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RESEARCH REPORT

Evolution of the digestive enzymes and bacterial changes of the gastrointestinal tract of the *Artemia urmiana* during growth period

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Abstract

As the digestive enzymes and gastro-intestinal (GI) bacterial community contribute to the health and nutrition of the organism, this study aims to evaluate the ontogeny of bacterial population and digestive enzymes activities in the GI tract of Artemia urmiana from nauplii to the adult stage. *Artemia* cysts were hatched under standard conditions and stocked at a density of 20 nauplii ml^{-1} for 15 days. Samplings for growth, bacterial and enzymatic analysis were collected on days 1, 5, 10 and 15 of the experiment. The results indicated that the GI tract was sterile at the time of hatching. *Artemia* GI tract was active after hatching by protease (0.282 ± 0.001 U mg^{-1} protein min^{-1}), lipase (0.182 ± 0.001 U mg^{-1} protein min^{-1}) and amylase (0.295 ± 0.001 U mg^{-1} protein min^{-1}) secretion and increased during the experiment. In addition, a significant relation ($p < 0.05$) was observed between *Artemia* total length and the activity of digestive enzymes (lipase ($r^2 = 0.98$) amylase ($r^2 = 0.97$) and protease ($r^2 = 0.98$). A significant relation ($p < 0.05$) was observed between total aerobic bacteria and digestive enzyme secretion (lipase, $r^2 = 0.83$; amylase, $r^2 = 0.66$ and protease, $r^2 = 0.84$) too. Such a relation was observed between total *Bacillus* spp. count and digestive enzyme activities (lipase $r^2 = 0.82$; amylase $r^2 = 0.79$; and protease; $r^2 = 0.74$). These results suggest that in addition to the chemical composition of food, total length, GI bacteria enzyme secretion and the interaction of these factors contribute in digestive enzymes ontogeny during the growth period.

Key Words: *Artemia*; health; nutrition; digestion

Introduction

Some aquatic organisms, consume bacteria as food and gastrointestinal (GI) flora can be a reflection of bacterial community swallowed with food or as food. Bacteria population can play an important role as food by providing micronutrients such as essential fatty acids, vitamins, or digestive enzymes (Olafsen, 2001).

The GI flora is affected by several factors such as season (Irianto and Austin, 2002), species (Cahill, 1990), diet, environmental conditions and the growth stage (Hansen and Olafsen, 1999). The microbial ecology of the GI tract of a variety of freshwater and marine organisms has been investigated (Denev *et al.,* 2009; Zarkasi *et al.,* 2016; Zhu *et al.,* 2016). In addition, some researchers have studied the probiotic properties of some indigenous and non-indigenous bacteria in aquaculture, but information on *Artemia* larval development (ontogeny) is scarce and no work has been published concerning the bacterial changes of *Artemia* gut during development.

Investigations of nutritional requirements and feeding ecology of marine invertebrates requires a deep consideration of basic digestive physiology. (Patel *et al.,* 2012). Structure and the physiology of the digestive system of crustaceans and other marine invertebrate undergo important ontogenetic changes with adaptation to different food types (Andrés *et al.,* 2010) and developmental stages (Veróonica and Gimenez, 2013). Fulfilment of nutritional requirements of marine invertebrates, including crustaceans, plays a critical role during larval development. Since digestion is considered as a key factor in metabolism, it may be essential in determining the availability of nutrients required for biological functions (McConaugha, 1985). Thus, the

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Table 1 Physico chemical parameters of Artemia hatching and rearing water (mean ± SD)

Agent	Temperature (ºC)	DO (mgL^{-1})	Salinity(gL^{-1})	pH
Hatching	29±1	4±1	35±2	8.3±0.5
Rearing	29±1	6±1	60±2	8.3±0.5

study of various aspects of the digestion physiology during ontogeny is essential to have a promising larviculture (Applebaum *et al.,* 2001).

Most of the reports on digestive enzymes in crustaceans have been confined to adult specimens; interactions between variations in gut morphology, digestive enzyme activities, and diet throughout early stages of life cycle are thoroughly unknown. Changes in digestive enzyme activity during development have been reported in relatively few crustacean species *Penaeus setiferus* (Lovett and Felder, 1990) *Artemia* spp, (Gawlicka *et al.,* 2000), *Macrobrachium rosenbergii* (Kamarudin *et al.,* 2011), *Litopenaeus vannamei* (Wei *et al.,* 2014). Despite digestive enzymes secreted by indigenous bacteria of GI are of high importance (Suzer *et al.,* 2008; Wang *et al.,* 2017), we found no study concerning the role of bacteria in enzymatic development of crustaceans. Ceccaldi (1989) stated that digestive enzymes of crustacean species are diversified into proteases, among which trypsin is the major one, lipases and esterases, amylases, maltases and chitinases are also well-represented. Determination of digestive enzyme profiles in puerulus, postpuerulus, juvenile and adult stages of the spiny lobster Jasusedwardsii was made to find out the ontogenetic alterations in digestive proficiencies. A variety of enzymes was observed in juvenile and adult lobsters, exhibiting their potential to exploit different diets. According to the results, lobster was found out to be carnivorous (Johnston, 2003) as dietary protein played more essential role than carbohydrate sources.

Gawlicka and co-workers in 2000 determined Activities of trypsin, amylase, lipase and alkaline phosphatase in metamorphic larvae *Hippoglossus hippoglossus* and in their *Artemia* prey to estimate the importance of exogenous enzymes for Atlantic halibut larvae. The calculated contribution of enzyme activities derived from *Artemia* prey to the relatively high levels of enzyme activity in the digestive system of metamorphic larvae was less than 10 % for all enzymes except amylase, for which the contribution was estimated to be more than 50 %. Based on the available literature, there is no information on GI bacterial ontogeny and the interaction between GI bacteria and digestive enzymes, this survey can open new viewpoint of the nutritional biology of *Artemia* and other crustaceans.

Recently, *Artemia urmiana* (Günther, 1890) population as an indigenous species in Urmia Lake (in the North-west of Iran) has been exposed to main challenges including drought and salinity increment (Motlagh *et al.,* 2012). Therefore, indoor mass-culture of *Artemia* can alleviate the pressure of aquaculture industry on natural stocks to collect following products (cyst and biomass). Regarding the critical role of GI microflora and digestive enzymes on the health and nutrition of aquatic larvae, the aim of present study was to evaluate the digestive enzymes (protease, lipase and amylase) and bacterial ontogeny of the GI tract of the *A. urmiana* from nauplius to the adult stage as a vital point of *Artemia* mass culture.

Materials and Methods

Hatching and rearing of Artemia urmiana

Nauplii of *A. urmiana* were hatched in the laboratory in cylindrical containers (2 L) of saltwater and maintained on a 12:12 light:dark cycle, under standard condition (Sorgeloos 1986). *Artemia* nauplii were transferred into 60 L polyethylene tanks with the density of 20 nauplii ml^{-1} and reared for 15 days (Shahnaz Jabari et al., 2015). Physicochemical properties of hatching and rearing water, including temperature, dissolved oxygen, salinity and pH (Table 1) were monitored daily according to standard methods (Agh, 2007) for hatching and rearing periods.

Artemia urmiana *feeding*

During the first five experimental days, nauplii were fed with backers yeast (*Saccharomyces cerevisiae*) (Lavens and Sorgeloos, 1996). From the second day, after hatching 1.25 mg of baker yeast per 1000 nauplii in 400 ml saline water (35 gL^{-1}) at 28 ºC, the distribution of solutions in rearing water was facilitated by passing them through a 150 micron mesh. From the sixth day onwards, the *Artemia* were fed a diet including white wheat flour (11.24 %) and equal amounts of soybean meal and chickpea flour (44.38 %) provided by Behparvar Co. (Iran). Feeding was performed three times a day with a four-hour interval. The dietary chemical composition was analyzed using the standard methods (Peterson and Martin-Robichaud, 1983). The crude protein, crude fat, dry matter and ash values were (12 % ± 0.93), (55 % ± 1.2), (97.5 % ± 0.77) and (5 % ± 0.35), respectively. Table 2 shows the feeding schedule of *Artemia*.

Growth monitoring

Biometry of *Artemia* was performed on the first, fifth, tenth and fifteenth experimental days for the evaluation of growth. Sampling was conducted by removing five samples of water with 10 ml volumes from each tank and measuring the total length of *Artemia* by a micrometer (0.01 mm).

Table 2 *Artemia* feeding schedule over the experiment

Experimental days	1	2	3	4	5	6	7	8	9	10	11-14
Feed amount (g L^{-1})	-	0.02	0.03	0.05	-	0.05	0.06	0.062	0.07	-	0.07
Yeast	-	0.02	0.03	0.05	-	0.037	0.03	0.0155	-	-	-
Dry food	-	-	-	-	-	0.0125	0.03	0.0465	0.07	-	0.07

Microbial analysis

The total aerobic bacteria count and total Bacillus count in the Gl of *Artemia* (CFU g^{-1} *Artemia*) were estimated on first, fifth, tenth and fifteenth days. A 70 % alcohol was used for washing nauplii, and the sterile saline water (35 g NaCl L-1) was used for rinsing them to eliminate the sticking bacteria from their body surface. Five ml of sterile saline water was gradually added to the samples in order to homogenize them. Then, ten times serial dilution were prepared, and mediums Bacillus cereus Agar and Marine agar 2216 (HiMedia©) were used to count the total aerobic bacteria and Bacillus, respectively (Rengpipat *et al.,* 1998). Bacillus cereus Agar was incubated at 30 °C for 24 h, and Marine agar was incubated at 29 °C for 24 h.

Enzymatic assays

Samplings for determining the activities of protease, lipase and amylase were conducted on first, fifth, tenth and fifteenth experimental days. The samples were first washed with cold fresh water and rinsed. Then, the samples were stored in 15-ml falcon tubes and instantly transferred to a freezer (-80 ºC) (Paiva-Maia *et al.,* 2013). The samples were defrosted in laboratory conditions to extract the enzymes. The homogenates of the whole animal body were used in all evaluations. Homogenization of the extracts prepared in physiological saline solution (0.9 % NaCl) was performed by adding saline solution which obtained a total volume of 1.6 ml per sample. The homogenized solutions were centrifuged at 5,000*g* for 5 min. The enzymatic evaluations were conducted using the supernatants. The hydrolysis of casein at pH 8 was used to estimate the protease activity (Paiva-Maia *et al.,* 2013), starch was used as the substrate to measure the amylase activity (Worthington, 1991). In order to determine the lipase activity, olive oil emulsion substrate-Gum Arabic was used and the thawed samples were titrated at room temperature (Worthington, 1991).

Statistical analysis

All percentage data were transformed using the arcsine method. The Leaven test was used to confirm the homogeneity of variance, and the Kolmogorov-Smirnov test was used to determine the normality of data (Zar, 1999). The data were analyzed using one-way ANOVA. Duncan multiple range test was applied in order to find out if there were any significant differences among the treatments ($p < 0.05$). Regression relations were conducted using SPSSTM version 19 (SPSS Inc., Chicago, USA).

Results

The changes of total length in Artemia urmiana

As shown in Figure 1, there are significant differences between total length of *A. urmiana* during sampling days. The significantly highest total length (3.5 mm) was observed in the day 15.

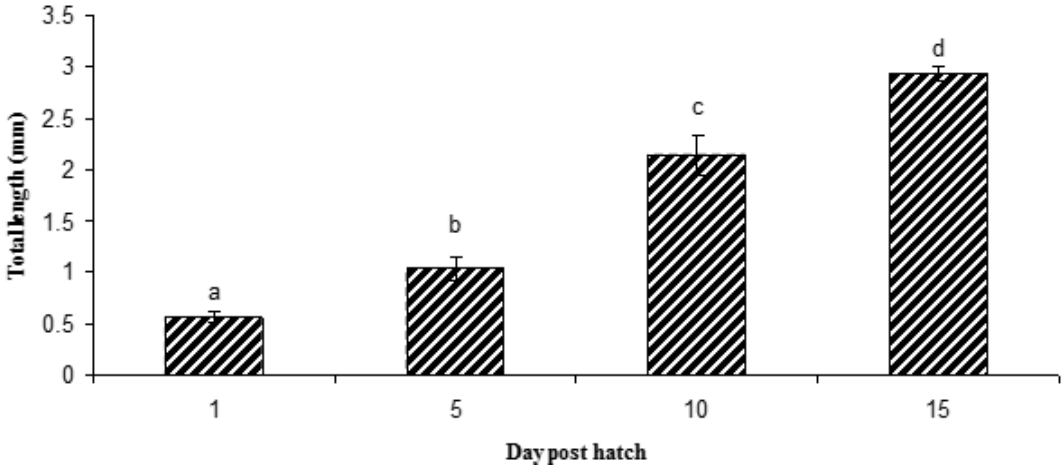


Fig. 1 The mean (±SD) total length (mm) in *A. urmiana* during sampling times with three replicates. Different letters indicate significant differences ($p < 0.05$).

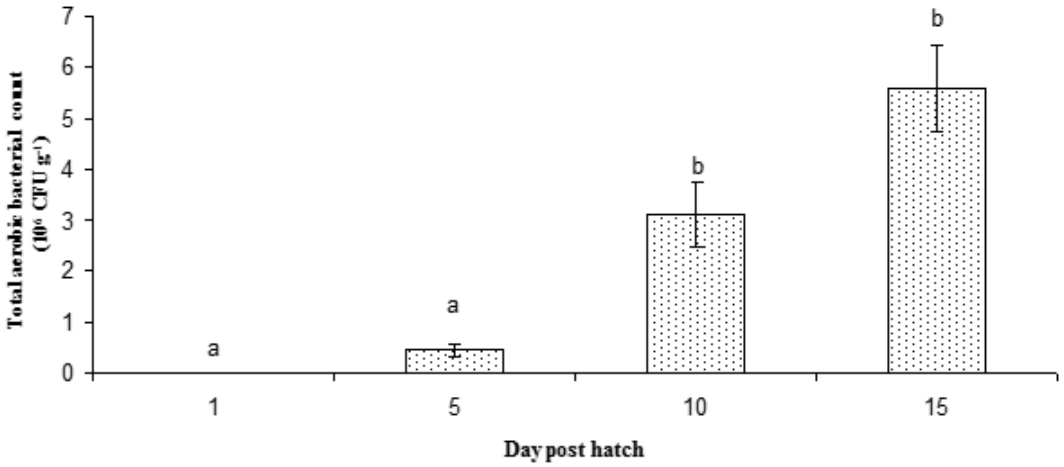


Fig. 2 The mean (±SD) total aerobic bacterial count (×10^6 CFU g^{-1}) in *A. urmiana* GI during sampling times with three replicates. Different letters indicate significant differences ($p < 0.05$).

The ontogeny of the GI bacterial in Artemia urmiana

The changes of the total aerobic bacterial count

Total number of aerobic bacteria in the GI of newly hatched *Artemia* nauplii showed that the GI was sterile and free of bacteria. As shown in Figure 2, the total number of aerobic bacteria shows a significantly ($p < 0.05$) increasing trend, up to the fifteenth day of the experiment. The average number of total aerobic bacteria in the gut showed significant differences between the fifth, tenth and fifteenth day of the experiment ($p < 0.05$). The results revealed no significant difference between the tenth and the fifteenth day.

The changes of the Bacillus *spp. count*

No *Bacillus* was reported in *Artemia* GI at the first day of sampling but count increased later throughout the experiment. The final comparison between average *Bacillus* spp. count shows a significant increase ($p < 0.05$) from zero (in day one) to 3.8 ± 0.8×104 (in day 15) (Fig. 3).

The ontogeny of digestive enzymes activities in Artemia urmiana

The changes in protease, amylase and lipase activities during the experiment are shown in Figure 4. On the first day, the specific activities (U min^{-1}) of protease, amylase and lipase in the *Artemia* gut were reported to be 0.282 ± 0.07, 0.295 ± 0.05 and 0.182 ± 0.02, respectively. The lipase activity was higher than the other enzyme activities ($p < 0.05$) on day five. However, on the tenth and fifteenth days, significant differences were observed among all enzymes, and lipase exhibited the highest activity ($p < 0.05$). At the beginning of the experiment, amylase showed the highest activity with no significant difference with the others, however over the time amylase activity decreased and lipase activity was increased.

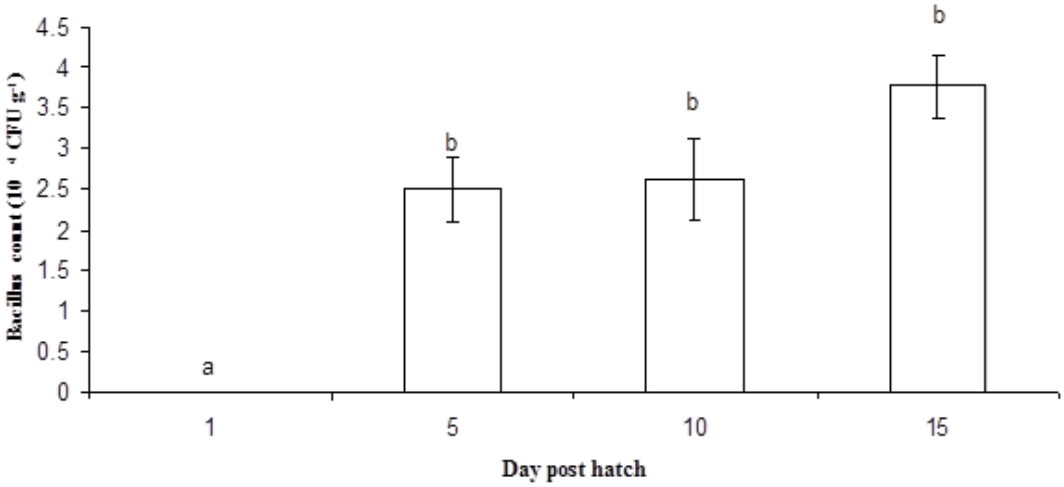


Fig. 3 The mean (±SD) *Bacillus* spp. count (×10^6 CFU g^{-1}) in *A. urmiana* during sampling times with three replicates. Different letters indicate significant differences ($p < 0.05$).

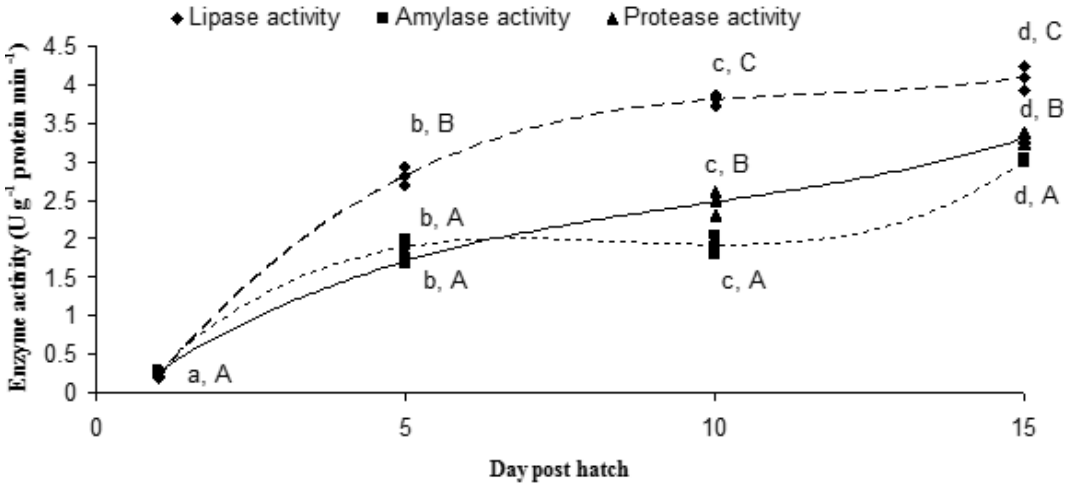


Fig. 4 The mean (± SD) activities (U mg protein^{-1} min^{-1}) of digestive enzymes (lipase, amylase and protease) in *A. urmiana* during sampling times with three replicates. Different letters indicate significant differences ($p < 0.05$). Small letters (a - d) and capital letters (A - C) show differences during day post hatch and among enzyme activities during a specific day, respectively.

Polynomial models in Figure 5 indicated that, there were significant difference ($p < 0.05$) between polynomial models of length and digestive enzyme activities including lipase ($r^2 = 0.98$), amylase ($r^2 = 0.97$) and protease ($r^2 = 0.98$). As well, Figure 6 significant differences were found ($p < 0.05$) between polynomial models of the age of *Artemia* (Day Post Hatch) and digestive enzymes including lipase activity ($r^2 = 0.99$), amylase activity ($r^2 = 0.99$) and protease activity ($r^2 = 0.99$). Significant differences were found ($p < 0.05$) between polynomial models of total aerobic bacteria count and digestive enzymes including lipase activity ($r^2 = 0.83$), amylase activity ($r^2 = 0.66$) and protease activity ($r^2 = 0.84$) (Fig. 7). In addition, as shown in Figure 8, there were significant differences between ($p < 0.05$) polynomial models of total *Bacillus* spp. count and digestive enzymes including activities of lipase, amylase and protease ($r^2 = 0.82$, 0.79 and 0.74, respectively).

Discussion

The ontogeny of the GI bacteria in Artemia urmiana

Ontogeny of the bacterial flora of the GI tract of freshwater and marine organisms have not been studied yet and this issue requires more research (Safari *et al*., 2014; Wang *et al*., 2017). In the current study, no bacteria were detected in GI on the first day post-hatch which confirms the other researchers finding (Ringø and Gatesoupe, 1998). An increasing trend of total aerobic bacteria was observed. In fact, the total number of aerobic bacteria increased significantly with *Artemia* growth and development of the attachment sites in the GI. This trend was also observed in total *Bacillus* count as representative beneficial bacteria. This could be due to dietary consumption. In the present study, the *Artemia* consumed *S. cerevisiae* for the first 5 days of the experiment. Several researchers had proved the dietary effects on the intestinal microflora. The effect of the food sequence on the population of intestinal microbiota in larvae and juveniles of *Sparus aurata* has been observed by weaning the early juveniles of this fish (Savas *et al*., 2005). *Vibrio* and *Pseudomonas* were found out as the dominant genera after feeding juveniles with a compound diet depending on culture methods (Silva *et al.,* 2011). Unfortunately, few studies have been conducted on *Artemia* ontogeny, which requires further research. Moreover, quantifying the main bacteria family throughout each ontogeny stage and using DGGE technique are recommended as the future research topics.

The ontogeny of digestive enzyme activities in Artemia urmiana

One of the most important factors affecting commercially successful larval production is survival rate that is affected by various factors-mainly, larval rearing period and larval nutrition (Shields, 2001; Turkmen *et al.,* 2017). Digestion of food to provide nutrients for growth, maintenance, motion, and reproduction is a very important function in an organism (Hernández and Murueta, 2009). The intake of sufficient amount of nutrients and their efficient digestion by an organism depends on its ability to select food and the capacity of its digestive enzymes (Bautista 1983; Silva *et al.,* 2010). It is thus important to understand the profile of these digestive enzymes, as this can be used as a tool to develop food specific to the metabolic needs of species (Pavasovic *et al.,* 2004). However, we need to standardize specific histochemistry methods for further studies on live feed production industry including *Artemia*.

Extended published records have focused on invertebrate digestive enzyme synthesis, regulation, and evaluation, including crustaceans (Le Vay *et al.,* 2001; Muhlia-Almazán *et al.,* 2008; Andrés *et al.,* 2010;

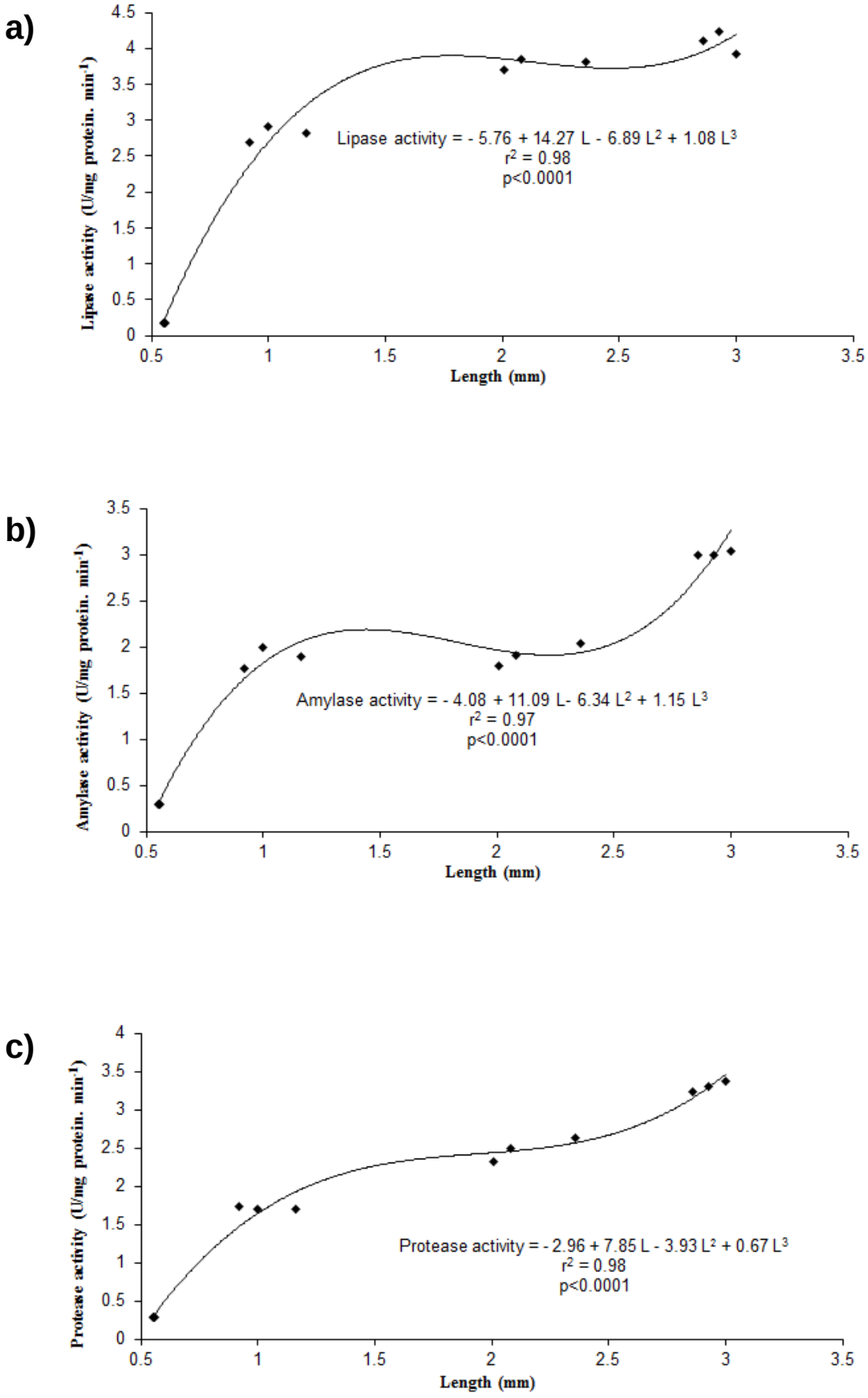


Fig. 5 Polynomial model fitting (a) lipase activity (U/mg protein. min^{-1}), (b) amylase activity (U/mg protein. min^{-1}) and (c) protease activity (U/mg protein. min^{-1}) to length (L; mm) in *A. urmiana* with three replicates.

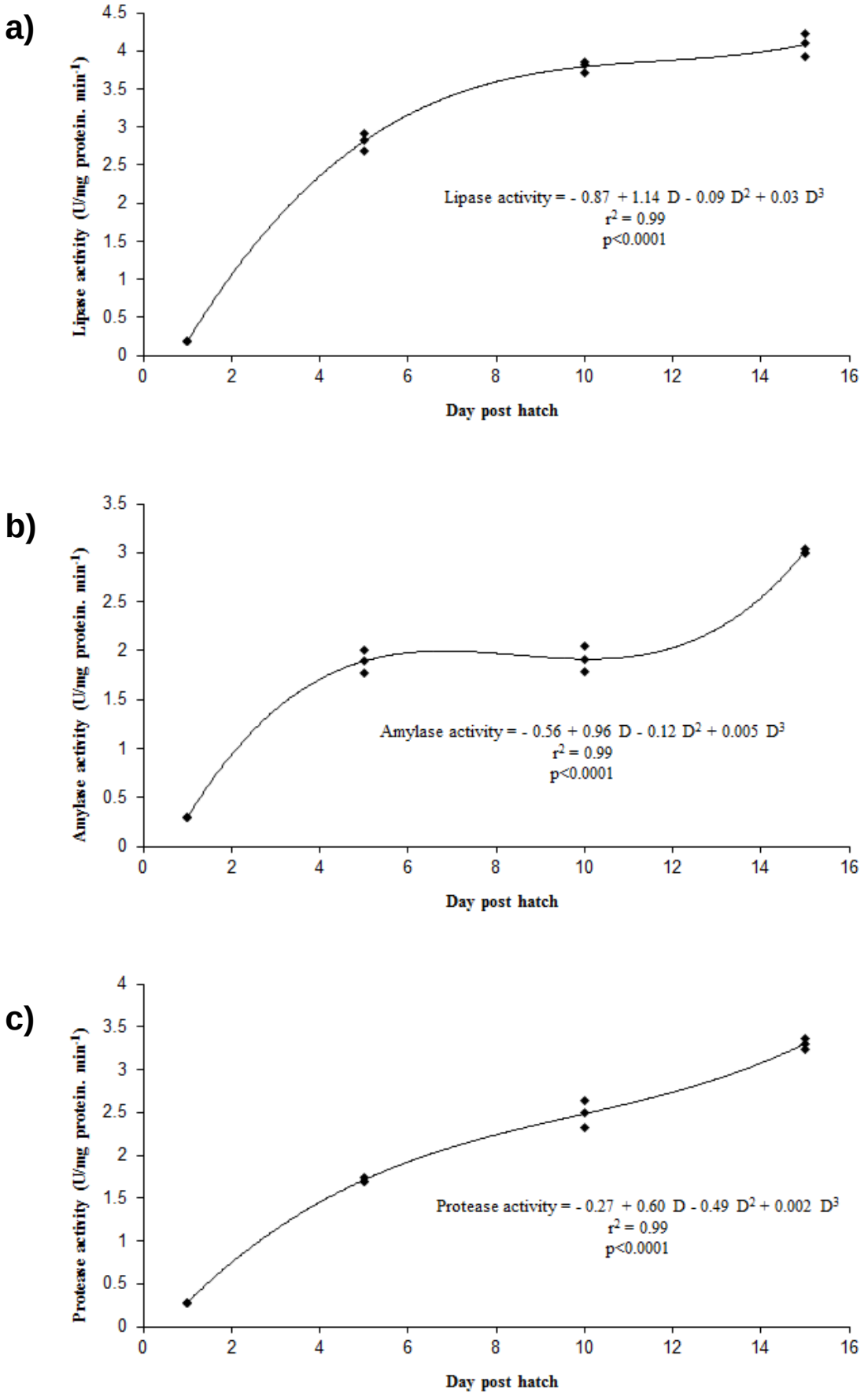
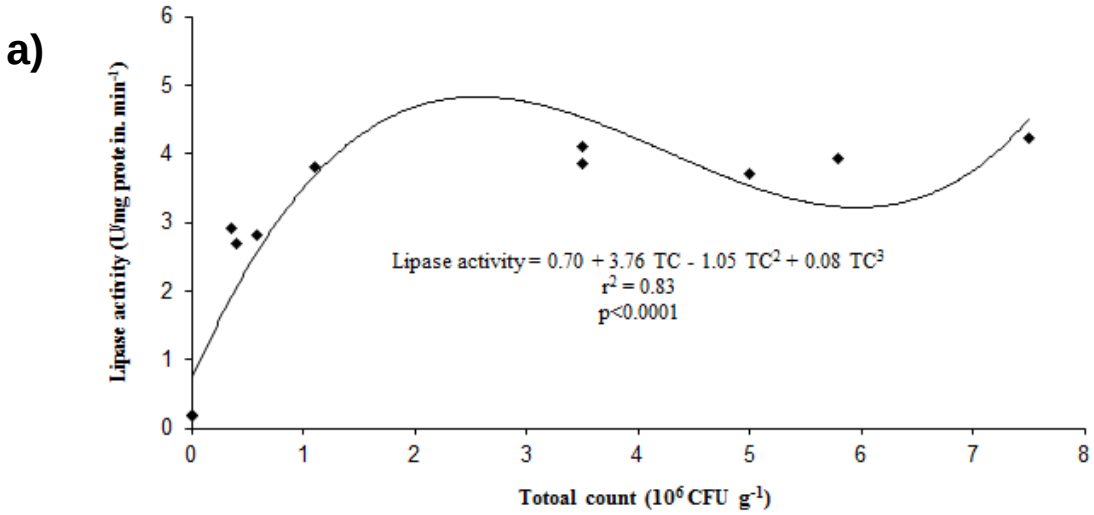
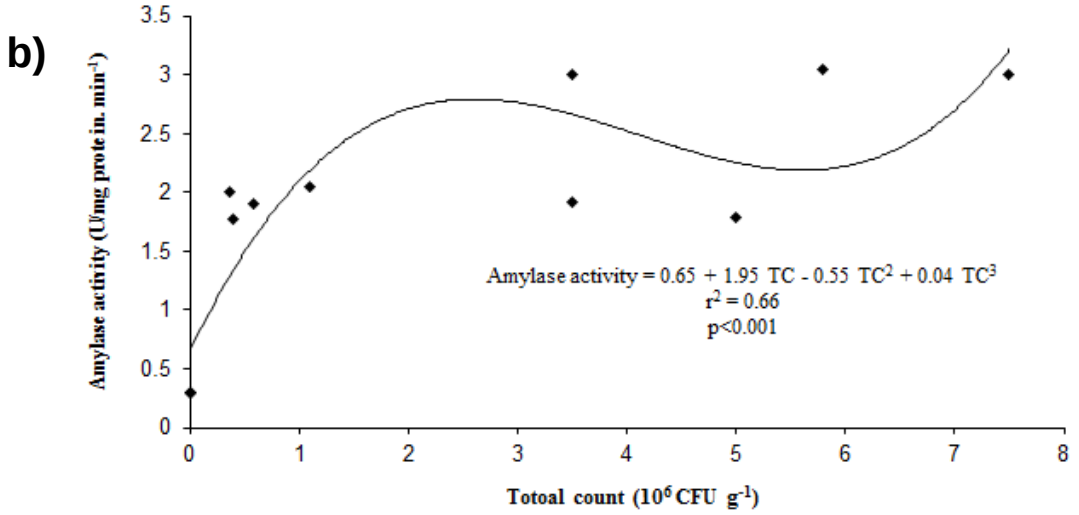


Fig. 6 Polynomial model fitting (a) lipase activity (U/mg protein. min^{-1}), (b) amylase activity (U/mg protein. min^{-1}) and (c) protease activity (U/mg protein. min^{-1}) to day post hatch (D) in *A. urmiana* with three replicates.





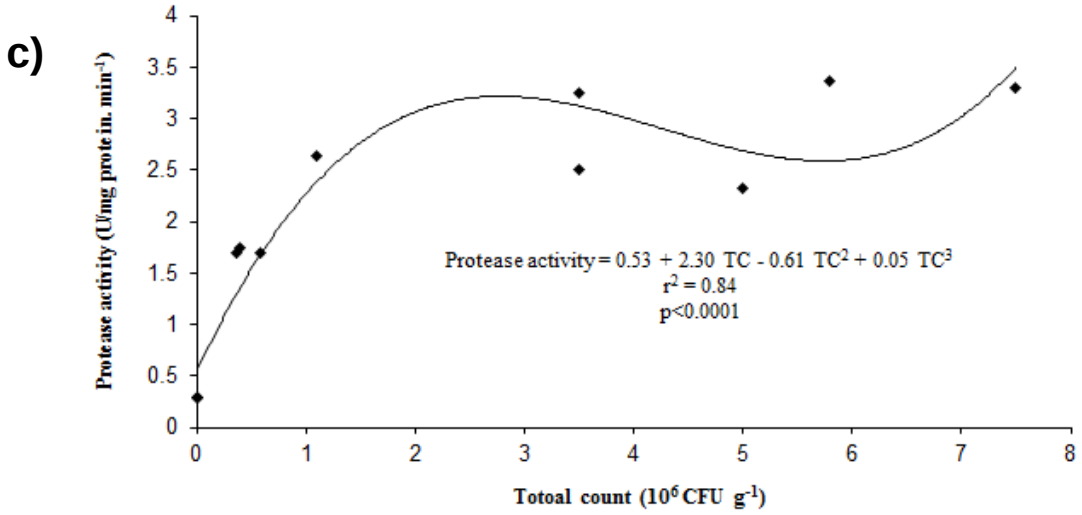
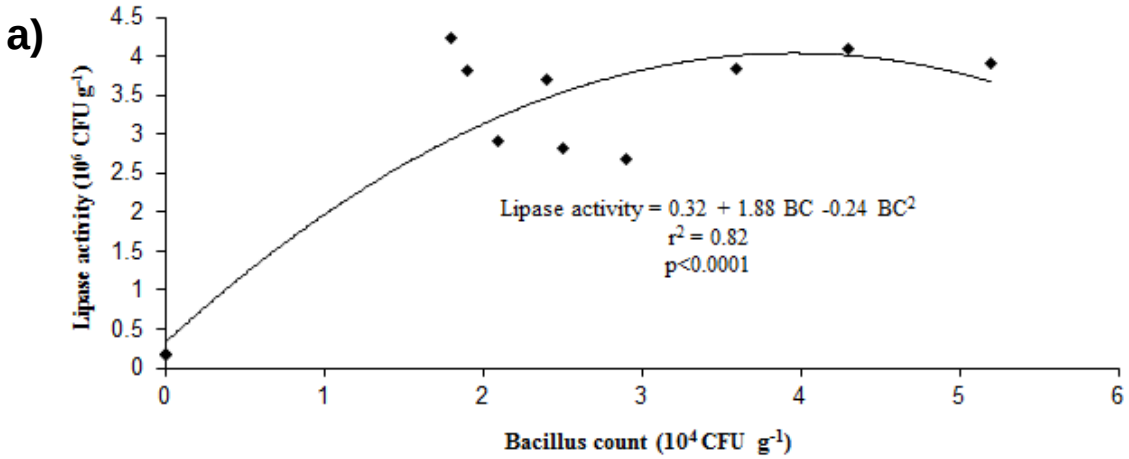
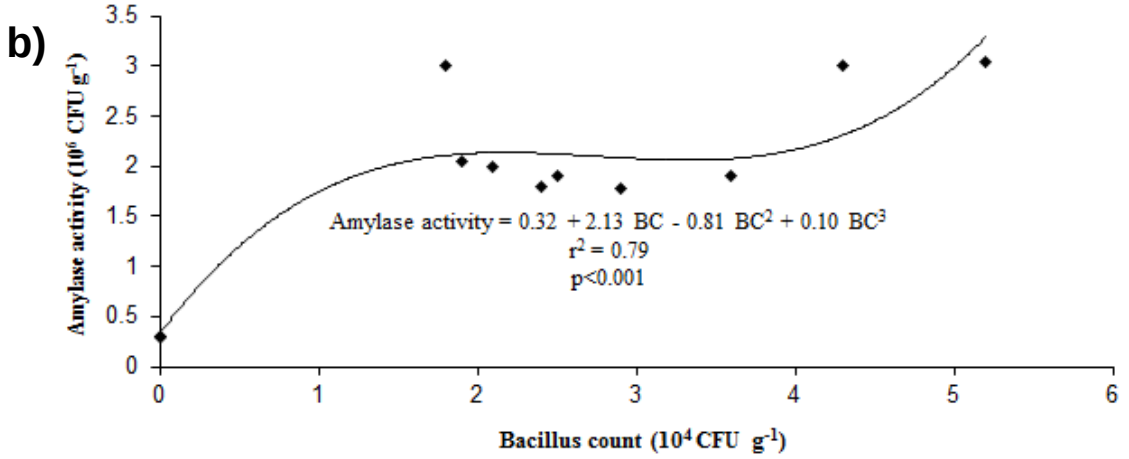


Fig. 7 Polynomial model fitting (a) lipase activity (U/mg protein. min^{-1}), (b) amylase activity (U/mg protein. min^{-1}) and (c) protease activity (U/mg protein. min^{-1}) to total count (TC; x 10^6 CFU g^{-1}) in *A. urmiana* with three replicates.





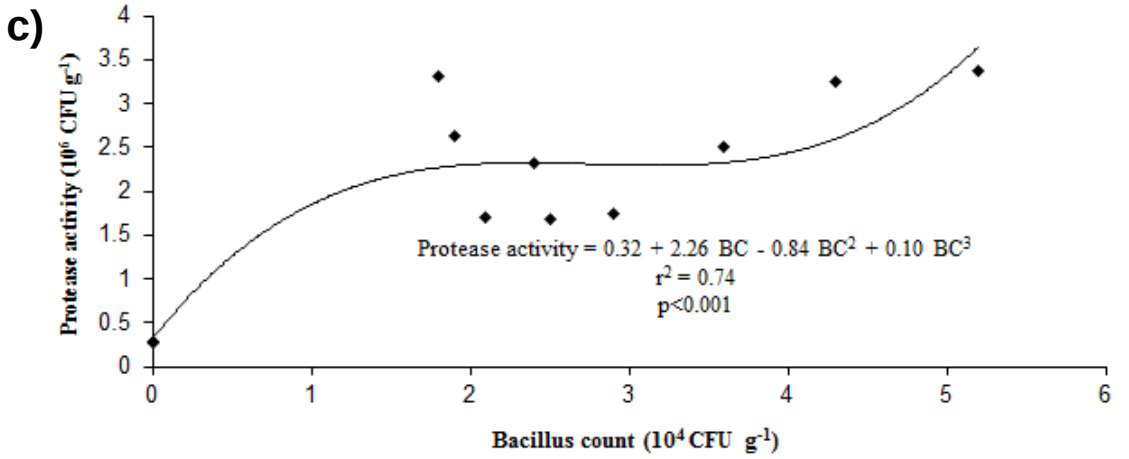


Fig. 8 Polynomial model fitting (a) lipase activity (U/mg protein. min^{-1}), (b) amylase activity (U/mg protein. min^{-1}) and (c) protease activity (U/mg protein. min^{-1}) to *Bacilus* spp. count (BC; x 10^4 CFU g^{-1}) in *A. urmiana* with three replicates.

Veróonica and Gimenez, 2013; Wei *et al.,* 2014). Assessment of the digestive enzymes activity can be used as an indicator of larval growth rate, food acceptance and the digestive capacity. Several workers have reported the activity of proteases, amylases, and lipases in crustaceans (Figueiredo *et al.,* 2001). But no report was found about *A. urmiana* digestive enzymes (protease, lipase and amylase) ontogeny during development.

It has shown that many crustacean species with economic importance possess the necessary enzymes for the hydrolysis of carbohydrates; however, amylase has been poorly studied in crustaceans (Pavasovic *et al.,* 2004). The results of the current experiment showed that *A. urmiana* nauplii digestive system is active starting from hatching in production and secretion of digestive enzymes. These findings were in agreement with Andrés *et al.* (2010) which indicated that all assayed enzymes were active from hatching time in spider crab, while Lovett and Felder (1990) reported that in *Penaeus setiferus* no activities were present for pepsin and lipase in some developmental stages. Maximum activities of protease, lipase and amylase were recorded in the fifteenth day of the experiment for *A. urmiana* which agrees with peak activities for all enzymes occurred during late zoea or early mysis larval stages of *P. setiferus*. This discrepancy can be species-specific, due to sampling methods and the definition of critical ranges of digestive enzyme activities during sample preparation and final optical density (OD) reading.

The activity of lipase was higher than others during the test that is consistent with other studies (Johnston, 2003). The high lipase activity in all *Artemia* stages reflects the importance of lipid as an energy reserve in crustaceans (Icely and Nott 1992; Johnston, 2003) and its key role as an energy substrate for *J. edwardsii puerulus* (Jeffs *et al.,* 1999, 2001, 2002), *H. americanus* (Sasaki *et al.,* 1986) and *Artemia*. According to Johnston (2003), a significant change in lipase-specific activity during different post hatch days confirms that lipid utilization is not consistent throughout development. Although the lipase activity was higher than other assessed enzymes, such an increment will not necessarily demonstrate higher absorption of fat in the digestive tract.

Results indicated that by shifting diet to a rich protein diet on the fifth day, the secretion of protease increased significantly. These findings are confirmed by other researchers who treated *A. urmiana* with different diets and reported that chemical composition of diets can alter the activities of digestive enzyme secreted by *Artemia* (Bami *et al.,* 2011). Similar results were obtained in the investigation of the ontogenetic changes in digestive enzymatic capacities of the spider crab (*Maja brachydactyla*) (Andrés *et al.,* 2010). On the other hand, some hypothesis on considering diet as a non-essential factor for ontogenic changes in the activities of digestive enzymes may arise. Instead, some developmental changes in enzyme synthesis or a secondary effect of change in the function and approximate size of the midgut throughout its distinction may be responsible for the ontogenic alteration of digestive enzyme activities (Lovett and Felder, 1990).

Results reported there was a significant relationship between *Artemia* total length and the protease activity which it may suggest that larger *Artemia* tends to consume foods that contain more protein content, like certain protozoa and bacteria. A similar trend was detected in ontogenetic changes in digestive enzyme activity of the spiny lobster (*Jasus edwardsii*) that revealed a strong relationship between total enzymes activity and carapace length juvenile and adult lobsters (Johnston, 2003). In the present study, we observed significant polynomial models between total length and digestive enzymes activities. These findings are in agreement with Ribeiro and Jones (2000) which stated that the evolution of trypsin and lipase activity in *Fenneropenaeus indicus* showed a dependence on the length of the post larvae, therefore the enzymatic response increases with size and evolutionary stages in *F. indicus*. Measurement of amylase and trypsin activities during different evolution stages of San Francisco Lake *Artemia* showed that the secretion of these enzymes depends on nutritional requirements of *Artemia*, and also the amount, chemical composition and the ingestion of food (Harris *et al.,* 1986). These researchers also have reported lots of changes in the activity of these enzymes in different developmental stages. These enzymes activity can fluctuate depending on the age and type of food.

Since the beneficial bacteria of GI tract like *Bacillus* spp. are capable of secreting digestive enzymes (Moriarty, 1998), it is likely that a part of the enzyme activity in the GI was associated with the bacteria. These suggest that in addition to chemical composition of food (Bami *et al.,* 2011), total length, evolutionary stages, GI bacteria enzyme secretion (Motlagh *et al.,* 2012), the interaction among these factors (Augusto E *et al.,* 2012) can influence on digestive enzymes activity during growth period. However, these findings may open a new area in live feed production industry.

The results of this study demonstrated the role of GI bacteria and other factors in the secretion of digestive enzymes. The accurate discovery of the bacterial role in producing digestive enzymes may result in the selection of probiotics more compatible with crustaceans and more accurate application of prebiotics. However, it requires a precise identification of bacterial communities and their role in the production and secretion of each digestive enzyme. Nowadays, new methods such as NGS and DGGE have been introduced to obtain such an intention. Apparently, more authentic information of digestive tract micro-biota and its relationship with the host will result in the expansion of more efficient micro-biota strategy to improve the health and production of crustaceans.

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