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Original Article

Digestive enzyme activity during ontogenetic development and effect of live feed in green catfish larvae (*Mystus nemurus* Cuv. & Val.)

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Abstract

Mystus nemurus is one of the economically important freshwater species fish for aquaculture in Thailand. The aim of this study was to describe the development of proteolytic enzymes (pepsin, trypsin and chymotrypsin), carbohydrase (α-amylase) and lipase, and the influence of live feed during early stage of larval development. Enzymatic assays were conducted from day 1 to day 45 after hatching in larvae fed the following feed sequence: *Moina* sp. during 3-15 day after hatching (DAH), co-feeding between *Moina* sp. and powder feed during 16-29 DAH and artificial diet during 30-45 DAH. Three samples of larvae were collected before morning feeding on days 1, 3, 5, 7, 9, 12, 15, 20, 25, 30, 35, 40 and 45. All enzymes were detected at low level as early as hatching and remained constant until 20 DAH. Significant increases of pepsin, trypsin, chymotrypsin and α-amylase activity were detected during 25-45 DAH ($p<0.05$) whereas lipase activity showed an increase during 30-45 DAH, which was related to an increased growth of fish larvae at 30 DAH onward ($p<0.05$). Live feed, *Moina* sp., significantly increased enzyme activity of trypsin, chymotrypsin and α-amylase of fish larvae during 3-15 DAH in comparison with those of unfed fish larvae ($p<0.05$).

Keywords: green catfish larvae, digestive enzymes, enzyme activity, live feed

1. Introduction

Mystus nemurus (Cuvier & Valenciennes) or green catfish is one of the economically important freshwater species for aquaculture in Thailand, Malaysia and in other part of Southeast Asia, because of its taste, non-bony flesh and high protein value.

The digestive system of early fish larvae is extremely immature comprising a straight intestine and a small pancreas with a lack of functional stomach so that the digestion before the differentiation of the stomach depends mainly on pancreatic enzymes. There are indications that the simple morphological structure of the digestive tract correlates with a low production of enzymes (Dabrowski, 1979) and result in poor digestion of artificial diets and their dependence on live food organisms (Srivastava *et al*., 2002). Therefore, if we know the presence and levels of activity of main digestive enzymes during larval development which indicate the ability to digest dietary composition on appropriate time, we can use the information to determine the appropriate timing to wean fish larvae with artificial feed. The knowledge of appearance of key enzymes in the gut of cultivable species in aqua-

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culture is essential to understand age specific formulation of feeds that contributes to rapid and efficient growth rates (Tengjaroenkul *et al*., 2002). However, very little is known about the pattern of digestive enzymes of green catfish larvae during early stages of development. Besides, it is equally important to know the influence of live feed, *Moina* sp., on activity of digestive enzyme of fish larvae during early stage of green catfish. The aim of this experiment was to study the development of main digestive enzyme activity of green catfish larvae from larval stage until juvenile stage (1– 45 day after hatching, DAH) and to determine the effect of live feed on main digestive enzymes activity in 1-15 DAH of green catfish larvae.

2. Materials and Methods

Experiment 1: Development of main digestive enzyme activity of green catfish larvae from larval stage until juvenile stage (1– 45 DAH).

2.1 Larval rearing

Newly hatched fish larvae were obtained from Songkhla Inland Fisheries Research and Development Center, Thailand. The larvae were stocked in a 60x40x25 cm^3 aquarium at 25 fish/l. Larvae were fed *Moina* sp. at the density 10 individual/ml from day 3-15 after hatching, co-feeding between *Moina* sp. and powder feed from day 16-29 after hatching and pelleted feed from day 30-45 after hatching 2 times a day.

2.2 Sampling and processing

Triplicate samples of larvae (100 larvae or 0.3 g) were collected randomly before morning feeding at day 1, 3, 5, 7, 9, 12, 15, 20, 25, 30, 35, 40 and 45 after hatching for monitoring growth and enzyme extraction. Whole larvae were used in the preparation of a crude enzyme extract from day 1-20 after hatching and the mid portion (stomach and intestine) of the larvae was used from day 25-45 after hatching. Pooled fish larvae and midgut organs were rinsed with distilled water and immediately frozen in liquid nitrogen, then homogenized with 2.5 volumes (v/w) of 0.5 mM Tris buffer pH 7.5 under low temperature (4°C), and then centrifuged at 12,879 ×g for 30 min at 4°C twice. Supernatant was collected for crude enzyme and stored at -70°C.

2.3 Analysis of digestive enzymes activity

Samples were analyzed for protein concentration by modified Lowry's Method (1951) using 1 mg/ml bovine serum albumin (BSA) as a standard curve.

Pepsin activity was determined according to method of Anson (1938) using 1% hemoglobin as a substrate. The buffer used was glycine-sodium chloride-HCl (pH 2). The mixture consisted of 1% (w/v) solution of 0.5 ml hemoglobin, 0.4 ml buffer and 0.1 ml of crude enzyme. All mixtures were incubated for 10 min in water bath at 37°C and the reaction stopped by adding 1.5 ml of 5% trichloroacetic acid (TCA) and left at room temperature for 1 hour. The samples were then centrifuged at 2,236 ×g for 10 min and the absorbance of supernatant recorded at 280 nm. Tyrosine was used as the standard curve and 1 enzyme unit was equivalent to 1 mmole tyrosine released in 1 minute. The specific enzyme activity was expressed as unit per milligram of protein (Unit/mg protein).

Trypsin activity was measured with N_α-benzoyl-DL-arginine-ρ-nitroanilide hydrochloride (BAPNA). 1 mM BAPNA was dissolved in 1% dimethyl sulfoxide 0.05 M Tris-HCl, pH 8.2, 0.02 M $CaCl_2$. Chymotrypsin activity was measured using 0.2 mM succinyl-$(ala)_2$-pro-phe-ρ-nitroanilide (SAPNA) as a substrate in 0.05 M Tris-HCl, pH 8.0, 0.02 M $CaCl_2$. The incubation consisted of 900 ml of substrate and 100 ml crude enzyme for 10 min at 37°C, then stopped the reaction with 100 ml of 30% TCA. The absorbance was recorded at 410 nm. Trypsin and chymotrypsin activity units were defined as 1 mmol ρ-nitroaniline released per minute, using 8800 $M^{-1}.cm^{-1}$ as a extinction coefficient of ρ-nitro-aniline at 410 nm (Erlanger *et al*., 1961). Activity units were calculated using the following equation:

$$\text{Activity units} = \frac{(\text{Abs}_{410}\text{nm-blank})/\text{min} \times 1000 \times \text{total volume of reaction mixture}}{8800\text{M}^{-1}.\text{cm}^{-1} \times \text{volume of enzyme} \times \text{mg protein in reaction mixture}}$$

The specific enzyme activity was expressed as milli-unit per milligram of protein (mUnit/mg protein).

Amylase activity was assayed with 1% (w/v) starch solution as substrate (Rick and Stegbauer, 1974). The 1% starch solution was prepared in phosphate buffer (pH 6.9). The reaction mixture was incubated at 37°C for 10 min. Then dinitrosalicylic acid (DNS) was added to stop the reaction and the preparation kept in a boiling water bath for 5 min. After cooling, the reaction mixture was diluted with distilled water and absorbance recorded at 540 nm. Activity was determined from the maltose standard curve and specific activity expressed as mmole of maltose released per milligram of protein at 37°C (Unit/mg protein).

Lipase activity was assayed according to the method of Winkler and Stuckman (1979), following the release of ρ-nitrophenol (ρ-NP) from ρ-nitrophenyl palmitate (ρ-NPP). A freshly prepared substrate was pre-warmed at 37°C and incubated with the enzyme extract for 15 min at 37°C. The reaction was stopped with 2 M sodium carbonated and the absorbance was recorded at 410 nm against enzyme-free control. Enzyme specific activities are reported as unit of activity per milligram of protein, with one enzyme unit representing 1 mmol of ρ-nitrophenole liberated during 1 minute of hydrolysis using the following equation. The extinction coefficient of r-nitrophenol is 15,000 $M^{-1}.cm^{-1}$

Activity units =

$$\frac{(Abs_{410}\text{nm-blank})/\text{min} \times \text{total volume of reaction mixture}}{15,000 M^{-1}.cm^{-1} \times \text{volume of enzyme} \times \text{mg protein in reaction mixture}}$$

Experiment 2: The Effect of age and live feed on larval digestive enzymes activity during 1-15 day after hatching.

Newly hatched larvae were stocked in a 60x40x25 cm^3 aquarium at 25 fish/L. Larvae fish were fed from the mouth opening (day 3) with live feed (*Moina* sp.) two times a day. At the sampling time (day 1, 3, 5, 7, 9, 12 and 15 after hatching), fish larvae were divided into two groups (20 liter aquaria, 3 replicate), the first group without feeding, the second group still fed with *Moina* sp. at the density of 10 ind./ml. Sampling was conducted 1 hour after feed in order to evaluate the effect of the age and diet in the production of digestive enzymes. Pooled samples of each group of larvae (0.3 g) were collected for enzymatic assays and samples were taken at the same hour in the morning. Crude enzyme extraction and determination enzyme activity using the same method as in Experiment 1.

2.4 Statistical analysis

Results presented are mean ± SD, (n=3). In Experiment 1, data were analyzed using one-way ANOVA and difference among means were compared by Duncan's multiple range test. Two-way ANOVA following Student-Newman-Keuls multiple range tests was used in Experiment 2 when significant differences were found at a 0.05 level.

3. Results

3.1 Growth

Growth of green catfish larvae during 1-45 DAH is presented as wet average body weight per larvae related to day after hatching (Figure 1). Body weight showed a slow increase from day 1-25 after hatching ($p>0.05$). From 30-45 DAH, weight increased rapidly and significantly ($p<0.05$) which can divide the growth into 2 phases and the relationship between body weight and age was exponential with an R^2 of 0.965.

3.2 Specific enzyme activity

Specific enzyme activity of green catfish larvae during development was slow during first phase (1-25 DAH) and increasing in the second phase (30-45 DAH). All proteolytic enzymes; pepsin trypsin and chymotrypsin were detected at low level as early as hatching and remained constant until 20 DAH except pepsin activity which was significantly increased on 5 DAH ($p<0.05$) and decreased from day 7-20 DAH. After that they increased abruptly, especially pepsin and chymotrypsin. But, when specific pepsin activity was increased on 40-45 DAH, the specific activity of trypsin and chymotrypsin decreased. The highest specific pepsin activity was recorded on 45 DAH (22.45±0.62 Unit/mg protein), and the highest trypsin and chymotrypsin activities on 35 DAH (565.64±29.05 and 79.70±0.03 mUnit/mg protein respectively) (Table 1 and Figure 2).

The specific carbohydrase enzyme activity, α-amylase, was detected at the lowest level at hatching, increased on 5 DAH, and then decreased constantly from 7-20 DAH. After that the specific α-amylase activity showed an increasing trend from 25 to 45 DAH, and the maximum specific activity was recorded on 45 DAH (6.20±1.14 Unit/mg protein) (Figure 3).

The specific lipase enzyme activity was detected at the lowest level at hatching and remained constant until 25 DAH ($p>0.05$). After that it increased abruptly to the maximum level on 30 DAH (58.54±3.68 mUnit/mg protein) and then decreased significantly to a lower level on 35-45 DAH (Figure 4).

3.3 Effect of live feed on digestive enzyme activity

Specific digestive enzymes activities of fed and unfed larvae group were assayed between 1-15 DAH on whole body

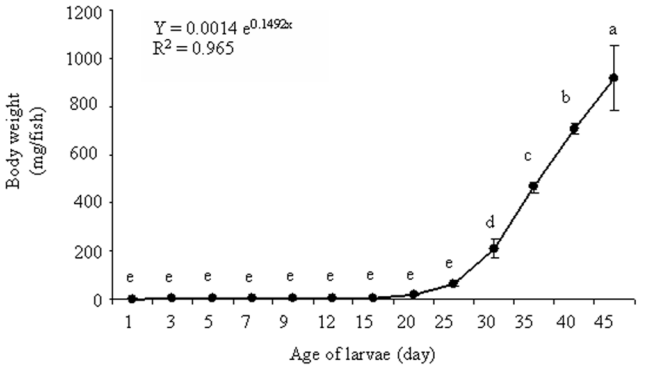

Figure 1. Growth of green catfish during 1-45 DAH. Mean±SD (n=3) with different superscripts are significantly different ($p<0.05$).

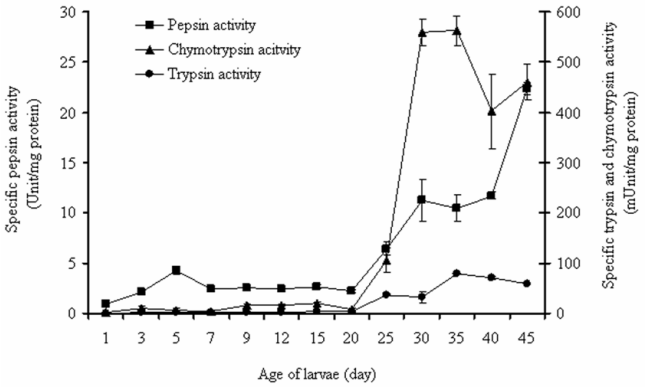

Figure 2. Specific activity of proteolytic enzymes; pepsin, trypsin and chymotrypsin, during larval development, 1-45 DAH.

Table 1. Specific enzyme activity of proteolytic enzymes; pepsin, trypsin and chymotrypsin, during larval development, 1-45 DAH.

Age of larvae (DAH)	Pepsin[1] (Unit/mg protein)	Chymotrypsin (mUnit/mg protein)	Trypsin (mUnit/mg protein)
1	0.91±0.05^{f}	1.49±0.29^{e}	0.16±0.10^{e}
3	2.13±0.21^{ef}	10.52±3.37^{e}	1.29±0.28^{e}
5	4.26±0.43^{d}	6.94±2.30^{e}	1.52±0.12^{e}
7	2.42±0.16^{e}	4.87±0.79^{e}	1.84±0.70^{e}
9	2.57±0.16^{e}	15.38±3.29^{e}	2.95±0.20^{e}
12	2.41±0.30^{e}	15.29±2.00^{e}	2.56±0.29^{e}
15	2.67±0.20^{e}	20.05±1.44^{e}	4.00±0.24^{e}
20	2.21±0.15^{ef}	8.38±1.64^{e}	3.75±0.36^{e}
25	6.43±0.69^{c}	105.54±23.51^{d}	35.97±1.70^{d}
30	11.22±2.04^{b}	480.51±138.25^{a}	32.02±11.56^{d}
35	10.40±1.31^{b}	562.64±29.05^{a}	79.70±0.03^{a}
40	11.67±0.35^{b}	402.84±74.07^{c}	70.63±3.15^{b}
45	22.41±0.62^{a}	460.87±35.54^{b}	59.14±1.58^{c}

[1]Mean±SD (n=3 of larva were pooled). Mean values with the same superscript letter in the same row are not significantly different (p>0.05).

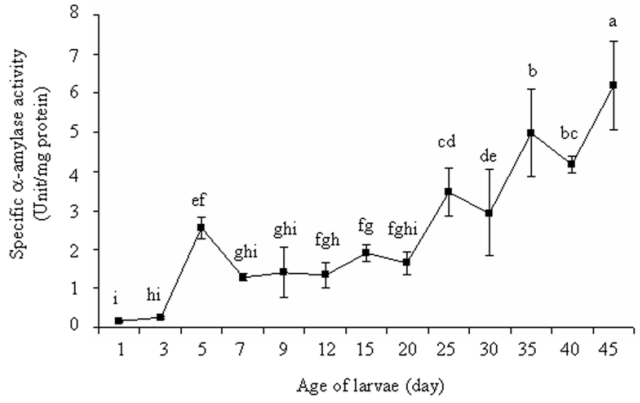

Figure 3. Specific activity of α-amylase during larval development, 1-45 DAH. Mean±SD (n=3) with the same superscript letter are not significantly different (p>0.05).

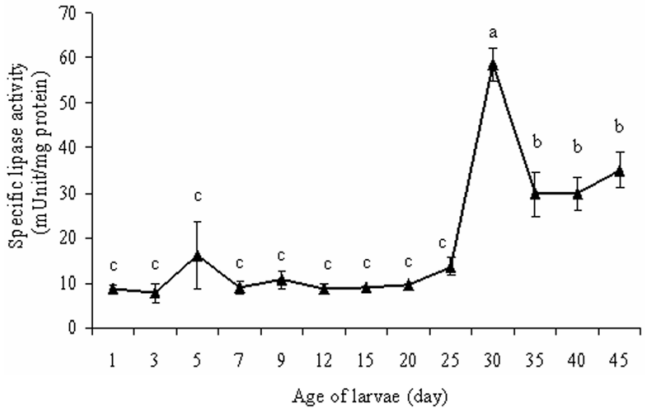

Figure 4. Specific enzyme activity of lipase during larval development, 1-45 DAH. Mean±SD (n=3) with the same superscript letter are not significantly different (p>0.05).

homogenates. Table 2 presents the data of enzyme specific activityin terms of main effects of age, *Moina* sp. and the *Moina* sp. x age interaction. The presence of *Moina* sp. increased the specific enzyme activity of trypsin, chymotrypsin and α-amylase, whereas pepsin and lipase activity was strongly influenced by age. In addition, the result showed that age and *Moina* sp. had an interaction effect on α-amylase activity (p<0.05).

4. Discussion

Growth of green catfish larvae exhibited a pattern similar to those found in other fish species such as European seabass (Infante and Cahu, 1994), yellow croaker (*Pseudosciaena crocea*) (Ma *et al*., 2005), yellow catfish (*Pelteobagrus fulvidraco*) (Yang *et al*., 2010), white seabass (*Atractoscion nobilis*) (Galaviz *et al*., 2011) and spotted rose snapper (*Lutjanus guttatus*) (Galaviz *et al*., 2012). It was slow at the first phase (1-25 DAH) then dramatically increased in the second phase (30-45 DAH) and the relationship between body weight and age was exponential.

Enzymes involved in the digestion of proteins, lipid and carbohydrate were present at low level in green catfish larvae as early as hatching in accordance with the results found in striped bass (*Morone Saxatilis*)(Baragi and Lovel, 1986), turbot (*Scophthalmus maximus* L.) (Cousin *et al*., 1987), gilthead seabream (*Sparus aurata*) (Moyano *et al*., 1996), European seabass (*Dicentrarchus labrax*) (Infante and Cahu, 2001), haddock (*Melanogrammus aeglefinus*) and cod (*Gadus morhua*) (Perez-Casanova *et al*., 2006). Pancreatic enzyme activities, works from trypsin, chymotrypsin, lipase and α–amylase, have been detected as early as hatching and even before mouth opening because of the presence

Table 2. Two-way ANOVA tests of significance for the interactions of age and *Moina* sp. on specific enzymes activity of green catfish larvae during 1-15 day after hatching, $p<0.05$.

Age of larvae (Day)	Proteolytic enzyme activity						Carbohydrase		Lipase	
	Pepsin (Unit/mg protein)		Trypsin (mUnit/mg protein)		Chymotrypsin (mUnit/mg protein)		α-amylase (Unit/mg protein)		Lipase (mUnit/mg protein)	
	Unfed fish	Fed with *Moina* sp.	Unfed fish	Fed with *Moina* sp.	Unfed fish	Fed with *Moina* sp.	Unfed fish	Fed with *Moina* sp.	Unfed fish	Fed with *Moina* sp.
1	0.75±0.05	ND*	0.33±0.33	ND	0.99±1.26	ND	0.07±0.03	ND	9.90±0.86	ND
3	2.81±0.11	3.36±0.04	4.04±1.53	7.32±2.38	8.90±0.97	33.46±4.50	0.24±0.05	0.46±0.06	12.84±0.51	14.52±0.44
5	3.17±0.10	3.43±0.08	5.16±2.57	6.19±1.93	10.94±0.74	36.95±12.69	0.81±0.15	1.10±0.60	13.29±4.76	15.08±2.82
7	4.27±0.35	3.78±0.30	2.26±1.13	4.57±1.54	12.40±2.16	29.10±15.54	0.41±0.07	1.63±0.33	14.54±0.59	15.89±1.45
9	4.02±0.08	3.98±0.18	4.84±0.78	7.33±1.35	16.57±1.07	41.60±4.19	0.72±0.11	1.57±0.68	15.62±0.41	16.25±0.62
12	3.77±0.02	3.50±0.36	3.86±0.85	10.99±2.60	15.77±3.52	29.44±1.95	0.83±0.14	0.84±0.43	6.08±2.58	8.44±0.63
15	5.26±2.12	4.16±1.46	4.76±1.88	6.98±0.89	24.51±6.44	34.32±13.99	1.19±0.28	1.02±0.28	13.28±3.27	16.88±3.33

Effect	*p*	*p*	*p*	*p*	*p*
Age	0.010	0.550	0.149	0.005	0.000
Moina sp.	0.173	0.001	0.000	0.001	0.226
Age x *Moina* sp.	0.100	0.167	0.242	0.012	0.601

*ND = not detected

of the pancreas (Ma *et al*., 2005). Nevertheless, trypsin activity found at hatching may come from the hatching gland in mother fish (trypsin-like hatching enzymes) (Noting *et al*., 1999).

The pepsin activity was detected at hatching which is similar to yellow catfish (*Pelteobagrus fulvidraco*) (Wang *et al*., 2006), haddock (*Melanogrammus aeglefinus*) and cod (*Gadus morhua*) (Perez-Casanova *et al*., 2006) However, in many fish species, pepsin activity was detected at a later stage for example, day 28 for red porgy (Suzer *et al*., 2007), day 22 for red drum (Lazo *et al*., 2007), day 24 for European seabass (Infante and Cahu, 1994), day 10 for white seabass (Galaviz *et al*., 2011) and day 25 for spotted rose snapper larvae (Galaviz *et al*., 2012). Perez-Casanova *et al*. (2006) using the same method with this study suggested that the appearance of pepsin-like enzyme activity prior to the appearance of pepsinogen transcripts and appearance of gastric gland is likely the result of the measurement of other acidic proteases, particularly aspartic proteases belonging to the pepsin family (A1). This family includes enzymes such as the lysosomal cathepsin D. The biochemical technique used in this study has been used in other studies for the determination of cathepsin D with only a minor modification in the pH of the reaction (Anson, 1938). Cathepsin D is involved with the cellular degeneration of proteins and this enzymes is present in a variety of fish tissues including liver and muscle (reviewed in Nielsen and Nielsen, 2001 cited by Perez-Casanova *et al*., 2006). From this study the pepsin activity may be established on day 5 when specific pepsin activity was significantly increased ($p<0.05$) correlating with the recent histological study of Hug *et al*. (2012), which detected the morphological differentiation of the stomach of green catfish larvae at day 5 after hatching when gastric glands appearing on day 7, indicating the presence of gastric enzyme. Chen *et al*. (2002) also reported the appearance of gastric glands on 5 DAH in *Mystus macropterus* while the pepsinogen granules appeared in gastric glands at 3 DAH in yellow catfish (Yang *et al*., 2010). In African catfish larvae, the stomach appearance was at day 6 after hatching and gastric acid secretion started on day 6, leading to a pH below 3.3 in the stomach on day 7 after hatching (Verreth *et al*., 1992). The age at appearanceof gastric glands was earlier in fish of Siluriformes than those of other Orders (Yang *et al*., 2010).

An increase in specific pepsin activity on 25-45 DAH had an effect on the reduction in serine protease (trypsin and chymotrypsin) activity on 35-45 and 35-40 DAH, respectively. This phenomenon was similar to those reported in *Lates calcarifer* (Walford and Lam, 1993), red porgy (Suzer *et al*., 2007), orange-spotted grouper (*Epinephelus coioides*) larvae (Eusebio *et al*., 2004) and malabar grouper (*E. malabaricus*) (Fujii *et al*., 2007). A relationship between the marked decrease in the specific activity of protease and metamorphosis was reported by Tanaka *et al*. (1996). The activity of pepsin-like enzyme increased markedly as metamorphosis proceeded, while trypsin–like enzyme and amylase activities dropped significantly in Japanese flounder (Fujii *et al*., 2007). Additionally, tryptic activity of Asian seabass was markedly reduced after the differentiation of the gastric gland (Walford and Lam, 1993). Accordingly, the observed decrease in trypsin and chymotrypsin might be due to the increased acid digestion by pepsin activity and the maturity of digestive organ. In

addition, the transition feeding from co-feeding to artificial diet during 30-45 DAH might be related to the increase of pepsin activity. Wang *et al*. (2006) suggested that dietary protein level can induce pepsin gene expression of yellow catfish larvae at the transcription level.

The specific chymotrypsin activity of green catfish larvae was higher than the trypsin activity, which was similar to the findings in dourano (Vega-Orellana *et al*., 2006), dover sole (Clark *et al*., 1986) and carp (Rathore *et al*., 2005) larvae indicating the main alkaline proteolytic enzyme. However, in European seabass (Eshel *et al*., 1993) and gilthead seabream (Chong *et al*., 2002) trypsin is the main alkaline proteolytic enzyme during the early stage.

The explanation of the declining or constant specific enzyme activities occurring during 1-20 DAH using whole body for analysis is not due to a diminution in enzyme synthesis but is a consequence of growth, development of new organs, and an increase in tissue proteins (Wang *et al*., 2006). This is because the reported specific enzyme activity is the ratio of activity per mg protein, which does not reflect a lowering in digestive capacity (Infante and Cahu, 2001).

Specific amylase activity showed a constant increase from day 25 onwards while lipase activity showed an increase at 30 DAH and a significant decrease on 35-45 DAH. This may be due to the transition feeding from co-feeding on live feed and powder feed to artificial diet and the maturity of digestive organ.

The exogenous enzyme from live feed plays an important role in assisting in digestive process in fish or crustacean larvae (Dabrowski, 1982). Munilla-Moran *et al*. (1990) estimated that live food contributes significantly 43-60% protease, 78-88% esterase and 89-94% amylase into the digestive system of *Scopthalmus maximus* larvae (cited by Kamarudin *et al*., 2011). The effect of live feed in early stage feeding of green catfish larvae is demonstrated in this study. *Moina* sp. assisted the larval digestive processes via a contribution of the trypsin, chymotrypsin and amylase activity, whereas pepsin and lipase activities were not affected. The results are in line with Lauff and Hoffer (1984), who showed that about 70% of the trypsin activity in the intestine of *Coregonus* sp. larvae was derived from *Moina* sp.

5. Conclusions

The present study showed that all main digestive enzymes were detected at low level as early as hatching and remained constant until 20 days; after that the activities significantly increased corresponding with an increased growth of the fish larvae.This indicates that fish larvae have good protein and carbohydrate utilization while lipid utilization was delayed. Live feed, *Moina* sp., assists the larval digestive process via a contribution of trypsin, chymotrypsin and amylase activity during the early larval stage.

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