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Temperature and pH Characteristics of Amylase and Lipase at Different Developmental Stages of Siamese Fighting Fish (*Betta splendens* Regan, 1910)

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ABSTRACT

Temperature (20-80°C) and pH (2-12) profiles of amylase and lipase at three stages (10-day-old, 1.5-month-old and 3-month-old) in Siamese fighting fish (*Betta splendens* Regan, 1910) were studied. At least nine amylase activities were observed during development: pH 7 at 40°C, pH 9 at 30°C and 50° C, pH 8 and pH 11 at 50°C in females and pH 8 and pH 11 at 40°C and 60°C in males. At the younger stage (≤ 1.5 month old), activity at pH 7 and 40°C, and at pH 9 and 30°C and 50°C was observed. At the older stage (≥ 1.5 month old), amylase activity at pH 8 and pH 11 in the temperature range of 40-60°C was found. Lipase had at least five levels of activity: pH 7 at 20°C and 40°C, pH 8 at 20-40°C and 60°C, and at pH 11, where the activity peak disappeared in the maturing stage (3 month old) in both sexes. Amylase had very low specific activity at the 10-day-old stage, while lipase had a high specific activity, similar to older stages. The most suitable pH to determine amylase and lipase activity in Siamese fighting fish was pH 8, whereas a temperature of 50°C was appropriate for amylase and 40°C was suitable for lipase, regardless of sex and age. This information is a prerequisite for future studies of the *in vitro* digestibility evaluation of nutrient utilization in Siamese fighting fish.

Key words: Siamese fighting fish, *Betta splendens*, digestive enzymes, amylase, lipase

INTRODUCTION

The Siamese fighting fish (*Betta splendens* Regan, 1910), widely distributed in Southeast Asia, is a native species of ornamental and sport fish in Thailand. It is distinctly sexually dimorphic; adult males are distinguished by larger bodies with larger and longer fins than females. Males with long fins are the most expensive and provide the highest income among the exported ornamental fish of Thailand (Wiwatchaisaet, 2000). The fish is in high demand worldwide, resulting in over exploitation of fish populations through breeding and farming. In natural conditions, the fish feeds on live food, such as mosquito larvae and water fleas. In aquaculture, these live food sources and chicken/duck yolks are mainly used for broodstock conditioning and

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during the entire developmental stages (Sermwatanakul and Bowonsupakijkul, 2001). However, the quality and the quantity of the live food and the yolks, as well as the risk of inducing contaminated pathogens are not under control, and a substantial culture of live prey stock has to be maintained through extensive labor efforts. Therefore, the use of artificial feeds with optimized nutritional quality is needed in aquaculture. The utilization of nutrients by aquatic animals depends on digestive enzymes (Areekijseree *et al*., 2006; Rungruangsak-Torrissen, 2007; Rungruangsak-Torrissen and Fosseidengen, 2007; Supannapong *et al*., 2008; Rungruangsak-Torrissen *et al*., 2009). Studying amylase activity has facilitated the development of more rapid and accurate *in vitro* carbohydrate digestibility assay (Areekijseree *et al*., 2006; Supannapong *et al*., 2008) and has been used as an indicator for carnivorous feeding habits (Hofer and Schiemer, 1981). The aim of this study was to characterize amylase and lipase in the digestive tract of *B. splendens* at three representative stages: juveniles at the 10-day-old stage (completely developed digestive tract and having predatory behavior), the 1.5-month-old stage (sexually identified for individual rearing) and the 3-month-old stage (maturing stage). Lipase was included because dietary lipid requirements vary among species based on feeding habit, stage of life and habitat (Biswas *et al*., 2009). Fish of the long-finned form were the subject of this study, due to their high economic importance. The work could provide important prerequisite knowledge for future studies of digestive enzyme expression during the life cycle, as well as for future development of artificial feed formulations for different stages of *B. splendens*.

MATERIALS AND METHODS

Fish preparation

B. splendens at three different stages, 10-day-old (48.75 ± 1.00 mg, 6.63 ± 0.56 mm), 1.5-month-old (males: 413.33 ± 125.16 mg, 30.31 ± 3.21 mm; females: 329.00 ± 62.55 mg, 27.33 ± 1.54 mm) and 3-month-old (males: 1,190.48 ± 142.81 mg, 53.79 ± 3.62 mm; females: 648.57 ± 105.80 mg, 34.49 ± 1.93 mm) were sampled in triplicate using a completely randomized design (CRD). The experiment was carried out on a farm in Nakorn Pathom province, which is a main production area in Thailand for exported fish (Sermwatanakul and Bowonsupakijkul, 2001). At the farm, the newly hatched larvae were reared in cement tanks (75 cm diameter × 35 cm height) each containing 25 L of water. In each tank, five females were pooled (~400 larvae per female). The water volume was increased every few days and up to 150 L within one week, when the fish were 10 days old after hatching. The fish were reared in this volume of water until they were 1.5 months old. After that, they were reared individually in 200 ml glass bottles until 3 months old. The water temperature changed slightly in the range 27-29°C. During the first week, the larvae were fed twice daily with cooked chicken egg yolks dissolved in water following by paramecium and then small-sized water fleas. From 10 days old to 1.5 months old, the fish were fed on water fleas and after that, they were fed on mosquito larvae until maturation at 3 months. The biochemical composition of the common diets used for culturing Siamese fighting fish are shown in Table 1. The fish were starved for 2 h prior to sampling. Both males and females were studied at 1.5 and 3 months old. Females were identified by visual observation of an ovipositor, the most visible small white spot at the anus. Pooled samples of about 2,000 juveniles at 10 days old, and 100 males and 100 females at each of 1.5 and 3 months old from each replicate were collected for enzyme analyses.

Enzyme study

Enzyme extraction

The enzyme extracts from *B. splendens* were prepared from the whole bodies of 10-day-

Table 1 Biochemical composition in percent on wet weight basis of the common diets used for culture of *B. splendens*. The analyses were performed as described by AOAC (2005).

Diet	Moisture	Protein	Carbohydrate	Lipid	Fiber	Ash
Water flea	85.64	8.00	4.02	0.89	0.68	0.77
Mosquito larva	73.42	13.99	6.43	3.39	0.74	2.03
Chicken egg yolk	48.06	15.72	4.48	29.40	nd	2.34
Duck egg yolk	41.74	16.50	1.40	38.11	nd	2.25

nd = not detected.

old juveniles, from the digestive area of 1.5-month-old fish and from the digestive tracts of 3-month-old adults. The enzyme extracts were performed according to Rungruangsak-Torrissen (2007). Briefly, the samples were homogenized on ice in 50 mM Tris-HCl buffer pH 8 containing 200 mM NaCl (1:1 w/v). The homogenate was centrifuged at 4°C at 10,000× g for 20 min and the supernatant was collected and kept at 80°C for later determinations. The total protein content of the crude enzyme extract was determined based on Lowry *et al*. (1951).

Amylase specific activity

Amylase activity was determined by measuring the increase in reducing sugar from the starch solution, using the 3,5-dinitrosalicylic acid (DNS) method according to Areekijseree *et al*. (2004) based on Bernfeld (1951). The crude enzyme extracts were incubated with starch solution in various 100 mM buffers containing 6 mM NaCl at different pH levels. The buffers used were glycine-HCl for pH 2, citrate phosphate buffer for the pH range 3-5, phosphate buffer for the pH range 6-8, $NaHCO_3$-Na_2CO_3 buffer for the pH range 9-10, Na_2HPO_4-NaOH for pH 11 and KCl-NaOH for pH 12. For the pH profile study, the reaction mixture was performed at ambient temperature and at various pH levels (2-12). For the temperature profile study, the reaction mixture was performed at optimal pH conditions and at various temperatures (20-80°C). The amount of maltose released was calculated from a maltose standard curve. The amylase specific activity was expressed as μmol of maltose produced h^{-1} mg protein^{-1}.

Lipase specific activity

Lipase activity was assayed according to the method of Winkler and Stuckmann (1979), following the release of *p*-nitrophenol from *p*-nitrophenyl palmitate (*p*NPP). The reaction mixture contained 10 μl of crude enzyme extracts, 200 μl of 0.01 M *p*NPP (dissolved in isopropanol at 37°C) and 800 μl of 0.2 M buffer. For pH and temperature profiles, the reaction mixtures were incubated at various pH (2-12) and temperature (20 80°C) levels, as described above. The reaction was performed for 15 min and then stopped by adding 250 μl of 1 M Na_2CO_3. The supernatant was obtained by centrifuging at 10,000× g for 15 min and the absorbance was measured at 410 nm. Lipase-specific activity was expressed as the increase in absorbance at 410 nm h^{-1} mg protein^{-1}.

Statistical analysis

Estimates of the mean and standard deviation were calculated. Analysis of variance (ANOVA) at the 95% significance level was performed, and multiple comparisons were analyzed by Duncan's multiple range test (DMRT). Regression analysis was carried out on concentration values to obtain standard curves.

RESULTS

Characterization of amylase

Total specific activity of amylase increased with age in both males and females (Figure 1A). Males seemed to have lower amylase

specific activity than females at 1.5 months, while at 3 months, the males had significantly higher ($P < 0.05$) amylase-specific activity than females. At least nine levels of amylase activity were observed. At high amylase activity, four optimal pH levels (7, 8, 9 and 11) were detected. The amylase activity with optimal pH levels of 7 and 9 were observed in 10-day-old juveniles and 1.5-month-old males, while the amylase activities with optimal pH levels of 8 and 11 were observed in 3-month-old males and in females at both 1.5 and 3 months (Figure 1A). The temperature profile study revealed that the neutral amylase isoform with optimal pH 7 showed optimal temperature characteristics at 40° C and the alkaline amylase isoform with optimal pH 9 had optimal temperatures of 30°C and 50°C.

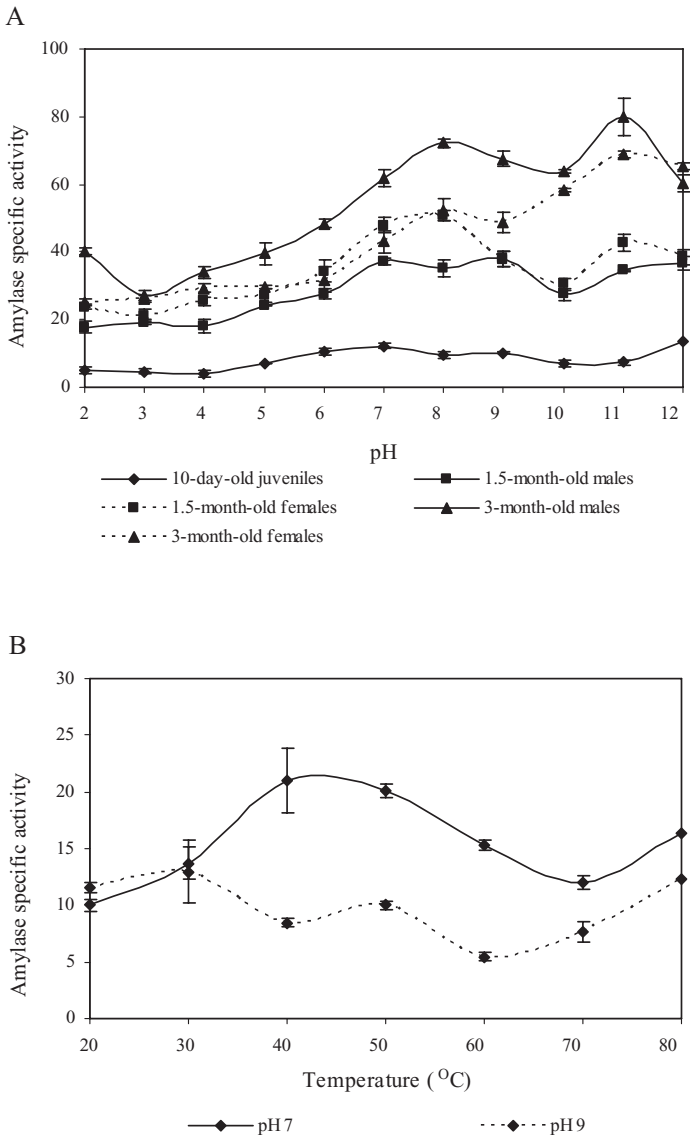

Figure 1 Amylase specific activity (μmol maltose h^{-1} mg $protein^{-1}$) at various stages of development of *B. splendens*: (A) pH profiles at ambient temperature; and (B) temperature profiles at optimal pH levels in 10-day-old juveniles.

These conditions were observed in young fish, namely 10-day-old juveniles (Figure 1B) and 1.5-month-old males (Figure 2A). The alkaline amylase with optimal pH 8 showed different temperature characteristics between adult males and females, with the optimal temperature being 50°C in females at both 1.5 months (Figure 2A) and 3 months (Figure 2B), and at 40°C and 60°C in males at 3 months (Figure 2B). For alkaline amylase with optimal pH 11, the males showed a broad range of optimum temperature (40-60°C at 1.5 months (Figure 2A) and at 40°C and 60°C when 3 months old (Figure 2B)), while the females showed an optimal temperature of 50°C, indicating similar characteristics to the amylase at optimal pH 8 (Figure 2B).

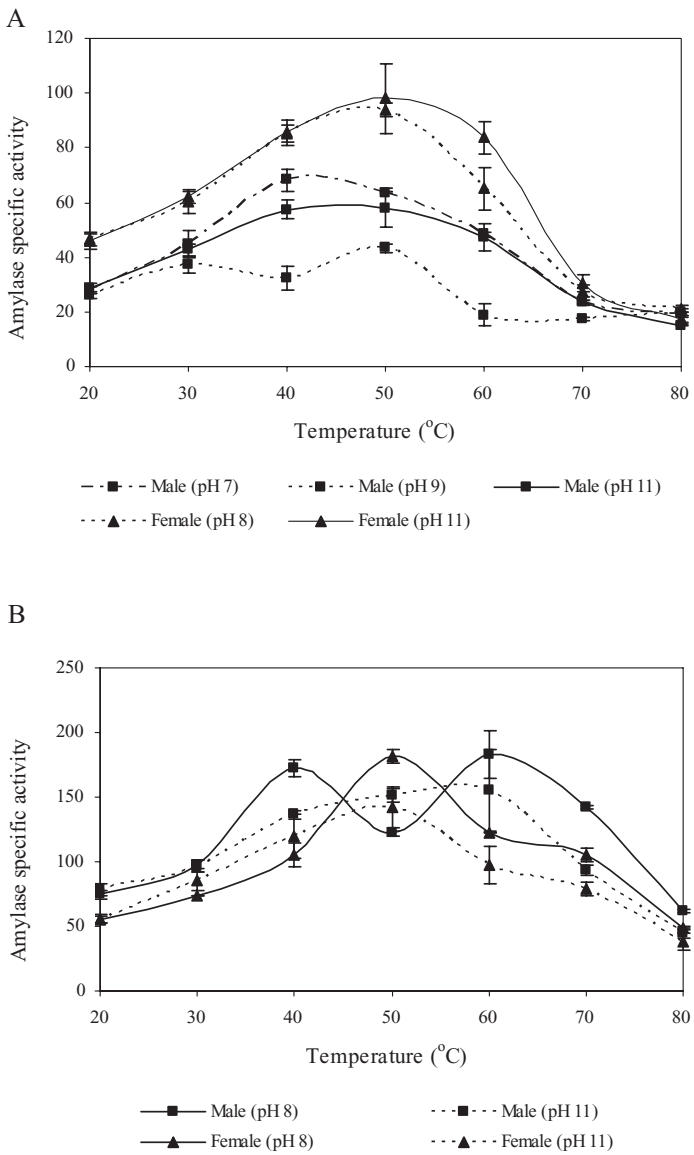

Figure 2 Temperature profiles of amylase specific activity (μmol maltose h^{-1} mg $protein^{-1}$) of *B. splendens* at different optimal pH levels at: (A) 1.5 months; and (B) 3 months.

Characterization of lipase

Specific activity of lipase was lower in acidic conditions than in neutral and alkaline conditions (Figure 3A). At least five lipase activities were observed. At high lipase activity, three optimal pH levels (7, 8 and 11) were detected. Neutral lipase with optimal pH 7 and the alkaline lipase with optimal pH 11 were observed in 10-day-old juveniles (Figure 3A). At 1.5 months, alkaline lipase isoforms with optimal pH levels of

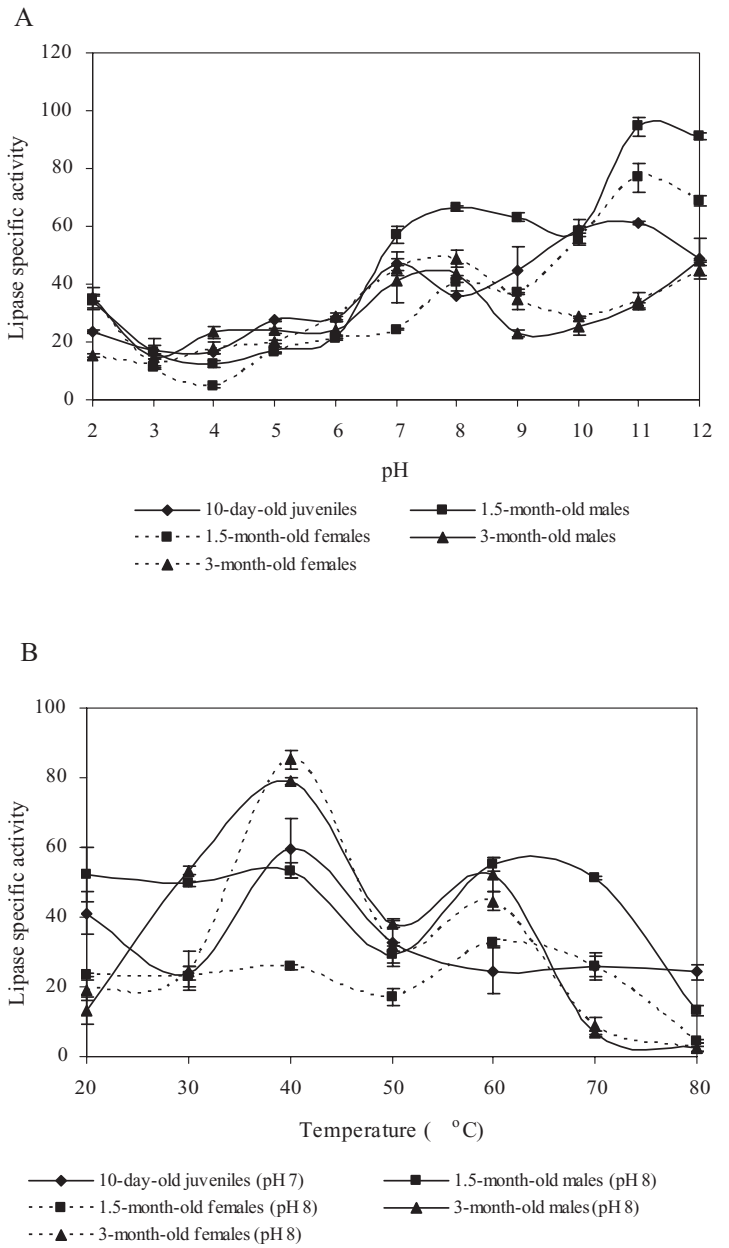

Figure 3 Lipase specific activity (increase in absorbance at 410 nm h^{-1} mg $protein^{-1}$) at different stages of development of *B. splendens*: (A) pH profiles at ambient temperature; and (B) temperature profiles at optimal pH levels.

8 and 11 were found in both males and females (Figure 3A). As the fish became older (3 months), alkaline lipase with optimal pH 8 was still observed, while the peak of the isoform with optimal pH 11 disappeared, regardless of sex (Figure 3A). The neutral lipase condition showed optimal temperatures at 20°C and 40°C with activity still being observed at 80°C (Figure 3B). In both sexes, the alkaline lipase with optimal pH 8 showed optimal temperatures in the range 20 40°C and at 60°C, and the specific activity at 1.5 months was significantly higher ($P < 0.05$) in males than in females, while the specific activity at 3 months was similar between the two sexes (Figure 3B). The temperature profile of the lipase with optimal pH 11 was not investigated.

DISCUSSION

Amylase specific activity in Siamese fighting fish increased with age, similar to amylase expression observed in red drum (Lazo *et al.,* 2007) and thick-lipped grey mullet (Zouiten *et al*., 2008). The four pH characteristics of amylase observed in Siamese fighting fish indicated that the isoforms with optimal pH levels of 7 and 9 were developed in the early life stage and the isoforms with optimal pH levels of 8 and 11 were developed at a later stage. At 1.5 months, females seemed to develop faster than males, as a high amylase specific activity was observed at pH levels of 8 and 11 ($P < 0.05$), while the profile in males was similar to the 10-day-old juveniles with optimal pH level of 7 and 9 (Figure 1A). Temperature optima varied (30-60°C) during development, and the amylase isoform with optimal conditions at pH 11 and 50°C (Figure 2) has been observed also in freshwater pearl mussel (Areekijseree *et al*., 2004; Supannapong *et al*., 2008). Amylase activity dominated in the pancreas and intestine (Natalia *et al*., 2004) under alkaline conditions close to the pH range in these organs (Chakrabarli *et al*., 1995), indicating alkaline amylase is the major enzyme involved in carbohydrate digestion. Amylase activity varied during development and depended on the nutritional habits (Hidalgo *et al*., 1999), and niche segregation (Chakrabarti *et al*., 1995) of each sex. The most suitable conditions observed for studying amylase activity in general during the life cycle of Siamese fighting fish were at pH 8 and 50°C. The assayed temperature for males at maturation could be changed to 40°C or 60°C (Figure 2B) if optimal activities required it. The optimal assayed conditions for amylase activity varied with each species. The optimal conditions were pH 7 and 40°C in freshwater pearl mussel (Areekijseree *et al*., 2004; Supannapong *et al*., 2008), while they were at neutral pH (7-7.5) in sea bream and turbot, and at an acidic pH (4.5-5) in redfish with an optimal temperature range of 35 45°C for the three species (Munilla-Moran and Saborido-Rey, 1996).

Siamese fighting fish require substantial lipase activity to effectively digest the high dietary lipid intake from live organisms, such as water fleas (0.89% lipid content, Table 1) and mosquito larvae (3.39% lipid content, Table 1), which were the live diets used in this experiment. Other diets commonly used during the larval stage are chicken and duck egg yolks, which have a higher lipid content of 29.40 and 38.11%, respectively (Table 1). Carnivorous fish usually consume fat-rich diets, and high lipase activity could result from the exogenous lipase present in live diets (Morais *et al*., 2007). On the other hand, tilapia, with low intakes of lipids from plant-related diets, showed limited distribution and low levels of lipase activity along the digestive tract (Tengjaroenkul *et al*., 2000). Moreover, dietary lipid plays an important role in carotenoid biosynthesis, transportation and pigmentation in fish (Gouveia and Rema, 2005). High lipase activity in 10-day-old juveniles might be induced by the exogenous lipase from the water fleas, or could be related to the metabolism of lipid reserves in yolk accumulated before metamorphosis that were mobilized during the

morphological modification (Martinez *et al*., 1999). In addition, lipase specific activity exhibited in this early stage might be related to lipid breakdown from oocyte, or correspond to fatty acid requirements for larval development (Morais *et al*., 2006). Lipase specific activities were significantly higher ($P < 0.05$) in males than in females during on-growing (1.5 months), but similar at maturation (3 months). Increased lipase specific activity toward alkaline conditions (Figure 3A) corresponded with digestive physiology in the pancreas and intestine of carnivorous fish (Natalia *et al*., 2004). The three pH characteristics of lipase observed in Siamese fighting fish indicated that the isoforms with optimal pH levels of 7 and 11 developed in the early life stage, the isoform with optimal pH 8 was developed later, and the peak of the isoform with optimal pH 11 disappeared in the late stage of maturation, regardless of sex (Figure 3A). The neutral lipase with optimal pH 7 showed optimal temperatures at 20°C and 40°C (Figure 3B), similar to the characteristics observed in red drum larva (Lazo *et al*., 2007). The alkaline lipase with optimal pH 8 showed an optimal temperature range of 20-40°C and at 60°C in both sexes (Figure 3B). These lipase characteristics were similar to those detected in freshwater pearl mussel (Areekijseree *et al*., 2002). The most suitable conditions observed for studying lipase activity during the life cycle of Siamese fighting fish should be at pH 8 and 40°C, which are close to the pH (6.5-7.5) and temperature (26-30°C) range in their natural habitat (Meenakarn *et al*., 1988).

Variations in the specific activity of the digestive enzymes could be affected by the nutritional qualities and the levels of dietary nutrients. The use of chicken egg yolk and the different live diets might have caused the variations in the observed enzyme specific activity levels at different life stages in this experiment. However, the effect was less for amylase than lipase, as chicken egg yolks, water fleas and mosquito larvae have a similar range of carbohydrate content, but vary in their protein and lipid contents (Table 1). Carbohydrate digestibility was more critical than lipid digestibility in the larval stage because the expression of amylase was very low (Figure 1A), while lipase expression was high (Figure 3A), compared to older stages. The modification of unavailable carbohydrate polymers in formulated feeds to enhance their digestibility and the addition of fatty acids to improve their quality might be of interest. It is important to study the characteristics of different digestive enzymes in each species in order to select the most suitable and practical conditions for future investigation of any impact on the expression of each digestive enzyme of interest during animal development, and a high temperature optimum is suitable for such a comparison study (Rungruangsak-Torrissen, 2007; Rungruangsak-Torrissen *et al*., 2007). The information will also be useful as prerequisite knowledge for developing artificial feed formulations through nutritional evaluation of diets by *in vitro* digestibility studies (Areekijseree *et al*., 2006; Supannapong *et al*., 2008), including the current study, with an optimal temperature close to natural habitat conditions being suitable.

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