Aquaculture 372–375 (2013) 62–69

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Ontogeny of the digestive enzymes in butter catfish *Ompok bimaculatus* (Bloch) larvae

Pravata Kumar Pradhan [a,*], Joykrushna Jena [a], Gopa Mitra [b], Neeraj Sood [a], Enric Gisbert [c]

[a] National Bureau of Fish Genetic Resources, Canal Ring Road, Dilkusha, Lucknow-226002, Uttar Pradesh, India
[b] Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar-751002, Odisha, India
[c] IRTA, Centre de Sant Carles de la Ràpita, Unitat de Cultius Aqüícoles, Crta. de Poblenou km 5.5, 43450 Sant Carles de la Ràpita, Tarragona, Spain

ARTICLE INFO

Article history:
Received 27 June 2012
Received in revised form 22 September 2012
Accepted 17 October 2012
Available online 22 October 2012

Keywords:
Butter catfish
Larviculture
Weaning
Digestion
Ontogeny

ABSTRACT

The digestive physiology of butter catfish was studied by assessing the activity of different pancreatic (trypsin, chymotrypsin, α-amylase and lipase), gastric (pepsin) and intestinal (alkaline phosphatase) enzymes from hatching until the juvenile stage (30 dph). Larvae were reared at 27 °C and fed with *Artemia* nauplii from 2 days post hatching (dph) until 10 dph, from 7–10 dph with *Artemia* nauplii and zooplankton (Cyclopoida) and from 10 dph onwards only with zooplankton. The assessment of the activity of digestive enzymes showed that enzymes involved in the digestion of proteins, lipids and carbohydrates were present in butter catfish larvae before mouth opening and increased after the onset of exogenous feeding, coinciding with the histological organization of the exocrine pancreas. The specific activity of most of the pancreatic enzymes increased until 15 dph and decreased thereafter coinciding with the increase of pepsin. A progressive shift in activity from alkaline (trypsin and chymotrypsin) to acid (pepsin) proteases indicated a change in the digestive physiology of the specimen, as alkaline proteases were no longer the main digestive enzymes involved in protein digestion after the onset of acidic digestion between 15 and 21 dph. The maturation of the intestine and the achievement of a juvenile-like mode of digestion were demonstrated by changes in enzyme activities from the exocrine pancreas and stomach that coincided with alterations in enzyme production occurring in the intestine (e.g. alkaline phosphatase). Considering the ontogenetic development of the digestive enzymes from the pancreas, stomach and intestine, butter catfish larvae might be weaned between 15 and 21 dph, as larvae have achieved the complete maturation of their digestive capacities. These results contradict previous recommendations, which were based solely on the histological organization of the gastric gland and histochemical properties of mucous cells from the stomach, to wean butter catfish larvae at earlier ages. These findings on the functional development of the digestive system in butter catfish would be useful to improve the actual larval rearing techniques for this promising catfish species from the Indian sub-continent.

1. Introduction

The ontogenetic development of digestive enzymes reflects the functional development of the digestive tract and digestive capacities of the organism, and consequently, it has been regularly used as a physiological biomarker to assess the nutritional status of fish at early life stages of development. A comprehensive analysis of the ontogenetic changes during the early life stages of fish may also help in identifying limiting factors during larval rearing, determining the appropriate time for weaning, optimizing the rearing technology and feeding practices with the developmental stage of the fish (Gisbert et al., 2008; Zambonino-Infante et al., 2009). The onset of the digestive functions and ontogenetic patterns of enzyme activity must be determined for each species in order to quantify and establish the capacity of larvae to digest and absorb different types of nutrients, either from live feed and/or compound microdiets (Gisbert et al., 2009). This will also assist in defining the nutritional requirements for several nutrients (Twining et al., 1983). Although the basic mechanisms of organ and system development are similar in all teleosts, there are considerable interspecific differences regarding the relative timing of differentiation, development, and functionality during early ontogeny (Treviño et al., 2011). Hence, there is a need to conduct specific studies on the ontogenesis of digestive system of fish for each species to better understand their nutritional physiology.

The description of the digestive system development just by means of histological and histochemical procedures is the first step for providing insights into the development of the digestive functions in fish larvae; however, since morphology does not always match functionality, studies based upon microscopy need to be complemented with biochemical ones in which the activity of the main digestive enzymes from the pancreas, stomach and intestine is assessed in order to provide

* Corresponding author. Tel.: +91 522 2442441; fax: +91 522 2442403.
E-mail address: pradhanpk1@gmail.com (P.K. Pradhan).

0044-8486/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquaculture.2012.10.024

evidence of digestive system functionality (see review in Lazo et al., 2011). The ontogenetic development of the digestive enzymes has been described in many marine and freshwater fish species (Chakrabarti et al., 2006; Kumar et al., 2000; Lazo et al., 2011; Zambonino-Infante et al., 2009 among others). Among them, several studies have evaluated the morpho-anatomical development of the digestive tract and accessory glands in several catfish species (de Amorim et al., 2009; Kozarić et al., 2008; Liu et al., 2010; Pradhan et al., 2012; Segner et al., 1993; Verreth et al., 1992; Yang et al., 2010). However, little is known about ontogenetic and dietary-induced changes in digestive enzyme production in different catfish species during early life stages of development outside of a few exceptions on sheatfish *Silurus soldatovi* (Liu et al., 2010) and striped catfish *Pangasianodon hypophthalmus* (Rangasin et al., 2012). Up to date, no study has been conducted on the ontogenetic development of digestive enzymes in butter catfish *Ompok bimaculatus* (Bloch).

Butter catfish, a fish species belonging to the family of Siluridae, has a very high consumer preference in the North Eastern, Eastern, and mainland states of India. The species is a delicacy in those areas and is one of the most expensive food fish species ($8–12 kg^{-1}). The high commercial value makes it a promising aquaculture species for the future. Further, the species is categorized as an endangered fish species according to the International Union for Conservation of Nature and Natural resources (IUCN) criteria and faces a high risk of extinction in the wild (CAMP, 1997), which raises the issue of the sustainable exploitation of this biological resource. It has recently been targeted as an important candidate for the diversification of freshwater Indian aquaculture and stock enhancement (NBFGR, 2011). Consequently, the aquaculture of this catfish species serves multiple purposes; i) diversification of Indian freshwater aquaculture; and ii) conservation and restocking programs. Independently of the final purpose of this activity, a reliable larval rearing technique must be developed to ensure consistent production of good quality fry. However, the high mortality rate during larval rearing is the most serious bottleneck at present for commercial production of this species. Therefore, the success and development of aquaculture activity devoted to butter catfish culture still demand improvements in rearing techniques, especially those affecting larval rearing practices such as the partial or complete replacement of live prey with a compound microdiet. Thus, in order to enhance the success of larval rearing of this catfish species and to facilitate overcoming one of the major bottlenecks of fish hatcheries, the description of the ontogeny of the larval digestive enzymes is a necessary tool. This information will be of value for synchronizing the larval stage of development and maturation of their digestive organs with the feeding protocol and rearing practices. Recently, Pradhan et al. (2012) has provided detailed information about the major morphological events in the development of the butter catfish digestive tract, which indicated a well-differentiated digestive system within the first 12 days after hatching (324 degree days). However, the presence of the structures which were found does not mean that they are functional (see review in Lazo et al., 2011). Hence, in the present study we have described the development of the main digestive enzymes from the pancreas, stomach and intestine in butter catfish reared under standard conditions from hatching to the early juvenile stage. This new information is expected to provide fundamental knowledge on the digestive physiology of this species at early life stages of development. The data will be very useful for improving actual larval rearing practices for butter catfish, one of the most interesting and promising fish species for diversification in freshwater aquaculture in the Indian sub-continent.

2. Materials and methods

2.1. Eggs and larval rearing

Larvae used in this study were obtained from a single pair of broodstock in which eggs from a mature female were fertilized with the sperm of a single male according to the procedures described in Pradhan et al. (2012). Fertilized eggs were washed thoroughly with clean water and placed in three 1800 L ($3\times1\times0.60$ m^3) cement incubators at 27.0 ± 1.1 °C with mild aeration. Incubation lasted for 23 ± 1 h and upon hatching, larvae were stocked in three fiberglass 3000 L tanks ($4\times1\times0.75$ m^3) at a density of 5 larvae L^{-1} and reared until the juvenile stage (30 days post hatch, dph) as previously described by Pradhan et al. (2012). In brief, larvae were fed to apparent satiation four times per day with newly-hatched *Artemia* nauplii (O.S.I. PRO 80™, Ocean Star International, Inc. USA) from mouth opening (2 dph) until 10 dph. From 7 dph onwards, zooplankton collected from nearby ponds, which consisted mainly of copepods (Cyclopoida), were also added to fish rearing tanks four times per day. After 10 dph, only zooplanktonic preys were given to butter catfish larvae. During the rearing period, water temperature, dissolved oxygen and pH values were maintained at 27.0 ± 1.1 °C, 6–8 ppm and 6.8–7.6, respectively. Fish were held under natural photoperiod according to the rainy season of the year (25°53′ N, 91°55′ E). Larval culture was performed in the Tripura State Government Hatchery at Agartala, Tripura (India).

2.2. Larval sampling and growth measurements

A random sample of 100 larvae was collected daily from 1 to 7 dph; 75 larvae every second day until 15 dph and 50 larvae every third day until 30 dph from the three rearing tanks, respectively. Larvae were sampled in the morning prior to food distribution in the rearing tanks and euthanized with an overdose of tricaine methanesulfonate (MS-222, Sigma), rinsed in distilled water and after water removal with a filter paper. Then, samples were frozen in liquid nitrogen and stored at −80 °C until further analysis. Growth measurements were obtained from a pool of 10 larvae per tank from each sampling day. Total length (TL) of larvae was measured individually using a scale to the nearest of 0.1 cm and wet body weight (BW) was assessed to the nearest of 0.001 g using an analytical microbalance (Metler Toledo AG245). The specific growth rate (SGR) was determined using the following formula: $SGR\ (\%\ day^{-1}) = [(\ln(F_{BW,\ TL}) - \ln(I_{BW,\ TL})\ t^{-1})]\times 100$; where F is the final growth in TL or BW at a given time (t), I is the initial size in TL or BW of fish, and t is the length in days of the considered period.

2.3. Sample processing

Whole larvae pools were homogenized for enzymatic assays for fish younger than 21 dph, since larvae were too small to dissect, while older fish were dissected in order to separate pancreatic and intestinal segments as described by Cahu and Zambonino Infante (1994). Dissection was conducted under a dissecting microscope on a prechilled glass plate maintained at 0 °C. For pancreatic (α-amylase, lipase, trypsin and chymotrypsin) and gastric (pepsin) enzymes, samples were homogenized with 5 volumes of ice-cold 0.9% NaCl solution (pH = 7.2). After homogenization the samples were sonicated for 30 s and centrifuged at 4 °C at 3300 ×*g* for 10 min and the supernatant was collected and stored at −80 °C.

Determinations of the intestinal brush border membrane enzyme were conducted according to Cahu et al. (1999). Samples were homogenized in 30 v/w fractions of Tris (2 mM)–mannitol (50 mM), pH 7.0 for 30 s using a motor driven glass Teflon homogenizer. Brush border extracts from enterocytes were prepared as described by Crane et al. (1979). Briefly, tissue homogenates were centrifuged at 9000 ×*g* for 10 min after the addition of 0.1 M $CaCl_2$ and sonicated for 1 min. Then, the supernatant was collected and centrifuged at 32,000 ×*g* at 4 °C. After that, the supernatant was discarded and the pellet was collected and suspended in 0.1 M KCl, 5 mM Tris–HEPES,1 mM DTT (dithiothreitol) solution at pH = 7.5 and stored at −80 °C until analysis of enzyme activity.

Trypsin (E.C.3.4.21.4) activity was measured with N-α-benzoyl-dl-arginine-p-nitroanilide (BAPNA) as substrate. BAPNA (1 mM in 50 mM Tris–HCl, pH 7.5, 20 mM $CaCl_2$) was incubated with the enzyme extract at 37 °C for 2 min. Absorbance was recorded at 410 nm (Erlanger et al., 1961). One unit of trypsin activity corresponded to 1 μmol of 4-nitroaniline liberated in 1 min per mL of extracellular enzymatic extract, based on the extinction coefficient of the substrate ($\varepsilon 410 = 8800\ M^{-1}\ cm^{-1}$). Chymotrypsin (EC. 3.4.21.1) activity was measured by using 0.1 mM Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SAPNA) in 50 mM Tris–HCl, pH 7.5, 20 mM $CaCl_2$. The enzyme preparation was incubated with substrate at 37 °C and monitored at 410 nm for 3 min. One unit of chymotrypsin activity corresponded to 1 μmol of substrate hydrolyzed in 1 min per mL of extracellular enzymatic extract, based on the extinction coefficient of the substrate ($\varepsilon 410 = 8800\ M^{-1}\ cm^{-1}$) (Erlanger et al., 1961).

α-Amylase (EC.3.2.1.1) activity was measured as described by Bernfeld (1955). The increase in reducing power of buffered starch solution was measured with 3,5 dinitro salicylic acid (DNSA) at 540 nm. The enzyme preparation was incubated with 1% starch solution (pH = 7.0) at 37 °C for one hour and reaction was stopped by adding 2 N NaOH. The α-amylase activity (U) was expressed as μg maltose liberated from starch per mg protein in sample per hour.

Lipase (EC.3.1.1.3) activity was assayed by the method of Colowick and Kaplan (1955). Emulsion of olive oil and 2% polyvinyl alcohol solution was used as the substrate. The liberated free fatty acids in the enzyme substrate complex were titrated with 0.02 N NaOH. One unit of lipase was defined as the amount of enzyme, which liberated one micromole of free fatty acid per minute (1 ml of 0.02 N NaOH is equivalent to 100 μmol of free fatty acids).

Pepsin (E.C.3.4.23.1) activity quantification was carried out following Worthington (1991) and based on Anson (1938). In brief, the enzymatic extract was mixed with the substrate (2% hemoglobin solution in 0.3 N HCl at pH = 2.0) and incubated for 10 min at 37 °C. The reaction was stopped with 5% trichloroacetic acid, and the assay tubes were centrifuged at 4000 ×*g* for 6 min at 4 °C. The absorbance of the supernatant was recorded at 280 nm. One unit of pepsin activity was defined as the μg of tyrosine released at 37 °C $min^{-1}\ mL^{-1}$, considering the extinction coefficient ($\xi 280 = 1250\ M^{-1}\ cm^{-1}$).

The activity of alkaline phosphatase (EC.3.1.3.1) was estimated as described by Bramley (1974) using p-nitrophenyl phosphate (p-NPP) as substrate dissolved in alkaline medium. The enzyme extract, was incubated at 30 °C in 1 M sodium carbonate (pH 10.4), 0.5 μM EDTA (pH = 10.4) and 3 μM magnesium acetate. The reaction was stopped by adding 0.5% (w/v) EDTA in 0.5 M NaOH after 30 min. Free p-nitrophenol from purified brush border extracts was measured at 420 nm. Enzyme activity (U) was expressed as μg p-NP liberated/mg protein/h. The protein content of supernatant solutions used for assessing digestive enzymes was measured following the method of Lowry et al. (1951) using bovine serum albumin as standard. All the enzymatic assays were run in triplicate using a Perkin Elmer Lambda25 UV/Vis Spectrophotometer, each replicate corresponding to a pool of larvae from a different rearing tank.

2.4. Statistical analysis

All data are expressed as the mean ± SD ($n = 3$). Digestive enzyme activity (total and specific activity) from larvae sampled at different ages was compared between sampling dates by means of a repeated measures ANOVA test (data normally distributed, Kolmogorov–Smirnov test; Zar, 1999) using IBM SPSS Statistics® version 16.0 (Armonk, New York, USA), followed by a post hoc Duncan's multiple-comparison test.

3. Results

3.1. Larval growth

Most relevant developmental changes in digestive system organs and accessory digestive glands, as well as in mean BW and TL of butter catfish from hatching to the juvenile stage (30 dph) are given in Fig. 1. Newly hatched larvae weighed 0.4 mg and measured 0.3 cm in TL; whereas at the end of the study, they measured 370.4 ± 58.04 mg and 4.52 ± 0.30 cm and the specific growth rates (% growth day^{-1}) for BW and TL were 23.9 and 9.5, respectively. Larval growth in weight and length showed rapid exponential growth during the study period ($y = 0.232\ e^{0.390x}$; $r^2 = 0.99$ and $y = 0.285\ e^{0.147x}$; $r^2 = 0.99$; $n = 320$, respectively).

3.2. Activity of digestive enzymes

Trypsin activity in butter catfish aged 1 dph was 0.48 ± 0.01 U mg^{-1} protein and remained stable until 4 dph (0.59 ± 0.01 U mg^{-1} protein). Since then, specific activity progressively increased reaching a peak at 15 dph (2.05 ± 0.04 U mg^{-1} protein). Between 15 and 18 dph, trypsin activity dropped by 71.8% (0.60 ± 0.03 U mg^{-1} protein) and since then, it progressively decreased until 27 and 30 dph to stabilize thereafter (0.08 ± 0.03 U mg^{-1} protein). Trypsin total activity remained stable between hatching and 4 dph (1.21 ± 0.15 U $larva^{-1}$), whereas it progressively decreased between 5 and 11 dph (0.35 ± 0.01 U $larva^{-1}$). Then, two peaks in trypsin total activity were observed between 13 and 27 dph, the first one occurring between 13 and 15 dph (0.93 ± 0.06 U $larva^{-1}$) and the second one between 21 and 24 dph (3.01 ± 0.32 U $larva^{-1}$). After then, total activity was sharply reduced by 3.9 times (0.71 ± 0.08 U $larva^{-1}$) (Fig. 2a).

No significant changes in chymotrypsin specific activity were observed between 1 dph (0.38 ± 0.01 U mg^{-1} protein) and 7 dph (0.54 ± 0.01 U mg^{-1} protein). Between 7 and 11 dph, chymotrypsin activity increased 4.9 times and reached a maximum value of 2.67 ± 0.008 U mg^{-1} protein. After then, chymotrypsin suffered a sharp decrease in activity between 11 and 18 dph (0.56 ± 0.01 U mg^{-1} protein), followed by a progressive decrease until 30 dph, when specific activity reached a minimum value of 0.02 ± 0.005 U mg^{-1} protein. Chymotrypsin total activity remained stable from hatching to 4 dph (0.95 ± 0.09 U $larva^{-1}$), whereas it decreased since then until 7 dph (0.37 ± 0.06 U $larva^{-1}$). Between 9 and 15 dph, total activity values of chymotrypsin recovered and were similar to those recorded at hatching (0.83 ± 0.09 U $larva^{-1}$). Total chymotrypsin activity peaked between 21 and 24 dph (3.24 ± 0.26 U $larva^{-1}$), whereas it sharply decreased until the end of the study at 30 dph (0.19 ± 0.06 U $larva^{-1}$) (Fig. 2b).

The activity of α-amylase in butter catfish at 1 dph was 45.98 ± 0.62 U mg^{-1} protein and remained stable until 3 dph (45.82 ± 0.83 U mg^{-1} protein). Later on, the α-amylase activity increased progressively until 11 dph (75.37 ± 0.28 U mg^{-1} protein) and between 11 and 15 dph, it increased by 45.2% and reached a maximum value of 109.44 ± 0.24 U mg^{-1} protein. After then, between 15 and 18 dph, the α-amylase activity dropped by 63.8% (39.65 ± 0.51 U mg^{-1} protein) and become stable between 21 and 24 dph (29.68 ± 0.77 U mg^{-1} protein). After 24 dph, the activity increased again progressively until the end of the study when it reached a value of 77.76 ± 0.87 U mg^{-1} protein. Total activity values of α-amylase progressively decreased from hatching to 18 dph, when α-amylase total activity (16.58 ± 1.75 U $larva^{-1}$) was 85.8% lower than those initially recorded (117.29 ± 5.21 U $larva^{-1}$). After 18 dph, α-amylase total activity sharply increased, reaching maximum values at the end of the study (30 dph, 708.6 ± 10.6 U $larva^{-1}$) (Fig. 2c).

The specific activity of lipase in butter catfish larvae at 1 dph was 0.03 ± 0.001 U mg^{-1} protein and remained stable until 9 dph

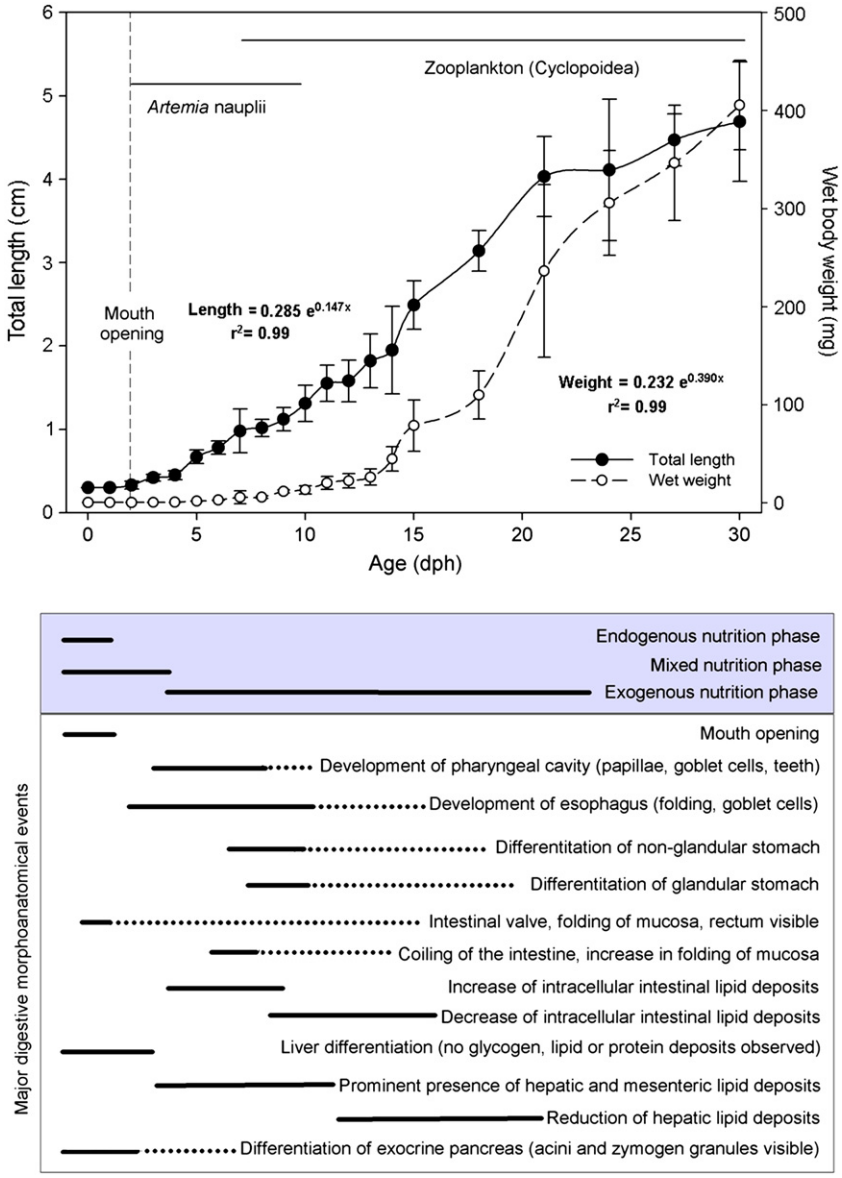

Fig 1. Growth in length and weight (mean ± SD) of butter catfish (*O. bimaculatus*) from hatching until the juvenile stage (30 days post hatch) reared under standard rearing conditions (27 °C). Main feed types and associated feeding schedule are indicated. A table summarizing the most relevant changes in the histological organization of the digestive tract and accessory digestive glands are also included (data redrawn from Pradhan et al., 2012). The solid line indicates the major digestive morphoanatomical events for different digestive organs and tissues. The dotted line shows that no relevant histological and histochemical modifications were observed although the organ and/or tissue increased in size and complexity.

(0.10 ± 0.001 U mg^{-1} protein). Between 9 and 15 dph, lipase activity increased progressively and reached a value of 0.25 ± 0.01 U mg^{-1} protein. Later on, between 15 and 18 dph, the lipase activity increased 3.2 times and reached a maximum value of 0.81 ± 0.01 U mg^{-1} protein. Between 18 and 24 dph, the specific activity of lipase dropped by 46.8% (0.43 ± 0.04 U mg^{-1} protein), followed by a progressive increase until 30 dph, when the specific activity reached a maximum value of 0.46 ± 0.02 U mg^{-1} protein. Lipase total activity was stable from hatching until 15 dph (0.10 ± 0.05 U $larva^{-1}$). Lipase total activity showed a 61.4-fold increase at 21 dph (6.29 ± 0.75 U $larva^{-1}$) followed by a 1.5 fold reduction at 24 dph, from that point on, total activity of lipase remained constant (4.00 ± 0.26 U $larva^{-1}$; Fig. 2d).

Pepsin activity was not detected until 15 dph in butter catfish larvae. The specific activity of pepsin increased progressively between 15 and 21 dph (0.65 ± 0.01 to 0.88 ± 0.01 U mg^{-1} protein) and between 21 and 24 dph, the specific activity increased 2.5 times and reached a value of 2.2 ± 0.27 U mg^{-1} protein. After then, the activity increased progressively until 30 dph when it reached a maximum value of 2.59 ± 0.02 U mg^{-1} protein, four times higher than when it was initially detected at day 15. No significant changes in total pepsin activity were detected between 15 and 21 dph (4.33 ± 0.99 U $larva^{-1}$), from that point on, total pepsin activity increased by 4.2 times at 24 dph (17.80 ± 0.41 U $larva^{-1}$) and remained stable until the end of the study (Fig. 2e).

The specific activity of the brush border alkaline phosphatase remained low from 1 dph (0.01 ± 0.009 U mg^{-1} protein) to 5 dph (0.04 ± 0.001 U/mg protein). Between 5 and 6 dph, the specific activity of this intestinal brush border enzyme increased three times, reaching a value of 0.12 ± 0.004 U mg^{-1} protein, whereas the activity remained stable until 13 dph (0.13 ± 0.006 U mg^{-1} protein). Between 13 and 21 dph, the alkaline phosphatase activity increased 8.6 times and reached a maximum value of 1.17 ± 0.004 U mg^{-1} protein. Between 21 and 24 dph, the specific activity dropped by 25% (0.88 ± 0.01 U mg^{-1} protein), followed by 48.6% decrease in activity between 24 and 30 dph (0.45 ± 0.01 U mg^{-1} protein). Total activity

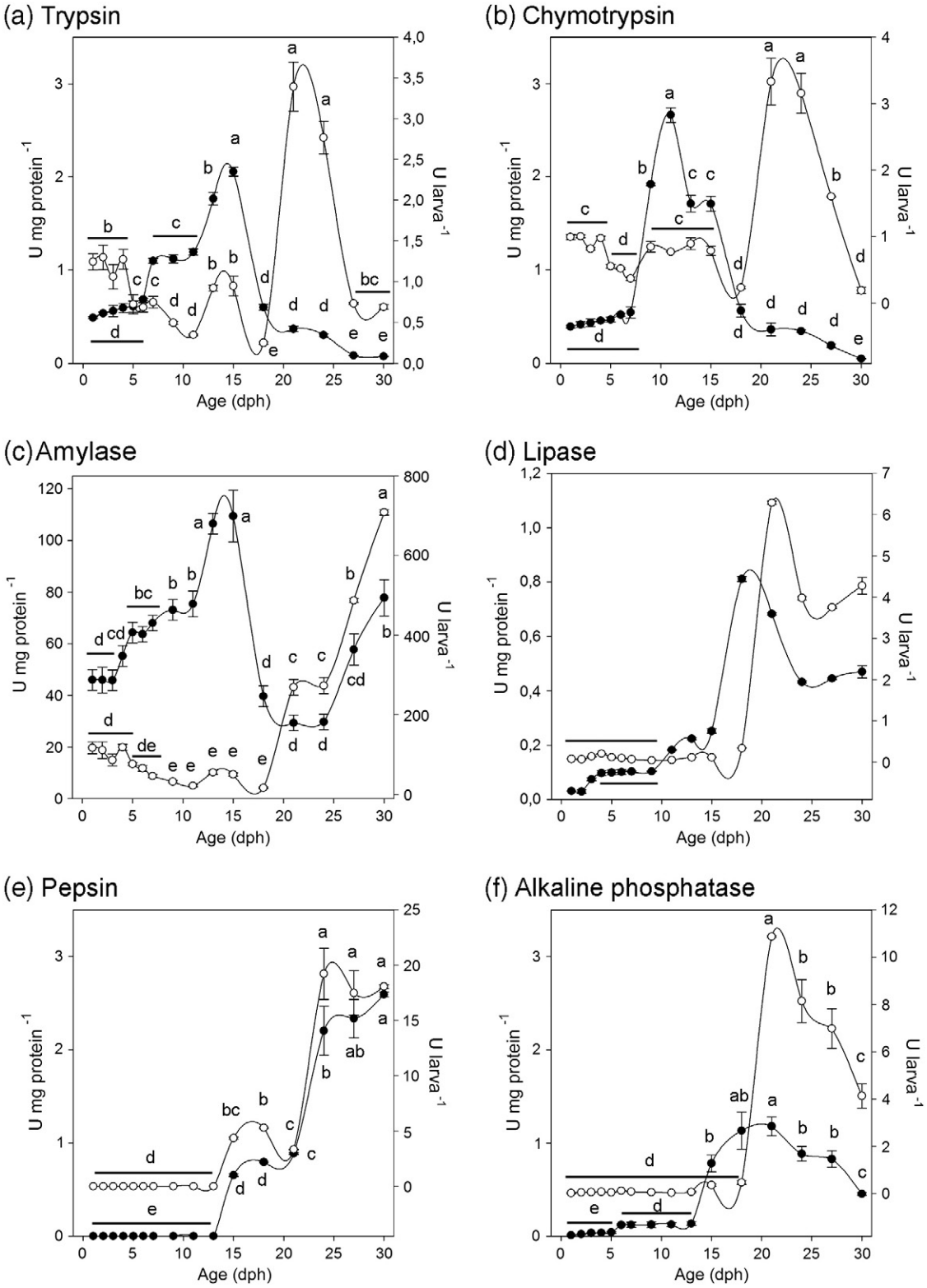

Fig. 2. Specific (●, U mg^{-1} protein) and total (○, U $larva^{-1}$) activity of pancreatic enzymes (trypsin, chymotrypsin, amylase and lipase), pepsin from the stomach and alkaline phosphatase from intestinal brush border in butter catfish (*O. bimaculatus*) from hatching to the juvenile stage (30 days post hatch). Different values of enzyme activity (mean ± SD, $n = 3$) with different superscript letters are statistically significant ($P<0.05$).

of alkaline phosphatase did not change from hatching until 18 dph (0.064 ± 0.03 U $larva^{-1}$), whereas it sharply increased 167 times at 21 dph (10.85 ± 1.46 U $larva^{-1}$) and since then, it progressively decreased until 30 dph (4.04 ± 0.94 U $larva^{-1}$) (Fig. 2f).

4. Discussion

Although the array of digestive enzymes in bony fishes is considered to be the same as that in other vertebrates, fish digestive

enzymes are less studied. The prevailing paradigm is that digestive enzyme activities in fishes are indicative of their feeding ecology and trophic niche in natural conditions, thus, correlating well with their diet (Faulk and Holt, 2009). In addition, the proper development of feeding protocols and diet formulations under fish larval culture conditions requires a deep knowledge and understanding of the digestive processes occurring during early ontogeny in order to synchronize different types of feeds (live prey and microdiets) with production of different digestive enzymes. Thus, the assessment of the presence and level of activity of digestive enzymes may be used as a comparative indicator of the rate of development of fish larvae, food acceptance, digestive capacity, as well as for survival and growth rate predictions. Three main steps are considered crucial in the maturation of digestive functions in fish larvae: i) achievement of pancreatic secretion; ii) onset of the brush border membrane enzymes in the intestine; and iii) development of the stomach and onset of acidic digestion (Lazo et al., 2011).

Comparing digestive enzyme activities between different studies is difficult because of differences in methodology, including differences in assay substrates, assay temperatures, instruments used for analysis, units of reported activity, and species and ontogenetic stages examined, as well as in the quantity and composition of diet (Chan et al., 2004; German et al., 2004; Peres et al., 1998 among others). Consequently, we decided to compare only the development of different digestive enzymes in butter catfish to those reported from different freshwater and marine fish species, but not to compare absolute activity values between different species. In this context, special emphasis has focused on other catfish species and other freshwater species with similar reproductive strategies and developmental patterns (Balon, 1986; Govoni et al., 1986).

Enzymes involved in the digestion of protein, lipid and carbohydrate were present in butter catfish larvae at hatching and before the onset of exogenous feeding. The developmental pattern of pancreatic, gastric and intestinal enzymes was closely related to the histological development of the digestive system previously described by Pradhan et al. (2012). The activity of alkaline proteases, trypsin and chymotrypsin, as well as that of α-amylase and lipase remained stable during the endogenous feeding phase, whereas only α-amylase activity was affected by the onset of exogenous feeding at 3 dph. Trypsin, chymotrypsin and lipase increased after a few days, which seem to be correlated with the full development of the exocrine pancreas (Pradhan et al., 2012).

During the larval stage and before the onset of acidic digestion, protein digestion occurs mainly by the action of alkaline proteases such as trypsin and chymotrypsin in combination with intestinal cytosolic peptidases. During this period, these enzymes have limited capacity for digesting macromolecules that are absorbed by pinocytotic activity of the enterocytes in the posterior intestine for their intracellular digestion (Zambonino-Infante and Cahu, 2001). Under present experimental conditions, the total activity of trypsin and chymotrypsin in butter catfish increased from 6 to 7 dph, and reached a maximum between 15 and 13 dph, respectively. The increase in the production of the studied pancreatic alkaline proteases was observed after the completion of the exocrine pancreas development (Pradhan et al., 2012) and later to that reported in sheatfish (Liu et al., 2010). Similar developmental changes in trypsin and chymotrypsin activities have been reported in other freshwater species (Comabella et al., 2006; Drossou et al., 2006; Kumar et al., 2000; López-Ramírez et al., 2011; Uscanga-Martínez et al., 2011) and marine fish species (Gisbert et al., 2009; Ribeiro et al., 1999 among others). The decrease in specific activity recorded from then until the end of the study may be attributed to an increase in total protein content, and not to a decrease in enzyme production, as indicated by total activity values which were maximal between 21 and 23 dph. From that point on, the specific and total activities of both alkaline proteases sharply decreased, which seems to be in agreement with the development of the gastric function and the increase in pepsin activity, and the transition from an alkaline to an acidic digestion mode. This fact has been reported in many studies describing the developmental changes of digestive enzymes in fish (see reviews in Rønnestad and Morais, 2008; Zambonino-Infante et al., 2009), although species-specific differences in the timing of this shift in the digestion mode occur depending on the life history pattern of each species (Lazo et al., 2011).

Generally, α-amylase activity is mainly detected soon after hatching in most species, whereas it progressively decreases in carnivorous marine fish species during larval ontogeny (Kuz'mina, 1996; Zambonino-Infante and Cahu, 2001; Zambonino-Infante et al., 2009). In butter catfish larvae, α-amylase activity was the highest among the studied pancreatic enzymes (ratio protease/carbohydrase activity), similar to other species fed on zooplankton (Kuz'mina, 1996). The specific activity of this carbohydrase progressively increased with age from the onset of exogenous feeding, peaking at 15 dph and increasing again after 27 dph, whereas total activity values increased from 18 dph. This activity pattern is similar to that reported in another catfish species, the striped catfish (Pangasiidae) (Rangasin et al., 2012), and several freshwater fish species like some cichlids (Mayan cichlid *Cichlasoma urophthalmus*, López-Ramírez et al., 2011; bay snook *Petenia splendida*, Uscanga-Martínez et al., 2011), Indian major carps (*Catla catla*, *Labeo rohita*, *Cirrhinus mrigala* Kumar and Chakrabarti, 1998), or acipenserids (Persian sturgeon *Acipenser persicus*, Bababei et al., 2011), where α-amylase activity increased in larvae after metamorphosing into juveniles. These results contrasted with those reported in sheatfish (Siluridae) where α-amylase activity decreased at 8 dph in larvae fed a mixture of daphnids and earthworms (Liu et al., 2010). However, the short duration (10 days) of the above-mentioned study hampered the ability to evaluate changes in the digestive enzymes in sheatfish at older stages of development. Therefore, it was not possible to know if this was a species-specific digestive trait of this catfish species, or just a change in α-amylase activity over a longer period of time. According to Uscanga-Martínez et al. (2011), high α-amylase activity levels at late larval and juvenile stages in these carnivorous and omnivorous species would enable fish to more adequately take advantage of nutrients through the hydrolysis of glycogen from zooplanktonic and zoobenthic types of prey, when these species shift their feeding habits. In addition, α-amylase activity also reflected the relatively high carbohydrate content (6–10%) in live zooplanktonic prey (Ma et al., 2005) used for feeding larvae, which might have differentially stimulated the synthesis and secretion of α-amylase during larval development.

The study of the lipases during the early life stages of development in fish is an important issue because it permits the activity of these enzymes to be related to the optimal utilization of lipids from food, and to define whether the types of lipids and the enrichment processes are adequate for the formulation of artificial diets (Uscanga-Martínez et al., 2011). In butter catfish, lipase activity was detected at hatching and remained constant until 9 dph. From that point on, total lipase activity increased until 18 dph, whereas it decreased until 25 dph and remained stable until 30 dph. The increase in the capacity of digesting lipids during development has also been described in some catfish species, like the sheatfish (Liu et al., 2010) and striped catfish (Rangasin et al., 2012), in other freshwater species (Mayan cichlid, López-Ramírez et al., 2011; bay snook, Uscanga-Martínez et al., 2011; catla, Kumar et al., 2000; Persian sturgeon, Bababei et al., 2011) and marine species (Zambonino-Infante et al., 2009). The reported changes in lipase activity in butter catfish larvae could not be attributed only to a developmental increase of the lipolytic capacities of this catfish species. Changes in lipase activity might also be attributed to changes in food quality and quantity, since the production and secretion of pancreatic enzymes are modulated by food composition (see reviews in Rønnestad and Morais, 2008; Zambonino-Infante et al., 2009). Considering that different dietary HUFA levels and lipid classes may affect the production

and secretion of lipase (Morais et al., 2004, 2006), the increase in lipase activity observed in butter catfish larvae after 11 dph may be attributed to a diet shift from non-enriched *Artemia* nauplii to copepods, which are richer in HUFAs, wax esters, phospholipids and triacylglycerols (Brett et al., 2009; Shields et al., 1999) which might also stimulate the production of lipase in the pancreas of fish larvae. This hypothesis is confirmed by the reduction of intestinal and hepatic fat deposits observed in butter catfish larvae during this developmental period (Pradhan et al., 2012).

In contrast to pancreatic enzymes, the activity of intestinal brush border membrane enzymes such as alkaline phosphatase is lowest at first feeding and subsequently increases with age. A decrease in the activity of intracellular peptidases (e.g. leucine-alanine aminopeptidase) concurrent to an increase in brush border enzyme activities is indicative of the full intestinal maturity of marine fish larvae (Cahu and Zambonino Infante, 1994). Under present experimental conditions, the activity of alkaline phosphatase in butter catfish increased moderately during the first 13 days, whereas it rose abruptly between 15 and 21 dph (a 8.6-fold increase in specific activity and a 167-fold increase in total activity). This sharp increase in activity is also concomitant with the decrease in lipid deposits in enterocytes and an important development of the intestinal mucosa in terms of villi size and number (Pradhan et al., 2012). The highly folded intestinal mucosa would increase the intestinal surface, indicating an increased importance of surface digestion, and the process of cell proliferation and differentiation in the bases and tips of the folds, respectively, would become more efficient (Kvåle et al., 2007). These findings are in agreement with many other studies on the digestive physiology of freshwater and marine fish larvae that reported an increase in intestinal brush border enzymes around the third and fourth weeks of life of the animal (Bababei et al., 2011; López-Ramírez et al., 2011; Uscanga-Martínez et al., 2011; Zambonino-Infante and Cahu, 2001). The decrease in the alkaline phosphatase specific activity found in this study after 21 dph should not be attributed to a lower activity of this digestive enzyme as fish were developing properly. This change in the specific activity of alkaline phosphatase might be due to a change in the sampling processing, as the digestive tract was dissected in large fish whereas in small ones the whole larva was homogenized, resulting in a change of protein content in the crude extract (Lazo et al., 2007). Although in the present study the activity of intestinal cytosolic enzymes was not analyzed, it seems plausible that the observed increase in alkaline phosphatase activity was coupled with a progressive decline of cytosolic enzyme activities, as this process characterizes the normal development of the intestine and maturation of the enterocytes in vertebrates (Zambonino-Infante et al., 2009).

The development of a functional stomach is one of the last steps in organogenesis of the digestive system in butter catfish (Pradhan et al., 2012). In the present study, pepsin activity was detected in butter catfish at 15 dph, although activity sharply increased after 21 dph and remained stable until the end of the study, showing that acid digestion in this species takes place during this period of time. Concomitantly with this increase in pepsin activity, a decrease in the activity of alkaline pancreatic proteases like trypsin and chymotrypsin was observed, confirming the acquisition of a juvenile-like digestion mode (Lazo et al., 2011; Rønnestad and Morais, 2008; Zambonino-Infante et al., 2009). These results indicate that the glandular stomach becomes functional in butter catfish after 21 dph and contrasts to the histological data previously reported (Pradhan et al., 2012). According to the former authors, non-glandular and glandular regions of the stomach in butter catfish were histologically differentiated at 11–12 dph. The authors also suggested that gastric glands might be active by then due to the presence of mucous cells producing and secreting neutral glycoconjugates in the epithelium lining the stomach lumen, since neutral muco-substances may protect the stomach from auto-digestion processes caused by HCl and enzymes produced by gastric glands (Sarasquete et al., 2001). Thus, as described in many species (Zambonino-Infante et al., 2009), these results confirm that the morphological development of gastric glands does not match their functionality, as HCl and pepsin production and secretion only take place between a few days or even several weeks after their histological differentiation. The onset of acidic digestion might be considered as an optimal point for weaning, since the adult-like mode of digestion becomes fully functional. Nevertheless, this is not supposed to be a definite point, since it has been shown in other fish species that weaning on dry feed can be done before acidic digestion is present (Cahu et al., 1998; Ostaszewska et al., 2005). In addition, the type of prey and the nutrient composition may influence the maturation of the digestive system (Zambonino Infante and Cahu, 2007) and consequently, the nutrient composition of dry diets has to be adapted to the digestive capacity of different developmental stages in order to effectively stimulate the secretion of digestive enzymes. Thus, as the functional development of the digestive system is a plastic developmental process that is dependent on the rearing conditions and the diet, a study with different feeding regimes and weaning points is needed for establishing the optimal weaning strategy for butter catfish larvae in the future.

5. Conclusions

The main digestive enzymes with the exception of pepsin were present before the onset of the exogenous feeding stage in butter catfish larvae, indicating an early functional development of the digestive system. Digestive processes during early stages of development were mainly based on pancreatic enzymes, as functional development of exocrine pancreas coincided with the transition from endogenous to exogenous feeding. The developmental changes in the activity of pancreatic and gastric proteases, coupled with the establishment of an efficient brush border membrane as indicated by the activity of alkaline phosphatase, indicated that butter catfish larvae shifted from alkaline to acidic digestion between 15 to 21 dph (405–567 degree days), which may be considered as the onset of the juvenile period (adult digestive mode).

Acknowledgments

This work was funded by the International Foundation for Science, Sweden (IFS grant A/4422-1) and part of the work was completed at IRTA (Sant Carles de la Ràpita, Spain) under an IFS travel grant. Valuable suggestions provided by Dr A.V. Suresh (Integrated Aquaculture International) during the study and practical assistance provided by Mr Tapan Kumar Das, hatchery manager are gratefully acknowledged. The authors also gratefully acknowledge State Fisheries Department, Government of Tripura for providing facilities to carry out this work in the state hatchery. We would like to thank K.B. Andree (IRTA-SCR) for revising the final version of this manuscript.

References

Anson, M.L., 1938. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. Journal of General Physiology 22, 79–89.
Bababei, S.S., Kenari, A.A., Nazari, R., Gisbert, E., 2011. Developmental changes of digestive enzymes in Persian sturgeon (*Acipenser persicus*) during larval ontogeny. Aquaculture 318, 138–144.
Balon, E.K., 1986. Saltatory ontogeny and evolution. Rivista di Biologia/Biology Forum 79, 151–190.
Bernfeld, P., 1955. Enzymes of carbohydrate metabolisms. Methods in Enzymology 1, 149–225.
Bramley, T., 1974. Treatment of immature mice with gonadotrophin: effect on some enzymatic activities of unfractionated ovarian homogenates. Biochemistry Journal 140, 451–460.
Brett, M.T., Müller-Navarra, D.C., Persson, J., 2009. Crustacean zooplankton fatty acid composition. In: Arts, M.T., Brett, M.T., Kainz, M.J. (Eds.), Lipids in Aquatic Ecosystems. Springer, New York, pp. 115–146.

Cahu, C., Zambonino Infante, J.L., 1994. Early weaning of sea bass *Dicentrarchus labrax* larvae with a compound diet: effect on digestive enzymes. Comparative Biochemistry and Physiology 109A, 213–222.
Cahu, C., Zambonino Infante, J., Escaffre, A.-M., Bergot, P., Kaushik, S., 1998. Preliminary results on sea bass (*Dicentrarchus labrax*) larvae rearing with compound diet from first feeding. Comparison with carp (*Cyprinus carpio*) larvae. Aquaculture 169, 1–7.
Cahu, C.L., Zambonino Infante, J.L., Quazuguel, P., Gall, M.M.L., 1999. Protein hydrolysate vs. fish meal in compound diets for 10-day old sea bass *Dicentrarchus labrax* larvae. Aquaculture 171, 109–119.
CAMP, 1997. Conservation assessment and management plan (CAMP) workshop for freshwater fishes of India. NBFGR, Lucknow . p. 327.
Chakrabarti, R., Rathore, R.M., Kumar, S., 2006. Study of digestive enzyme activities and partial characterization of digestive proteases in a freshwater teleost, *Labeo rohita*, during early ontogeny. Aquaculture Nutrition 12, 35–43.
Chan, A.S., Horn, M.H., Dickson, K.A., Gawlicka, A., 2004. Digestive enzyme activities in carnivores and herbivores: comparisons among four closely related prickleback fishes (Teleostei: Stichaeidae) from a California rocky intertidal habitat. Journal of Fish Biology 65, 848–858.
Colowick, S.P., Kaplan, N.O., 1955. Preparation and assay of enzymes. Methods in Enzymology, vol. 1. Academic Press Inc. Publishers, New York.
Comabella, Y., Mendoza, R., Aguilera, C., Carrillo, O., Hurtado, A., García, G.T., 2006. Digestive enzyme activity during early larval development of the Cuban gar (*Atractosteus tristoechus*). Fish Physiology and Biochemistry 32, 147–157.
Crane, R.K., Boge, G., Rigal, A., 1979. Isolation of brush border membranes in vesicular form from the intestinal spiral valve of the small dogfish *Scyliorhinus canicula*. Biochimica et Biophysica Acta 554, 264–267.
de Amorim, M.P., Campos Gomes, B.V., Martins, Y.S., Sato, Y., Rizzo, E., Bazzoli, N., 2009. Early development of the silver catfish *Rhamdia quelen* (Quoy and Gaimard, 1824) (Pisces: Heptapteridae) from the Saõ Francisco River Basin, Brazil. Aquaculture Research 40, 172–180.
Drossou, A., Ueberschär, B., Rosenthal, H., Herzig, K.H., 2006. Ontogenetic development of the proteolytic digestion activities in larvae of *Oreochromis niloticus* fed with different diets. Aquaculture 256, 479–488.
Erlanger, B.F., Kokowski, N., Cohen, W., 1961. The preparation and properties of two chromogenic substances of trypsin. Archives of Biochemistry and Biophysics 95, 271–278.
Faulk, C.K., Holt, G.J., 2009. Early weaning of southern flounder, *Paralichthys lethostigma*, larvae and ontogeny of selected digestive enzymes. Aquaculture 296, 213–218.
German, D.P., Horn, M.H., Gawlicka, A., 2004. Digestive enzyme activities in herbivorous and carnivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic effects. Physiological and Biochemical Zoology 77, 789–804.
Gisbert, E., Ortiz-Delgado, J.B., Sarasquete, C., 2008. Nutritional cellular biomarkers in early life stages of fish. Histology and Histopathology 23, 1525–1539.
Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y., Estévez, A., 2009. Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. Aquaculture 287, 381–387.
Govoni, J.J., Boehlert, G.W., Watanabe, Y., 1986. The physiology of digestion in fish larvae. Environmental Biology of Fishes 16, 59–77.
Kozarić, Z., Kuzir, S., Petrinec, Z., Gjurcvic, E., Bozic, M., 2008. The development of the digestive tract in larval European catfish (*Silurus glanis* L). Anatomia, Histologia, Embryologia 37, 141–146.
Kumar, S., Chakrabarti, R., 1998. Ontogenic development of amylase activity in three species of Indian major carps, *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*, in relation to natural diet. Asian Fisheries Science 10, 259–263.
Kumar, S., Sharma, J.G., Chakrabarti, R., 2000. Quantitative estimation of proteolytic enzyme and ultrastructural study of anterior part of intestine of Indian major carp (*Catla catla*) larvae during ontogenesis. Current Science 79, 1007–1010.
Kuz'mina, V.V., 1996. Influence of age on digestive enzyme activity in some freshwater teleosts. Aquaculture 148, 27–37.
Kvåle, A., Mangor-Jensen, A., Moren, M., Espe, M., Hamre, K., 2007. Development and characterisation of some intestinal enzymes in Atlantic cod (*Gadus morhua* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae. Aquaculture 264, 457–468.
Lazo, J.P., Mendoza, R., Holt, G.J., Aguilera, C., Arnold, C.R., 2007. Characterization of digestive enzymes during larval development of red drum (Sciaenops ocellatus). Aquaculture 265, 194–205.
Lazo, J.P., Darias, M.J., Gisbert, E., 2011. Ontogeny of the digestive tract. In: Holt, G.J. (Ed.), Larval fish nutrition. Wiley-Blackwell, Chichester, pp. 5–46.
Liu, W., Zhang, X.-M., Wang, L.-B., 2010. Digestive enzyme and alkaline phosphatase activities during the early stages of *Silurus soldatovi* development. Zoological Research 31, 627–632.
López-Ramírez, G., Cuenca-Soria, C.A., Alvarez-González, C.A., Tovar-Ramírez, D., Ortiz-Galindo, J.L., Perales-García, N., Márquez-Couturier, G., Arias-Rodríguez, L., Indy, J.R., Contreras- Sánchez, W.M., Gisbert, E., Moyano, F.J., 2011. Development of digestive enzymes in larvae of Mayan cichlid *Cichlasoma urophthalmus*. Fish Physiology and Biochemistry 37, 197–208.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265–275.
Ma, H., Cahu, C., Zambonino-Infante, J.L., Yu, H., Duan, Q., Le Gall, M.M., Mai, K., 2005. Activities of selected digestive enzymes during larval development of large yellow croaker (*Pseudosciaena crocea*). Aquaculture 245, 239–248.
Morais, S., Cahu, C., Zambonino-Infante, J.L., Robin, J., Rnnestad, I., Dinis, M.T., Conceicao, L.E.C., 2004. Dietary TAG source and level affect performance and lipase expression in larval sea bass (*Dicentrarchus labrax*). Lipids 39, 449–458.
Morais, S., Caballero, M.J., Conceição, L.E.C., Izquierdo, M.S., Dinis, M.T., 2006. Dietary neutral lipid level and source in Senegalese sole (*Solea senegalensis*) larvae: effect on growth, lipid metabolism and digestive capacity. Comparative Biochemistry and Physiology 144B, 57–69.
NBFGR, 2011. Proceedings of National Consultation on Species Prioritization for *Ex situ* Conservation and Freshwater Aquaculture; 17–18 September 2011. NBFGR, Lucknow . p. 22.
Ostaszewska, T., Dabrowski, K., Czumińska, K., Olech, W., Olejniczak, M., 2005. Rearing of pike-perch larvae using formulated diets — first success with starter feeds. Aquaculture Research 36, 1167–1176.
Peres, A., Zambonino-Infante, J.L., Cahu, C., 1998. Dietary regulation of activities and mRNA levels of trypsin and amylase in sea bass (*Dicentrarchus labrax*) larvae. Fish Physiology and Biochemistry 19, 145–152.
Pradhan, P.K., Jena, J.K., Mitra, G., Sood, N., Gisbert, E., 2012. Ontogeny of the digestive tract in butter catfish *Ompok bimaculatus* (Bloch) larvae. Fish Physiology and Biochemistry. http://dx.doi.org/10.1007/s10695-012-9655-8.
Rangasin, W., Areechon, N., Yoonpundh, R., 2012. Digestive enzyme activities during larval development of striped catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878). Kasetsart Journal: Natural Science 46, 217–228.
Ribeiro, L., Zambonino-Infante, J.L., Cahu, C., Dinis, M.T., 1999. Digestive enzymes profile of *Solea senegalensis* post larvae fed *Artemia* and a compound diet. Fish Physiology and Biochemistry 27, 61–69.
Rønnestad, I., Morais, S., 2008. Digestion. In: Finn, R.N., Kapoor, B.G. (Eds.), Fish larval Physiology. Science Publishers, Enfield, New Hampshire, US, pp. 201–262.
Sarasquete, C., Gisbert, E., Ribeiro, L., Vieira, L., Dinis, M.T., 2001. Glycoconjugates in epidermal, branchial and digestive mucous cells and gastric glands of gilthead sea bream, *Sparus aurata*, Senegal sole, *Solea senegalensis* and Siberian sturgeon, *Acipenser baeri* development. European Journal of Histochemistry 45, 267–278.
Segner, H., Roesch, R., Verreth, J., Witt, U., 1993. Larval nutritional physiology: studies with *Clarias gariepinus*, *Coregonus lavaretus* and *Scophthalmus maximus*. Journal of the World Aquaculture Society 24, 121–134.
Shields, R.J., Bell, J.G., Luizi, F.S., Gara, J., Bromage, N., Sargent, J.R., 1999. Natural copepods are superior to enriched *Artemia* nauplii as feed for halibut larvae (*Hippoglossus hippoglossus*) in terms of survival, pigmentation and retinal morphology: relation to dietary essential fatty acids. Journal of Nutrition 129, 1186–1194.
Treviño, L., Alvarez-González, C.A., Perales-García, N., Arevalo-Galan, L., Uscanga-Martínez, A., Marquez-Couturier, G., Fernández, I., Gisbert, E., 2011. A histological study of the organogenesis of the digestive system in bay snook *Petenia splendida* Gunther, 1862 from hatching to the juvenile stage. Journal of Applied Ichthyology 27, 73–82.
Twining, S.S., Alexander, P.A., Huibregste, K., Glick, D.M., 1983. A pepsinogen from rainbow trout. Comparative Biochemistry and Physiology 75B, 109–112.
Uscanga-Martínez, A., Perales-García, N., Álvarez-González, C.A., Moyano, F.J., Tovar-Ramírez, D., Gisbert, E., Márquez-Couturier, G., Contreras-Sánchez, W.M., Arias-Rodríguez, L., Indy, J.R., 2011. Changes in digestive enzyme activity during initial ontogeny of bay snook *Petenia splendida*. Fish Physiology and Biochemistry 37, 667–680.
Verreth, J.A., Torrelle, E., Spazier, E., Sluiszen, H.W., 1992. The development of a functional digestive system in the African catfish, *Clarias garipinus*. Journal of the World Aquaculture Society 23, 286–298.
Worthington, C.C., 1991. Worthigton Enzyme Manual Related Biochemical, 3th Edition. Freehold, New Jersey.
Yang, R., Xie, C., Fan, Q., Gao, C., Fang, L., 2010. Ontogeny of the digestive tract in yellow catfish *Pelteobagrus fulvidraco* larvae. Aquaculture 302, 112–123.
Zambonino Infante, J.L., Cahu, C.L., 2007. Dietary modulation of some digestive enzymes and metabolic processes in developing marine fish: applications to diet formulation. Aquaculture 268, 98–105.
Zambonino-Infante, J.L., Cahu, C., 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comparative Biochemistry and Physiology 130, 477–487.
Zambonino-Infante, J., Gisbert, E., Sarasquete, C., Navarro, I., Gutiérrez, J., Cahu, C.L., 2009. Ontogeny and physiology of the digestive system of marine fish larvae. In: Cyrino, J.E.O., Bureau, D., Kapoor, B.G. (Eds.), Feeding and Digestive Functions of Fish. Science Publishers Inc., Enfield, USA, pp. 277–344.
Zar, J.H., 1999. Biostatistical Analysis. Prentice Hall International, NJ, USA.