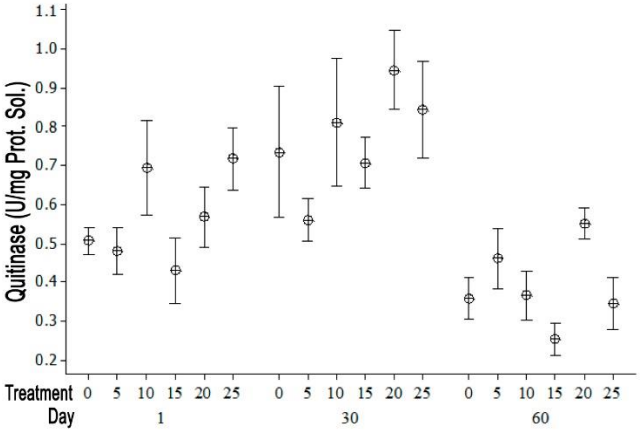

Figure 4. Chitinase activity in *Macrobrachium tenellum*: effect of the amount of chitin in the diet. Treatments are chitin amounts in diet (0%, 5%, 10%, 15%, 20%, and 25%). Sampling times: days 1, 30, and 60. Whisker lines are SE from the mean.

from days 30 to 60, when chitinase activity increases, activity of all other digestive enzymes decreased. Additionally, when chitinase activity declines, lipase and amylase activity increased, supporting the hypothesis of an inverse relation between chitinase and other digestive enzymes.

Another relationship was a decline in growth rate at high chitin content in the diet. The likely cause is an inability to absorb or metabolize big amounts of glucosamine if all dietary chitin was digested. This was observed in *M. rosengergii* (Kumar *et al.*, 2006) and *P. monodon* (Fox, 1993). These authors suggest that this response could be ameliorated with a diet rich in lipids, proteins, and minerals. In fish diets, containing chitin, Lindsay (1984) and Danulat (1986) report that chitinase activity could be increased with dietary protein. However, in crustaceans, this did not occur with diets containing purified chitin, but this chitinase activity has occurred in the basal region of the peritrophic membrane in the gut (Ceccaldi, 1989; Alexandre *et al.*, 2014). It is likely that the same mechanism occurs in *M. tenellum* because chitin is re-absorbed when chitinase dissolves the old exoskeleton into a more soluble form in the integument, which is common in decapods (Dall *et al.*, 1990; Chang, 1995; Shechter *et al.*, 2007; Stillman *et al.*, 2008).

In addition to chitin level in the treatments, variations in chitinase activity may be influenced by prawn physiological reactions to water conditions, such as temperature and dissolved oxygen, as reported by Alexandre *et al*. (2014) concerning enzyme activity in the whiteleg shrimp *Litopenaeus vannamei*. In *Macrobrachium nipponense* (De Haan, 1849), Zhang *et al*. (2014) found variations in chitinase activity by diet induced, but not from size, developmental stage, hierarchical position, molting stage or acclimation time.

In summary, diets containing 20% natural chitin produced the best growth of *M. tenellum*. Further research should focus in the study of how some variables such as temperature or photoperiod in the culture may affect chitin digestion causing differences in enzymatic activity, particularly chitinases. In addition, further research is needed in the relationship between the molting cycle and chitin levels in the diet to determine the impact of the chitin amount and quality on the molt cycle and growth.

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