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Ontogeny of endogenous and exogenous amylase and total protease activities in mud crab, *Scylla serrata* larvae fed live food

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ABSTRACT

This study aimed to measure the exogenous enzyme contribution derived from live feed to the endogenous amylase and total protease activities in the mud crab, Scylla serrata larvae at various stages of development. Crab larvae at various stages were fed live food (A) or inactivated live food (B): Enzyme activity of A group was the endogenous activity while [(A-B)/A] x 100 was the percent contribution of exogenous enzyme derived from live food. Amylase *activity level derived from live food were significantly different at various stages of development (P<0.05). Exogenous amylase activity started very low at Z1 (10.3%) increased to 23.3% at Z2 and to more than double at Z3. The activity decreased abruptly to less than half (22.1%) at Z4 and remained at almost similar levels at Z5, megalopa and CI stages (28.8, 25.0, and 17.5%, respectively). Total protease activity varied significantly among development stages. The contribution of live food-derived total protease activity was very high level (84.4%) at Z1 and decreased abruptly at Z2 (33.1%). It increased to a small peak at Z3 (47.0%), remained low at Z4 and Z5 (34.8 and 31.9%, respectively) and increased at the megalopa stage (43.2%) and decreased at its lowest level at CI stage (24.6%). Endogenous total protease activity reflected the reported anatomical development of the digestive system in S. serrata larvae*

INTRODUCTION

The mud crab, Scylla serrata is of commercial value and can be sourced from the natural habitat like in mangrove areas [1], estuaries [2] or given time, from the hatchery.

S. serrata larvae, along with the caridean and homarid larvae, do not have anterior midgut diverticulae (AMD) that penaeid shrimps possess. During the early larval stages in penaeid, digestive enzymes are released from the AMD rather than from the hepatopancreas (HP) to enhance digestive intensity. Having no alternative source of digestive enzymes, Scylla serrata larvae's digestive capabilities are very limited, exacerbated by the fact that their HP is underdeveloped at stages Z1-Z5 [3; 4]. To enhance digestive capability, one way is to increase the gut evacuation time (Serrano 2012 in press) and to digest and assimilate a higher percentage of energy and nutrients from their prey [5].

At very early stages of development, crustacean larvae in general are ill-equipped for digesting food materials. They lack a fully functional digestive system and this could be one reason of very high mortality rate. In portunid crab

Portunus sanguinolentus (Herbst), mortality is 28% during the first zoeal stage itself and gradually increases thereafter [6]. The long eyed swimming crab *Podophthalamus vigil* (Fabricius) exhibits a very high 97% mortality from the zoea 1 to megalopa [7].

Crustaceans generally lack the necessary enzymes for the breakdown of food particles. In *S. serrata* larvae, specific activities of trypsin, amylase, leucine aminopeptidase varied among different developmental stages [8]. Carnivorous fish and shellfish larvae are hypothesized to rely on the enzymes present in live food organisms to assist digestion, and this has been demonstrated in penaeid shrimp [9]. Several authors have pointed out the importance of live organisms as a first food, suggesting that fish and shellfish larvae could utilize the enzymes in the food to improve digestion until the digestive tract becomes completely differentiated and developed [10; 11]. In fish, this hypothesis has not been uniformly accepted [12]. Another hypothesis is the possibility of the products of prey autolysis stimulating secretion of pancreatic trypsinogen and/or activating endogenous zymogens [13] which is hypothesized by Kumlu and Jones [14] in crustaceans. In contrast, high levels of enzyme activity have been detected in penaeid larvae right after hatch [15].

In this light, this study aimed to measure the exogenous enzyme contribution derived from live feed (i.e. rotifer and *Artemia*) to the endogenous amylase and total protease activities in the mud crab, *Scylla serrata* larvae at various stages of development.

MATERIALS AND METHODS

Broodstock and larvae

The broodstock females, whose progeny were used in these experiments, were identified as *S. serrata* according to the description of Keenan *et al*. [16]. Mature females were purchased from Roxas City, Capiz, Philippines which were collected from the wild. Preparation, rearing and feeding of broodstocks were as described previously [8]. When eggs hatched after several days of incubation, strongly phototactic and schooling first stage zoea (Z1) larvae were captured, concentrated using plankton net and were transferred to incubation and hatching tanks.

All larvae used in this experiment were transferred to experimental tanks without prior feeding and were starved for 4 h prior to the initiation of the feeding trial to ensure they were ready to ingest feed. At this point, enzyme assays were done to measure the baseline activity levels of the digestive enzymes.

Dietary treatments

Dietary treatment 1 was live food and Dietary treatment 2 was live feeds with their enzymes inactivated by heating to 100^0C for 5 min. In zoeal and megalopal stages, diet 1 was composed of freeze dried *Artemia* nauplii (OSI Brine shrimp eggs, USA) and rotifers (*Brachionus plicatilis*) in 1:1 ratio (w: w) microencapsulated with chitosan; diet 2 was enzyme-inactivated microencapsulated live feed. For juveniles, heat inactivated and fresh clam meat was used as feed. Prior to feeding, enzyme levels of test diets were measured.

Experimental setup consisted of three 20-L tanks per dietary treatment. The experimental lasted for 48 h for all zoea stages and 72 h for megalopa and juvenile crab. Digestive enzyme activities were quantified every 24 h. Exogenous digestive enzyme contributed by the live feeds was quantified following the formula:

% Enzyme Contribution = [(A-B)/A] X 100

where A is the enzyme activity of larvae fed diet 1 and B is the enzyme activity of those fed diet 2.

Crude enzyme preparation and protein determination

Pooled whole larvae or juvenile homogenates were used in all assays in triplicate. Samples were homogenized in 20 vol. of cold 50mM citrate phosphate buffer pH 7.0 at 1:20 ratio (w/v) in an Ultra Turrax homogenizer. The homogenates were centrifuged at 4000 rpm for 15 min at 4^oC and the supernatant was used as enzyme preparation. Total soluble protein was measured following the procedure of Lowry *et al.* [17] modified by Marichamy *et al.* [18] with bovine serum albumin as a standard. All enzyme assays were conducted within 4 h of homogenization and all samples for a single enzymatic assay were run in the same day. Blanks (i.e. absence of either enzyme or substrate) and controls (i.e. zero time reaction) were also run during the assay.

Enzyme assay

α-Amylase activity was assayed as described by Bernfield [19] modified by Mukesh *et al*. [20]. Briefly, the assay mixture consisted of 0.1 ml soluble starch solution, 0.5 ml of enzyme preparation and 0.5 ml homogenizing buffer. The reaction was stopped by adding DNS solution, the mixture heated for 5 min in boiling water, cooled in running water, diluted and optical density read at 546 nm. Amylase activity was expressed in terms of µg maltose liberated from starch min^{-1} mg $protein^{-2}$.

Total protease activity was measured using casein as substrate according to the methods of Walter [21] and Abirami *et al*. [22]. Reaction mixture consisted 0.75 ml of 1% (w/v) aqueous solution of casein, 0.1 ml of enzyme extract and 0.75 ml buffer in a final volume of 1.6 ml. After 1 h of the reaction, 2.25 ml ice-cold trichloroacetic acid (5%) was added, the mixture left at 2^{o}C for 30 min and the absorbance of the supernatant solution was read at 280 nm. One unit of total protease activity was expressed as µg of tyrosine produced min^{-1} mg^{-2} protein.

Statistical Analysis

Statistical analysis of the data was performed using a graph- statistical software package (Statistica, Stat Soft., Inc., USA). Homogeneity of variances and normality were tested (Levene's test and Shapiro–Wilk's test, respectively) prior to ANOVA. Differences between between arcsine-transformed percent contribution of enzyme activities derived from live food and developmental stage and their interaction were tested using one-way ANOVA. Post hoc analysis among groups after finding significant differences were performed by Tukey tests, with the level of significance preset at $P<0.05$. Data were reported as mean ± standard error.

RESULTS

Table 1 shows that amylase activity level derived from live food were significantly different at various stages of development ($P<0.05$). Exogenous amylase activity started very low at Z1 (10.3%) increased to 23.3% at Z2 and to more than double at Z3 (Fig. 1). The activity dropped abruptly to less than half(22.1%) at Z4 and remained at almost similar levels at Z5, megalopa and CI stages (28.8, 25.0, and 17.5%, respectively). Since the percent endogenous enzyme activity level was 100 minus percent exogenous levels, the pattern of change in one was the reverse pattern in the other as shown in Figs. 1 and 2.

Total protease activity varied significantly among development stages (Table 1). In contrast to amylase activity, the contribution of live food-derived total protease activity started at a very high level (84.4%) and dropped abruptly at Z2 (33.1%, Fig. 2). It increased to a small peak at Z3 (47.0%), remained low at Z4 and Z5 (34.8 and 31.9%, respectively) and increased at the megalopa stage (43.2%) and decreased at its lowest level at CI stage (24.6%).

Table 1: One-way analysis of variance (ANOVA) of exogenous enzyme contribution from live food at Z1, Z2, Z3, Z4, Z5, M and CI stages. Percent of contribution of exogenous enzymes were arcsine transformed before analysis.

Enzyme contribution	SV	*df*	SS	MS	*F* computed	Sig.
Amylase	Larval stage	6	0.474	0.079	22.614	0.000*
	Error	14	0.049	0.003		
Total protease	Larval stage	6	1.174	0.196	4.715	0.008*
	Error	14	0.581	0.041		

*SV=source of variation; df=degrees of freedom; SS=sum of squares; MS=mean squares; F=Fisher F statistic; *= significant differences exist (P<0.05)*

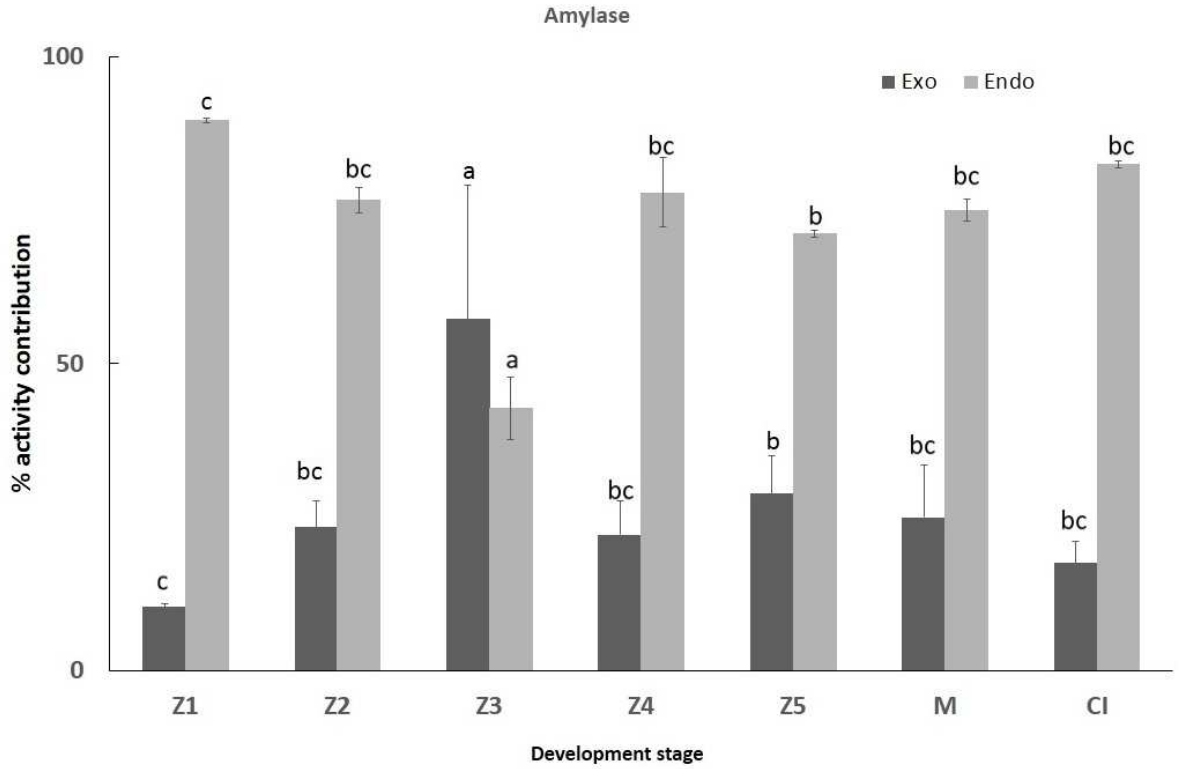
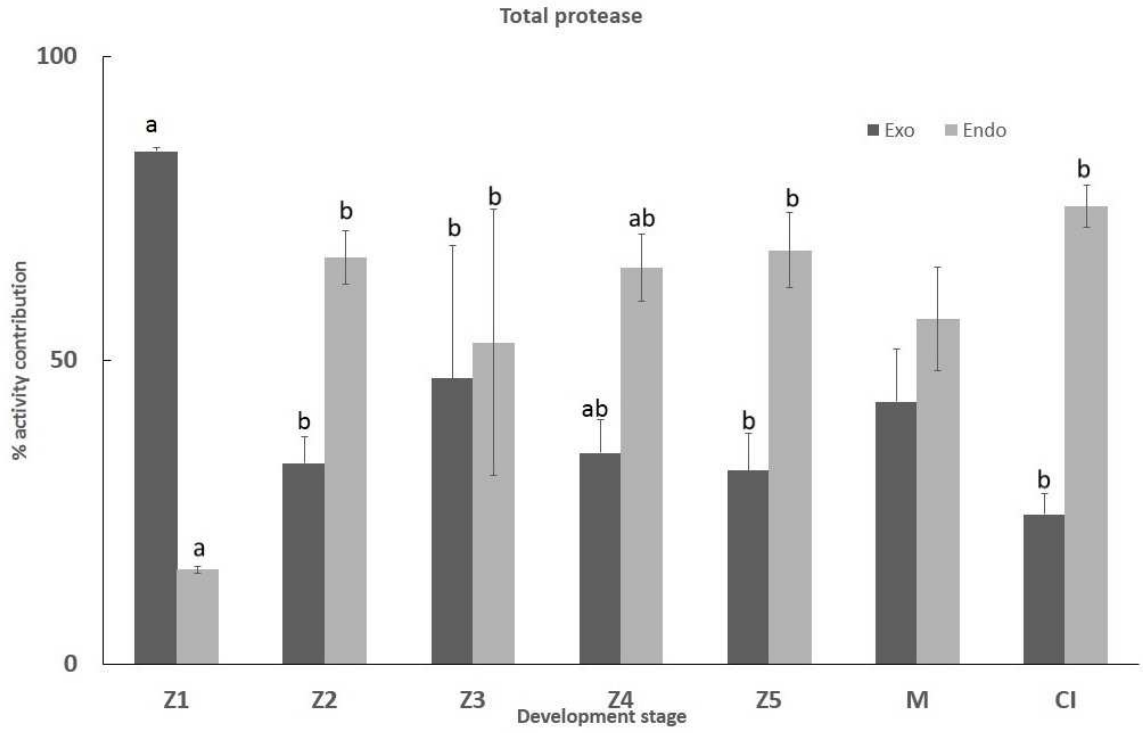


Fig. 1. Contribution (%) of exogenous and endogenous amylase activities (means, n=3) at various development stages in the mud crab *S.*



***serrata*. Different letters indicate significant differences (*P*<0.05).**

Fig. 2. Contribution (%) of exogenous and endogenous total protease activities (means, n=3) at various development stages in the mud crab *S. serrata*. Different letters indicate significant differences (*P*<0.05).

DISCUSSION

In the present study, *S. serrata* larvae, a brachyuran crab, appeared to be similar to caridean shrimps (*Palaemon* spp. and *Macrobarachium* spp) and homarid larvae (e.g. lobsters) in their inability to be reared with complete replacement of live diets with artificial diets. Live *Artemia* has been replaced completely with microparticulated diets in *Palaemon elegans* and *Macrobrachium rosenbergii* culture, but only from stage Z5/6 to PL stage [3; 4; 23]; this is also the case with *Scylla serrata* [24]. Genodepa *et al.* [25] have demonstrated 100% replacement of live food with microbound diet is possible when rearing *S. serrata* from the megalopa stage. It is clear from the present study that at early zoeal stages (Z1 to Z3), endogenous amylase activity was at the highest levels and the lowest total protease levels at Z1-Z2 and megalopa stages (exogenous activity derived from the live food had the opposite trend). This finding supports the hypothesis of Kumlu and Jones [23] that the inability of the early larvae of the caridean shrimp, homarid species and the crab is due to their low endogenous digestive enzyme activities during early larval stages.

Rotifers in the present study contributed considerable amount of total protease activities to the Z1 larvae (84%) but not enough amylase activity (10%). Except for the peak in amylase contribution at the Z3, contributions to amylase (23%-18%) and total protease (47%-25%) remained steady with development. Findings of the present study support the hypothesis of the importance of live food as sources of exogenous enzymes in all larval stages [26; 27]. This is in contrast with the findings of Jones *et al.* [5] and Kamarudin *et al.* [28] that contribution of exogenous enzymes from live *Artemia* nauplii to the digestion process of *M. rosenbergii* larvae and penaeid larvae has been measured and found to be insignificant. Lovett and Felder [29] report a very low contribution of enzyme activity from *Artemia* prey compared to that measured in larvae of *Peneaus setiferus*. Despite these contrary findings in fish and shellfish larvae, still the use of artificial microdiets for these species remain unsuccessful [30]. The author explains that this stems from the lack of understanding of the specificity of the enzymes that exist at different stages of larval development. The digestive capacities of larvae relative to the ingredients of a formulated feed may be species or stage-specific, as the present study showed. Digestive capacity parallels the anatomical development of the digestive system which in turn corresponds to changes in habitat and diet during metamorphosis [31]. The absence of digestive capacity can be attributed to either physical or chemical characteristics that are not compatible with the enzymatic capacity of fish and shellfish larvae [30]. Digestive capacity may also be based on the quality of food presented [32].

Early development stages in crustaceans, variations exist not only in the amount of enzyme but also in the type of enzyme present in the digestive system of the larvae [33]. Such different types of proteases, even if they are closely related, may show a different affinity for a given substrate. Pan *et al.* [34], for example, have found that *Artemia* nauplii autolysis is likely due to cathepsins present in these organisms, only slightly recognized in biochemical assays because of substrate affinity differences compared to serine proteases (e.g. trypsin, chymotrypsin). In the present study, total protease activity was measured and not a specific protease and thus, variations in the activities was attributed to all proteolytic reactions including cathepsins, serine proteases and other proteases.

In the present study, there was an increase in endogenous total protease activity (i.e. decrease in exogenous activity) from megalopa to CI, considering that the development of the digestive apparatus had advanced; at megalopa, the gastric mill is already a developed internal masticatory organ [35]. Fish, in contrast, exhibits a tendency of decreased trypsin activity after the stomach becomes functional [36], but in many cases, there is a delay in the increase in the protease activities following the completion of the development of the stomach [12]. In penaeid shrimp larvae, the level of trypsin activity is maximum at the megalopa stages and declines through metamorphosis [5; 37]. Other authors have shown that a correlation exists between the level of enzymes present and gastro-evacuation rate, and that the highest enzyme activity coincides with the shortest gut evacuation time [38]. Previous work in this laboratory did not agree with this observation; it was demonstrated that trypsin and leucine aminopeptidase in live-food-fed *S. serrata* larvae were highest at the CI stage [8] and yet the gut evacuation time was the longest (Serrano 2012, in press).

It is clear in the present study that endogenous protease activities was low only at Z1 and abruptly increased at Z2 remained high through the CI stage (exogenous activities were the reverse). This could be a reflection of the major changes of the digestive tract in this crustacean. Major changes in *S. serrata* larvae have been observed as the larvae

molt from Z3 to Z4, where the number of abdominal segments increase from 5 to 6, the gastric mill of the digestive system starts to develop [39], and the hepatopancreas becomes more functional [40].
Endogenous amylase activity was highest at Z1 then almost linearly decreased at Z2 and Z3 then increased at Z4 and remained high until CI (exogenous amylase activity showed the reverse pattern). In *Artemia*-fed *Hyas araneus*, a decapod crustacean, high amylase and trypsin activities are recorded [41] despite the fact that the diet is not rich in carbohydrates. The authors interpret this as a phylogenetic relic, suggesting that trypsin and amylase are co-regulated by a single factor.

In summary, live food-derived amylase activity was lowest at Z1 and maximum at Z3, the contribution abruptly decreased from Z4 through the CI stage. Exogenous total protease activity, in contrast, started at a very high level at Z1, dropped abruptly at Z2 and practically remained at that low levels from Z2 through the CI stage. Changes in the endogenous total protease activity reflected more the major development in the digestive system than did changes in the endogenous amylase activity.

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