Available online at www.sciencedirect.com
ScienceDirect
Aquaculture 265 (2007) 194–205
Aquaculture
www.elsevier.com/locate/aqua-online

Characterization of digestive enzymes during larval development of red drum (*Sciaenops ocellatus*)

J.P. Lazo [a,*], R. Mendoza [b], G.J. Holt [c], C. Aguilera [b], C.R. Arnold [c]

[a] *CICESE — Centro de Investigación Científica y de Educación Superior de Ensenada, Ensenada, B.C., México*
[b] *UANL — Laboratorio de Ecofisiología, Universidad Autónoma de Nuevo León, Monterrey, N.L., México*
[c] *UTMSI — Fisheries and Mariculture Laboratory, University of Texas at Austin-Port Aransas, Texas, USA*

Received 30 November 2006; received in revised form 23 January 2007; accepted 26 January 2007

Abstract

The digestive capacity of red drum larvae throughout development was evaluated by characterizing and quantifying digestive enzyme activities using biochemical and substrate-SDS-PAGE techniques with specific inhibitors. Results showed that red drum larvae posses a nearly complete set of alkaline proteases since first feeding (3 days post-hatch). Alkaline proteases played a major role in digestion during the first days of feeding, while acid proteases became more important toward the end of the larval period, concomitant with the appearance of a functional stomach. Enzymes for the digestion of proteins (trypsin-like), lipids (lipase) and carbohydrates (amylase) were already present in the larvae before exogenous feeding commenced, and their activity subsequently increased with age and length. At some stages of development, digestive enzymes of red drum larvae seem to have a temporal distribution mediated by underlying genetic mechanisms, rather than controlled by feeding activity. Intracellular digestion, measured as the activity of leucine–alanine peptidase, was high early in the larval stage and decreased as development progressed. In contrast, aminopeptidase activity, which is present in the intestinal brush border membrane and is indicative of intestinal maturity, was lowest at first feeding and subsequently increased with age. Using specific inhibitors the relative contribution of the alkaline proteases to the overall alkaline proteolytic activity was assessed. Interestingly, chymotrypsin relative contribution to alkaline proteolytic activity was higher than that for trypsin by 22 DAH. Substrate-SDS-PAGE analysis indicated that although the proteolytic enzymes measured were at least in part comprised of serine-type proteases, other types such as metalo-proteases may play a significant role in the digestive process in red drum larvae and should be further characterized.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Marine fish larvae; Digestive enzymes; Proteases; Lipase; Amylase

1. Introduction

The production of marine fish species for aquaculture depends greatly on the ability to successfully rear fish through the larval stage. Adequate nutrition is one of the principal factors influencing larval survival and depends on the effective ingestion, digestion and assimilation of diets containing the required essential nutrients.

Due to their rapid growth rates, larvae have high protein requirements and diets are usually designed to contain protein levels of 55–60% (Person-Le Ruyet, 1990). Since larvae lack a functional stomach (Govoni et al., 1986), they rely mainly on pancreatic and intestinal proteases to digest their food. Due to their importance,

* Corresponding author. Center for Scientific Research and Higher Education of Ensenada, Fish Nutrition Laboratory, Department of Aquaculture, P.O. Box 430222, San Diego, CA. 92143-0222, USA. Tel.: +1 52 646 1 75 05 00; fax: +1 52 646 1 75 05 72.
E-mail address: jplazo@cicese.mx (J.P. Lazo).

0044-8486/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2007.01.043

studies on the ontogenesis of digestive enzymes in fish larvae have focused primarily on pancreatic proteases. Trypsin is the only pancreatic enzyme that can activate other digestive enzymes (Corring, 1980), and thus may play an essential role in the digestive process of fish larvae (Hjelmeland et al., 1984). Following the initial digestion of proteins by trypsin and other endoproteases, exoproteases bound to the brush border further break down the peptides to amino acids before they can be absorbed from the gut lumen (Clark et al., 1987; Rust, 2002). In addition, the digestion of proteins by intracellular enzymes (e.g. catepsins, acid phosphatase and leucine–alanine peptidase) has been shown to occur in fish larvae and is thought to aid in digestion to compensate for the lack of a functional stomach (Georgopoulou et al., 1985; Govoni et al., 1986; Cahu and Zambonino Infante, 1995).

After proteins, lipids are the most abundant component of the natural foods of marine fish larvae. Large quantities of wax esters, phospholipids and triacylglycerols are present in copepods and other marine zooplankton (Shields et al., 1999), thus recent research has focused on several lipases and esterases in marine fish larvae (Hoehnereitan, 2001; Murray et al., 2003). Moreover, larvae exhibit essential requirements for some lipid constituents (e.g. highly unsaturated fatty acids). Carbohydrases are the least studied of the digestive enzyme groups in fish larvae, since there has been no essential requirement found for carbohydrates in fish (Rust, 2002), but are nonetheless important for the development of diets for commercial purposes where one of the goals is to minimize the cost of the feed. The utilization of carbohydrates as an inexpensive source of energy can help achieve this goal.

In the last two decades, interest in the culture of red drum (*Sciaenops ocellatus*) has increased due to its popularity as a sport and food fish. Several hatcheries in the southeastern U.S. and abroad (e.g., Mexico, Taiwan and Martinique) have been constructed to produce juveniles for commercial or restocking purposes (Thomas et al., 1995; Tucker, 1998). Although red drum larvae are able to ingest microdiets, limited success has been obtained when used as a sole food source, which has been attributed to inadequate ingestion, digestion and assimilation (Holt, 1993; Lazo et al., 2000a).

Recent studies have evaluated the effect of diet on digestive enzyme activity in red drum larvae (Buchet et al., 2000; Lazo et al., 2000a,b; Applebaum et al., 2001), but in order to develop successful microdiets designed to replace live prey a thorough understanding of the digestive capacity during ontogeny is needed. The onset of digestive enzyme activity, ontogenetic patterns and specific biochemical characteristics in combination with *in vivo* and *in vitro* ingredient digestibility must be determined in order to quantify and establish the capacity of larvae to digest and absorb different types of feed ingredients. Thus, the objectives of this study were to characterize and quantify digestive enzyme activity through larval development for this species. We utilized both well-established (spectrophotometric) and novel techniques (fluorometric, substrate-SDS-PAGE and specific inhibitors) to determine total protease activity as well as trypsin, leucine aminopeptidase, leucine–alanine peptidase, lipase and amylase activity.

2. Materials and methods

2.1. Larval rearing

Red drum (*S. ocellatus*) eggs were obtained from several spawns of broodstock maintained at the Marine Science Institute of the University of Texas at Austin in Port Aransas, Texas under controlled temperature and photoperiod (Arnold, 1988). Larvae were raised utilizing the rearing and feeding protocol described by Lazo et al. (2000a,b). Briefly, red drum larvae were stocked at a density of 13 larvae per L and reared at 28 °C in 150 L tanks equipped with internal biological filters. Following initiation of exogenous feeding (day 3 post-hatch) larvae were fed live prey consisting of HUFA enriched rotifers (*Brachionus plicatilis*) at concentrations of 3–5 ml^{-1} between days 3–12 and HUFA enriched brine shrimp (*Artemia fransiscana*) nauplii at 1–2 ml^{-1} thereafter. At least three tanks were used for each enzyme assayed.

2.2. Sampling and dissection

Larvae were collected prior to morning feeding at various times between hatching on day 1 and day 22. Depending on the enzyme assayed, larvae were sampled on all or some of the following days after hatching: 0, 2, 3, 4, 8, 10, 14, 18 and 22. Following collection, larvae were maintained in beakers without food for at least 1 h to allow any remaining food in the gut to be assimilated or excreted. Twenty-four larvae from each beaker were subsequently anesthetized and measured for standard length with a Wilde stereo-microscope using a Summa Sketch II digitizing tablet and Sigma Scan software. Since there is controversy as to whether enzymes or inhibitors present in the body tissue affect digestive enzyme measurements (Dabrowski, K., personal communication), digestive organs of individual larvae were dissected under a microscope by placing larvae on a

glass slide maintained at 0 °C. A cut was made at the boundary of the esophagus and intestine, and the whole intestinal segment, including the diffuse pancreas embedded in its outer surface, was removed. Samples for SDS-PAGE electrophoresis were freeze-dried utilizing a Freezone® 4.5-Liter Benchtop freeze dryer system (LABCONCO, Kansas City, MI) and stored at −60 °C until used.

2.3. Ontogeny of digestive enzymes

Frozen guts were homogenized in ice-cold Milli-Q water with a tissue grinder. Three replicates per tank (where one replicate consisted of eight guts) were assayed in duplicate for enzyme activity and protein content.

Total proteases activity were measured using 2% hemoglobin or 2% azocasein as substrates for acid and alkaline proteases, respectively, following the method described by Sarath et al. (1989). One unit of enzyme activity was defined as the amount of enzyme required to cause an increase in absorbance of 0.001 at 280 nm across a 1 cm path length.

For determination of trypsin, bile-salt-dependent-lipase (BSD-lipase) and ∝-amylase activity, gut homogenates were centrifuged for 30 min at 1700 ×*g* and temperatures near 0 °C. The supernatant was subsequently used for analyses of enzyme activity. Trypsin-like enzyme activity was measured using a highly sensitive fluorescence technique (Ueberschar, 1988) as described by Oozeki and Bailey (1995). Trypsin activities were calculated as the percent increase in fluorescence emission per min. BSD-lipase was estimated following the spectrophotometric method of Gjellesvik et al. (1992). The extinction coefficient used for *p*-nitrophenol at 400 nm and pH 7.4 was 163,000 M cm^{-1}. A unit of lipase activity (*U*) was defined as μmol 4-nitrophenil caproate hydrolyzed min^{-1}.

∝-Amylase activity was measured spectrophotometrically using an assay kit (Sigma, Procedure No. 577) adapted from the method reported by Rauscher et al. (1986). The extinction coefficient used for *p*-nitrophenol at 405 nm and pH 7.0 was 8.8 mM m^{-1} at 30 °C. A unit (*U*) of activity was defined as the amount of amylase which hydrolyzes 1 nmol of substrate in 1 min.

Leucine aminopeptidase (LAP) activity was estimated in uncentrifuged homogenates using L-leucine-*p*-nitroanilide (1.2 mM in DMSO) as substrate in 50 mM Tris–HCl buffer at pH 8.0, following the method of Appel (1974). Leucine–alanine peptidase (Leu–Ala peptidase) activity was measured using L-Leucyl-L-alanine as a substrate, following the method reported by Nicholson and Kim (1975). One unit of enzyme activity was defined as the amount of enzyme required to cause an increase in 1 unit of absorbance at 530 nm across a 1 cm path length.

Measurements of soluble protein content were completed using the bicinchoninic acid protein assay kit (Sigma, Procedure No. TPRO-562), following the adaptation of the method described by Smith et al. (1985). Protein concentrations were calculated using bovine serum albumin (BSA) as a standard.

2.4. Characterization of digestive enzymes

Three digestive enzymes, trypsin (an endoprotease), aminopeptidase (an exoprotease) and BSD-lipase (a lipolytic enzyme), were characterized by determining their relative activity as a function of pH and temperature. The effect of pH on enzyme activity was determined by substituting the Tris–HCl buffers used in the previously described assays with the following buffers; 0.2 M HCl–KCl (pH 2.0), 0.2 M Citrate-Phosphate (4.0–6.0), 0.05 M Tris–HCl (7.0–9.0) and 0.2 M Glycine–NaOH (pH 10.0). The effect of temperature on enzyme activity was measured at the optimal pH found for each enzyme assayed.

In addition, a further characterization of the alkaline proteolytic activity (pH 9) was performed using specific enzyme inhibitors, following the methodology of Alarcon et al. (1998). *N-p*-tosyl-L-Lysin chloromethyl ketone (TLCK), *N*-tosyl-L-phenyl chloromethyl ketone (TPCK) and EDTA were used to inhibit and quantify the relative contribution of trypsin, chymotrypsin and metalo-proteases, respectively.

2.5. Substrate-SDS-polyacrylamide gel electrophoresis (Substrate-SDS-PAGE) analysis

SDS-PAGE analysis of protein fractions from red drum gut homogenates was performed according to the discontinuous buffer system method of Laemmli (1970) as described by Alarcon (1997). The gels consisted of a 4% acrylamide stacking gel in 0.125 M Tris–HCl, pH 6.8, and a 12% acrylamide resolving gel in 0.375 M Tris–HCl, pH 8.8. Electrophoresis was performed at a constant voltage of 100 V (15 mA) per gel for 90 min at a temperature of about 4 °C. Samples for electrophoresis and the corresponding zymograms produced were prepared and assayed for protease activity (Substrate-SDS-PAGE) according to García-Carreño et al. (1993). The molecular weight (MW) of proteolytic enzymes was determined by interpolation using a plot of the log

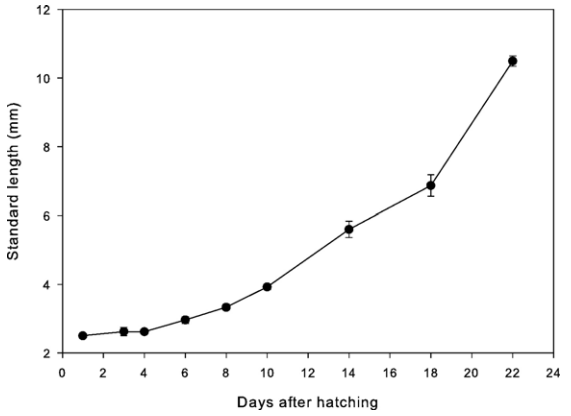

Fig. 1. Growth of red drum larvae fed live prey. Error bars represent S.E. of the mean SL at age of two growth trials ($n=12$).

MW of standards versus their relative mobility (Shi and Jackowski, 1998).

2.6. Characterization of protease types

Specific protease inhibitors were utilized to characterize protease types by Substrate-SDS-PAGE zymograms according to García-Carreño and Haard (1993). A 10 mM stock solution (dissolved in 1 mM HCl) of TLCK was used to inhibit trypsin-like proteases. A 5 mM stock solution (dissolved in methanol) of TPCK was used to inhibit chymotrypsin-like protease activity. Commercial trypsin (Type IX from porcine pancreas, Sigma–Aldrich Co, USA) and chymotrypsin (Type II from bovine pancreas, Sigma–Aldrich Co, USA) were used as controls.

2.7. Non-dissociating conditions (native) PAGE

Since aspartic proteases (e.g. pepsin) lose their activity under dissociating conditions (SDS-PAGE), they must be analyzed under native conditions (i.e., non-dissociating). The non-dissociating discontinuous page electrophoresis method described by Alarcon et al. (1998) was used to assay for acid proteases. The gels consisted of a 4% acrylamide stacking gel in 0.1 M Tris-phosphate, pH 5.5, and a 11% acrylamide resolving gel in 0.07 M Tris–HCl, pH 7.5. Since non-dissociating conditions separate proteins based on their size, charge and shape it is not possible to estimate accurate molecular weights of the separated fractions. Thus, no MWM were used. Characterization of aspartic proteases in zymograms was performed using Peptsatin A (Sigma–Aldrich Co, USA), a specific inhibitor for aspartic proteases. Pepsin from porcine stomach (Type A, Sigma–Aldrich Co, USA) was used as a control.

3. Results

3.1. Ontogeny of digestive enzymes

Fig. 1 shows the growth of red drum larvae during the experimental period. Digestive enzymes activities are presented as percent of total activity, total activity per gut as a function of age or as specific activity as a function of age. Alkaline protease activity expressed as percentage of total proteolytic activity found in red drum larvae represented 100% of the total activity found in dissected guts on days 4 and 8 (Fig. 2). This activity subsequently decreased with age. The contribution of acid proteases to total protease activity was small (<10%) on day 14 and increased substantially by day 22, when it represented 51% of the total protease activity measured. Non-acid or non-alkaline proteases (measured at pH 4 and 6, labeled as neutral proteases) accounted for 45% of the total activity observed on day 14. Alkaline protease activity (between pH 8 and 10) appeared earlier in development and remained higher than acid protease activity (pH 2) until day 22 (Fig. 3A). The specific activity of alkaline proteases peaked on day 4 following 1 day of feeding (Fig. 3B).

All specific digestive enzymes measured in this study were detectable in the larvae before the commencement of first feeding on day 3 (Figs. 3 and 4). Trypsin-like total activity increased with age (Fig. 3C). The specific activity of trypsin peaked on day 3, prior to first feeding, and subsequently decreased until day 14, when values stabilized at about 0.2 U/μg (Fig. 3D). The total activities of lipase and amylase remained low until larvae reached a standard length of about 4 mm (between day 10 and day

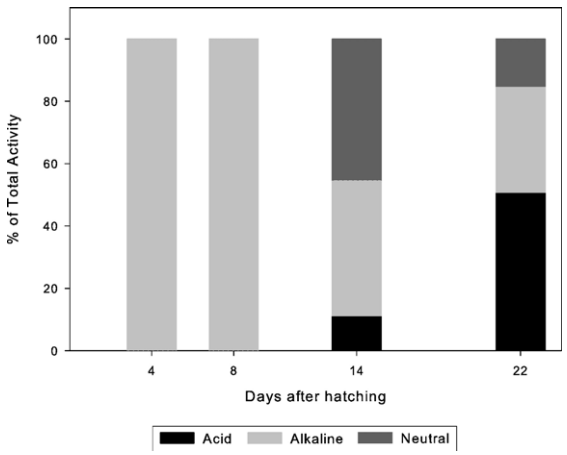

Fig. 2. Relative contribution of protease activity throughout development in red drum larvae. Acid represents activity measured at pH 2, neutral between pH 4 and 6 and alkaline between pH 8 and 10.

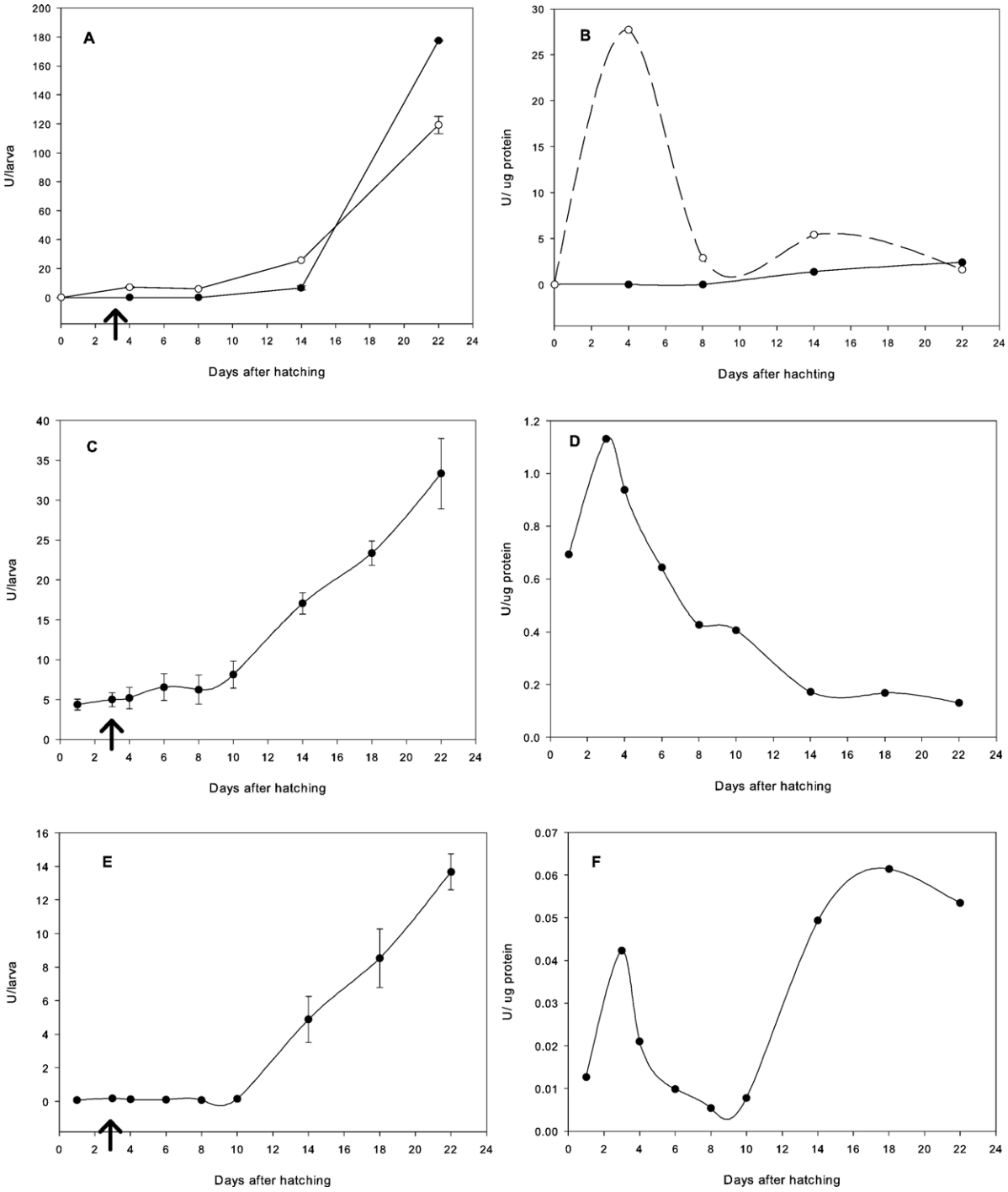

Fig. 3. Relationship between A. Total protease activity and age clear dots represent alkaline proteases and solid dots represent acid proteases, B. Total protease specific activity and age, C. Trypsin total activity and age, D. Trypsin specific activity and age, E. Lipase total activity and age and F. Lipase specific activity and age. Arrow represents initiation of exogenous feeding. Error bars represent S.E. of the mean ($n=3$).

12, Figs. 3E and 4A). Thereafter, total activity increased as a function of age. The specific activities of lipase and amylase exhibited two distinct peaks, one on day 3 (similar to trypsin) and one on day 18 (Figs. 3F and 4B). Specific activity of Leu–Ala peptidase was highest on day 4 and subsequently decreased with age (Fig. 4D).

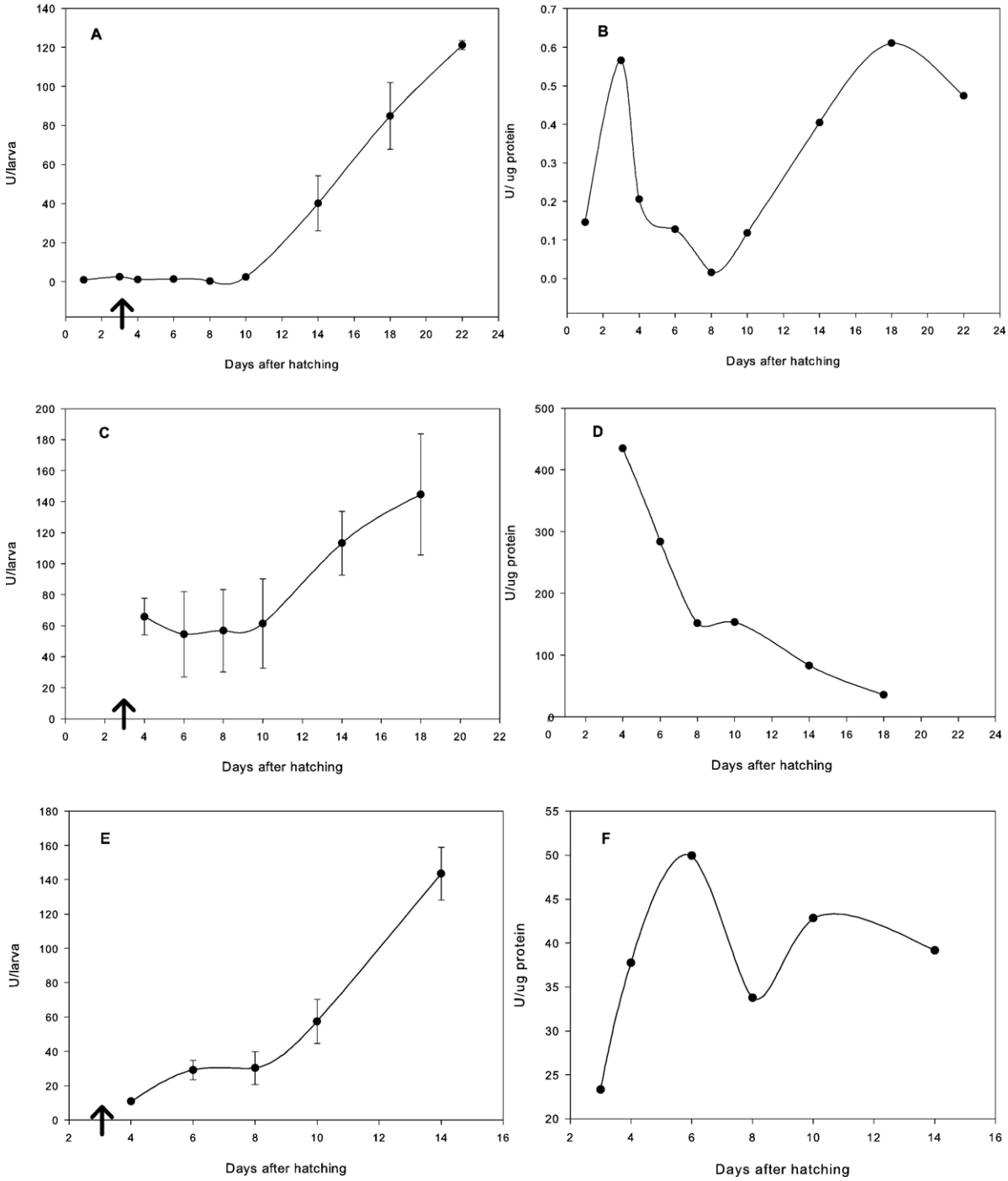

Fig. 4. Relationship between A. Amylase total activity and age, B. Amylase specific activity and age, C. Leu–Ala peptidase total activity and age, D. Leu–Ala specific activity and age, E. LAP total activity and age and F LAP specific activity and age. Error bars represent S.E. of the mean (n=3).

Similar to trypsin-like activity, LAP total activities increased with age (r^2=0.97, P<0.001) (Fig. 4E and F), although the highest peak in specific activity was not observed until day 6.

3.2. Characterization of digestive enzymes

The relative activity of trypsin, LAP and BSD-lipase was examined as a function of pH and temperature.

High trypsin-like activity was observed at alkaline pHs, with highest activity observed at pH 9 (Fig. 5A) using BAPNA as a substrate. Using L-leucine- *p*-nitroanilide as a substrate, highest aminopeptidase activity was found at pH 7.8 (Fig. 5C). Fig. 5E indicates that the optimum pH for BSD-lipase is around 8. Due to the low extinction coefficient of the product of the digestion of the substrate used (4-*p*-nitrophenol) below pH 6.5, the data obtained

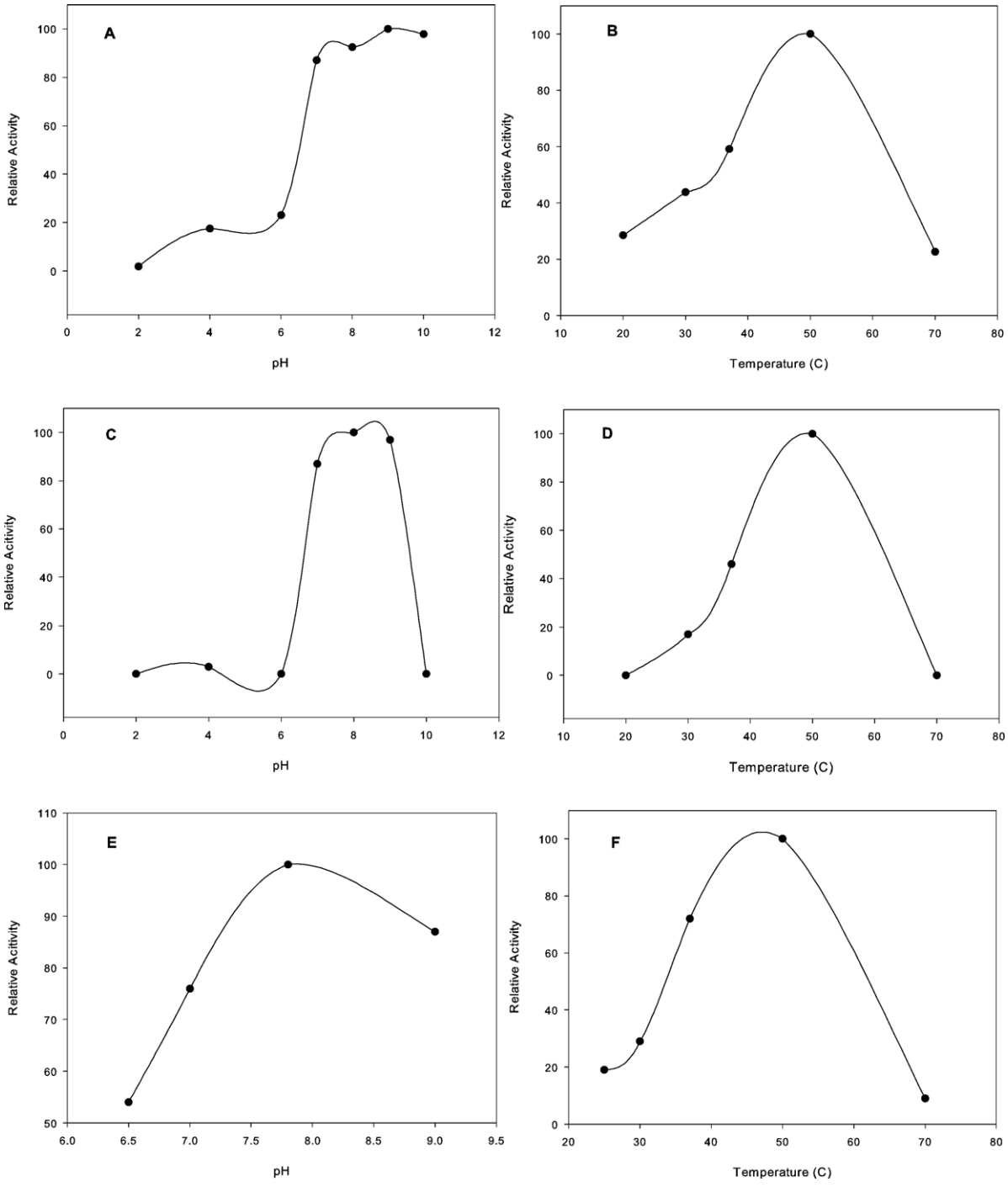

Fig. 5. Relationship between A. Trypsin activity and pH, B. Trypsin activity and temperature. C. Aminopeptidase activity and pH, D. Aminopeptidase activity and temperature. E. Lipase activity and pH, F Lipase activity and temperature.

below this pH cannot be used reliably. For all three enzymes examined the highest activity was observed at 50 °C.

The relative contribution of trypsin, chymotrypsin and metalo-proteases to total alkaline activity (pH 9) in 22 DAH larvae is presented in Fig. 6. Trypsin accounted for 26% and chymotrypsin for 30%, while metalo-proteases only 8%.

3.3. Substrate-SDS-PAGE and native-PAGE

The results obtained from the zymograms of alkaline proteases derived from gut homogenates of red drum larvae throughout development are summarized in Table 1. Alkaline proteolytic activity observed by the presence of distinct white bands against the dark background is presented by the symbol "+" in Table 1. Up to 8 characteristic bands can be observed with an estimated molecular weight ranging from 23 to 68 kDa. Alkaline proteolytic activity was detected at the commencement of exogenous feeding (day 3) as indicated by 5 bands between 27 and 42 kDa. A progressive increase in the relative activity as the larvae developed was observed (i.e. stronger intensity of the bands). An additional distinct proteolytic band of 68 kDa MW was first observed on day 18.

Additional characterization of alkaline proteases was accomplished by incubating the extracts with specific inhibitors such as TLCK for trypsin-like activity and TPCK for chymotrypsin-like activity. A comparison of the treated zymograms with non-treated ones helped to identify bands with a particular type of activity. Treatment with TLCK revealed that the bands with a MW of 23 and 68 kDa had trypsin-like activity (Table 1). Treatment with TPCK inhibited bands with a MW of 25 kDa, suggesting chymotrypsin-like activity (Table 1). TLCK and TPCK did not inhibit the 5 bands with a MW between 27 and 42 kDa present throughout larval development (Table 1).

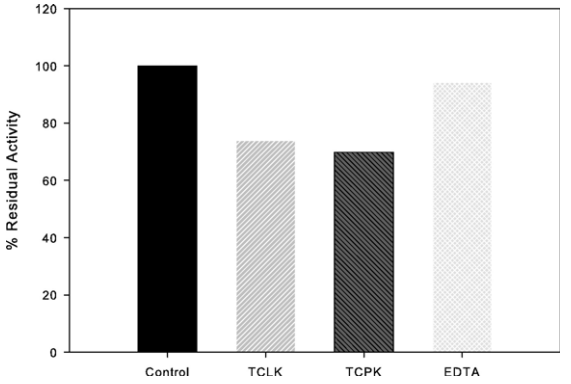

Fig. 6. Percent residual activity after incubation with enzyme specific inhibitors. Control (no inhibitor), TLCK (trypsin activity inhibitor), TPCK (chymotrypsin activity inhibitor) and EDTA (metalo-protease activity inhibitor).

Table 1
Protein fractions with caseinolytic activity (+) in substratre-SDS-PAGE zymograms of red drum larvae and their presumptive classification based on inhibitors

MW[a]	Inhibitor	Class	Days after hatching							
			3	4	6	8	10	14	18	22
68	TLCK[b]	Trypsin-like	−	−	−	−	−	−	+	+
65		?	+	+	+	+	+	+	+	+
41		?	+	+	+	+	+	+	+	+
34		?	+	+	+	+	+	+	+	−
29		?	+	+	+	+	+	+	+	+
27		?	+	+	+	+	+	+	+	−
25	TPCK[c]	Chymotr-like[d]	−	+	+	+	+	+	+	+
23	TLCK	Trypsin-like	?[e]	?	?	?	?	+	+	?
Rf[f]	Pepstatin	Pepsin-like	−	−	−	−	−	−	+	+

[a] Molecular Weight in kDa.
[b] TLCK = *N*- *p*-tosyl-L-Lysin chloromethyl.
[c] TPCK = *N*-tosyl-L-phenyl chloromethyl ketone.
[d] Chymotrypsin-like.
[e] Identification not clear, probably enzyme migrated beyond the front dye.
[f] Rf = Relative mobility, due to the non-dissociating conditions required for acid proteases it is not possible to properly determine MW.

Acid proteases activity was determined under non-denaturing conditions and was first observed in zymograms on day 22. Acid proteases were completely inhibited by Pepstatin A.

4. Discussion

The present study describes the temporal development of some digestive enzymes in larval red drum fed live prey and a further characterization of protease activity. Enzymes involved in the digestion of proteins, lipids and carbohydrates were present in red drum larvae before exogenous feeding commenced. Early detection of digestive enzymes has been reported for several species of fish larvae (Alliot et al., 1980; Baragi and Lovell, 1986; Cousin et al., 1987; Moyano et al., 1996; Baglole et al., 1998; Zambonino Infante and Cahu, 2001). Enzymes related to metabolism (digestion, absorption and transport) of proteins, lipids and carbohydrates from the egg and yolk reserves have been detected following fertilization and immediately after hatching (Tanaka, 1972; Segner et al., 1993; Perez-Casanova et al., 2006; Sveinsdottir et al., 2006).

Few studies to date have assess the relative contribution of the different digestive proteases throughout ontogeny. Although activities of specific proteases

have been studied widely, the use of specific substrates for each enzyme does not permit direct assessment of the relative contribution of each enzyme to the overall digestion. In this study we assessed the relative contribution of proteases throughout ontogeny using one common substrate, namely denatured hemoglobin (Sarath et al., 1989), using specific inhibitors or by varying the pH of the reaction. Alkaline proteases accounted for all proteolytic activity observed during the first days of feeding. The absence of a morphologically distinct and functional stomach is characteristic of most marine fish larvae and no acid secretion or pepsin digestion occurs before stomach development (Govoni et al., 1986). Instead, protein digestion is mediated by serine-type proteases (i.e. trypsin and chymotrypsin) at alkaline pHs (Fange and Grove, 1979; Smith, 1989; Zambonino Infante and Cahu, 2001). A progressive shift in relative activity from alkaline to acid proteases was observed during development, reflecting the temporal changes and stabilization of the different proteases, i.e. from trypsin to chymotrypsin to pepsin. Significant levels of pepsin-like activity, usually considered an indicator of a functional stomach, were not observed until day 22. This is in agreement with histological observations reported by Lazo (1999). Using specific inhibitors the relative contribution of the alkaline proteases to the overall alkaline proteolytic activity was assessed. Interestingly, chymotrypsin relative contribution to alkaline proteolytic activity was higher than that of trypsin by 22 DAH. This is in accordance with observations made by Applebaum et al. (2001) for red drum larvae and further supports the idea of ontogenetic shifts in enzyme activity associated with genetic and dietary shifts (Zambonino Infante and Cahu, 2001).

In general, the total activity of digestive enzymes increased with age and length, but while trypsin and aminopeptidase increased consistently, lipase and amylase remained low until the larvae reached a SL>4 mm. The differences in activity and patterns observed for the various digestive enzymes examined in red drum larvae are common among marine fish larvae. The time of first detection and activity levels of digestive enzymes have been reported to fluctuate for several marine fish species during the development of the digestive system (Pedersen, 1993; Zambonino Infante and Cahu, 1994; Moyano et al., 1996; Baglole et al., 1998; Perez-Casanova et al., 2006). For red drum, the transition period from endogenous to exogenous feeding (day 3 post-hatch) resulted in clear changes in digestive enzyme activity. With the exception of LAP, all enzymes measured showed a clear peak in specific activity prior to first feeding, followed by a decrease in activity. These results suggest that the process of enzyme production is mediated by underlying genetic mechanisms (Buddington and Diamond, 1989) and not induced by the diet, since high specific activity was observed before initiation of exogenous feeding. A marked decline in specific activity during certain periods of development has been observed in herring (*Clupea harengus*) (Pedersen, 1993) sea bass (*Dicentrarchus labrax*) (Zambonino Infante and Cahu, 1994) and sea bream (*Sparus aurata*) (Moyano et al., 1996). The decline in proteolitic activity may reflect major metabolic changes occurring during larval development, such as age or length related reduction in digestive enzyme synthesis or increased relative quantities of other soluble proteins. Additionally, a possible adjustment in larval digestive capacity was observed since LAP progressively increased during this period. This may reflect the end of macromolecular absorption and the onset of specific amino acid transporters. The latter mechanism may play a predominant role during red drum development, for gut total activities, tended to increase with age while specific activities decrease at some stages of development. Nevertheless, the former mechanism cannot be disregarded because lipase and amylase total activities were at a minimum on day 8. Similarly, Pedersen (1993) observed a decrease in the quantity of trypsinogen in herring larvae following initiation of exogenous feeding and a subsequent increase in later stages of development.

Following pynocytotic absorption, proteins can be digested by intracellular enzymes. Intracellular digestion has been shown to be active during early larval feeding and to subsequently decrease with the development of the digestive system (Watanabe, 1984; Govoni et al., 1986). Leu–Ala peptidase is primarily present inside cells (ca. 90%) and is characteristic of intracellular digestion (Cahu and Zambonino Infante, 1995). As expected, the specific activity of Leu–Ala peptidase in red drum was highest early in development. In contrast, aminopeptidase (LAP), which is present in the intestinal brush border membrane and helps to further cleave the peptide bonds of hydrolyzed proteins, was lowest at first feeding and subsequently increased with age. An increase in aminopeptidase specific activity with a concomitant decrease in Leu–Ala peptidase specific activity has been related to intestinal maturation in marine fish larvae (Cahu and Zambonino Infante, 1995).

The optimum pH observed for trypsin-like activity (around pH 9) is within the optimum range (pH 8–9) reported for fish trypsin (Overnell, 1973; Hjelmeland and Raa, 1982) and other vertebrates (Vonk and Western, 1984). LAP in red drum larvae had an optimum activity at pH 7.8, which is in agreement with the values of pH

7.4, 8.0 and 8.3 reported for carp (*Ciprinus carpio*), cod (*Gadus morhua*) and Dover sole (*Solea solea*), respectively (Overnell, 1973; Khablyuk and Proskuryakov, 1983; Clark et al., 1987). BSD-lipase optimum activity was observed at around pH 8.0, within the pH range of 7–9 reported for cod and sea bream (Gjellesvik et al., 1992). It is interesting to note that although these species live in different water temperatures and have different feeding behaviors digestive enzymes optimum pH are similar. Although the intestinal pH of red drum larvae was not measured in this study, values reported for marine fish larval guts early in development are in the neutral–alkaline range, i.e. pH 6.5–9 (Bengtson, 1993; Walford and Lam, 1993). If the pHs of red drum larval guts are within the alkaline region pH 7–9 (i.e. before the functional stomach is fully developed), these enzymes would be functioning close to their optimum pH. The timing and progressive adaptation of the digestive tract towards lowers pHs, coupled with changes in specific digestive enzyme activities is a valuable information to formulate adequate microdiets for weaning (Kolkovski, 2001).

The maximum activity observed for trypsin, LAP and BSD-lipase at temperatures of up to 50 °C are similar to those reported for alkaline proteases of the common dentex (*Dentex dentex*) and sea bream (Alarcon et al., 1998). These values may be of interest in terms of the comparative biochemistry of digestive enzymes among and between species (e.g. cool vs. warm water adapted species) and potential biotechnological applications. However, this has little relevance for practical aspects of fish larvae nutrition, since these temperatures are not found under physiological conditions.

Results obtained utilizing substrate-SDS-PAGE techniques are in accordance with those observed using biochemical assays in this study and those from histological preparations (Lazo, 1999). Early detection of proteolytic activity was shown on day 3 (prior to first feeding) with both substrate-SDS-PAGE and spectrophotometric techniques. An almost complete set of bands representing alkaline proteases was observed in zymograms of day three larvae, with no major changes until days 18 and 22. Similar results were obtained by Diaz et al. (1997) working with sea bream larvae, who observed a complete set of alkaline proteases by day 6 and no subsequent major differences in activity until day 30 after hatching.

The further characterization of proteolytic activity using specific inhibitors and substrate-SDS-PAGE revealed that the faint band with a MW of about 23 kDa had trypsin-like activity. Reported MW values for fish trypsin isozymes are in the range from 22 to 28 kDa (Hjelmeland and Raa, 1982; Vonk and Western, 1984; Moyano et al., 1996). In addition, the caseinolytic band with a MW of about 25 kDa was inhibited by the chymotrypsin-like inhibitor (TPCK). Chymotrypsin MW has been reported to be 24 kDa for Atlantic cod (Vonk and Western, 1984) and 28 kDa for sea bream (Alarcon, 1997) and 26 kDa for red drum (Applebaum et al., 2001). The presence of five bands ranging in MW between 27 and 42 kDa that where not inhibited by TLCK or TPCK suggests that they may not be proteases of the serine-type, but rather metalo-proteases such as carboxipeptidases or aminopeptidases. Reported MW values for fish carboxypeptidase A and B are within the range from 25 to 30 kDa (Vonk and Western, 1984).

The activities of acid proteases were not detected prior to day 22, as indicated by substrate-SDS-PAGE analysis. The aspartic protease inhibitor (Pepstatin A) completely inhibited acid proteases observed on day 22, confirming pepsin-like activity of that band. In addition, pepsin-like activity assayed *in vitro* was not significant until day 22. Both of these results are in agreement with histological development of the stomach, which indicated the initial appearance of gastric glands around day 22 (Lazo, 1999). It is important to corroborate pepsin-like activity detected using non-specific substrates with histological, histochemical or molecular techniques. Recently several authors have reported pepsin activity before the development of the gastric glands using hemoglobin as a substrate (Alvarez-Gonzalez et al., 2006; Perez-Casanova et al., 2006). Gastric glands are comprised of oxynticopeptic cells which in fish are responsible of producing both HCl and pepsin (Rust, 2002). As pointed out by Perez-Casanova et al. (2006), this activity is most likely not stomach pepsin, but some other acid proteases, present in the larval body (i.e., the authors used whole-body homogenates), such as lysosomal chatepsins. Correct assessment of the age at which the stomach becomes functional is important, since many authors use this point in development as a first approach to initiate weaning.

In summary, results from this study indicated that red drum larvae seem to posses a nearly complete set of alkaline proteases since first feeding. In relative terms, alkaline proteases seem to play a major role during the first days of feeding, while acid proteases become more important toward the end of the larval period, concomitant with the appearance of a functional stomach. At some stages of development, digestive enzymes of red drum larvae seem to have a temporal distribution mediated by underlying genetic mechanism rather than controlled by feeding activity. Intracellular digestion was high in the early larval stages and later decreased as

development progressed. Although the importance of serine proteases should not be understated, other types of enzymes, such as metalo-proteases might have a significant contribution to the digestive process in larval red drum. When possible it is recommended to corroborate patterns of digestive enzyme activity obtained using biochemical techniques with histological, histochemical, immunochemical or molecular techniques to avoid making incorrect conclusions.

References

Alarcon, F.J., 1997. Procesos Digestivos en Peces Marinos: Caracterizacion y Aplicaciones Practicas. Ph.D. Dissertation, Universidad de Almeria.

Alarcon, F.J., Diaz, M., Moyano, F.J., Abellan, E., 1998. Characterization of functional properties in two sparids; gilthead seabream (*Sparus aurata*) and common dentex (*Dentex dentex*). Fish Physiol. Biochem. 19, 257–267.

Alliot, E., Pastoureaud, A., Trellu, J., 1980. Evolution des activities enzymatiques dans le tractus digestif au cour de la vie larvaire de la sole. Variations des proteinogrammes et des zymogrammes. Biochem. Syst. Ecol. 8, 441–445.

Alvarez-Gonzalez, C.A., Cervantes-Trujano, M., Tovar-Ramirez, D., 2006. Development of digestive enzymes in California halibut *Paralichthys californicus* larvae. Fish Physiol. Biochem. 31 (1), 83–93.

Appel, W., 1974. In: Bergmeyer, H.U. (Ed.), Leucine Aminopeptidase Determination with L-Leucineamide as Substrate in Methods of Enzymatic Analysis. Academic Press, New York, pp. 954–958.

Applebaum, S.L., Perez, R., Lazo, J.P., Holt, G.J., 2001. Characterization of chymotrypsin Activity during early ontogeny of larval red drum (*Sciaenops ocellatus*). Fish Physiol. Fish Physiol. Biochem. 25, 291–300.

Arnold, C.R., 1988. Controlled year-round spawning of the red drum (*Sciaenops ocellatus*) in captivity. Contrib. Mar. Sci. 30, 65–70 (Suppl.).

Baglole, C.J., Goff, G.P., Wright, G.M., 1998. Distribution and ontogeny of digestive enzymes in larval yellowtail and winter flounder. J. Fish Biol. 53, 767–784.

Baragi, V., Lovell, R.T., 1986. Digestive enzyme activities in stripped bass from first feeding through larval development. Trans. Am. Fish. Soc. 115, 478–484.

Bengtson, D.A., 1993. A comprehensive program for evaluation of artificial diets. J. World Aquac. Soc. 24, 199–210.

Buchet, V., Infante, J.L.Z., Cahu, C.L., 2000. Effect of lipid level in a compound diet on the development of red drum (*Sciaenops ocellatus*) larvae. Aquaculture 184 (3–4), 339–347.

Buddington, R.K., Diamond, J.M., 1989. Ontogenic development of intestinal nutrients transporters. Annu. Rev. Physiol. 51, 601–619.

Cahu, C.L., Zambonino Infante, J.L., 1995. Effect of the molecular form of dietary nitrogen supply in sea bass larvae: response of pancreatic enzymes and intestinal peptidases. Fish Physiol. Biochem. 14, 209–214.

Clark, J., Macdonald, N.L., Stark, J.R., 1987. Leucine aminopeptidase in the digestive track of Dover sole (*Solea solea* L.). Aquaculture 61, 231–239.

Corring, T., 1980. The adaptation of digestive enzymes to the diet: its physiological significance. Reprod. Nutr. Dév. 20, 1217–1235.

Cousin, J.C.B., Baudin-Laurencin, F., Gabaudan, J., 1987. Ontogeny of enzymatic enzyme activities in fed and fasting turbot, (*Scophthalmus maximus*) L. J. Fish Biol. 30, 15–33.

Diaz, M., Moyano, F.J., Garcia-Carreno, F.L., Alarcon, F.J., Sarasquete, M.C., 1997. Substrate-SDS-PAGE determination of protease activity through larval development in sea bream. Aquac. Int. 5, 461–471.

Fange, R., Grove, D., 1979. In: Hoar, W.S., Randall, D.J., Brett, J.R. (Eds.), Digestion in Fish Physiology, vol. VIII. Academic Press, pp. 353–405.

García-Carreño, F.L., Haard, N., 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. J. Food Biochem. 17, 97–113.

García-Carreño, F.L., Dimes, L.E., Haard, N.F., 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. Anal. Biochem. 214, 65–69.

Georgopoulou, U., Sire, M.F., Vernier, J.M., 1985. Macromolecular absorption of proteins by epithelial cells of the posterior intestinal segment and their intracellular digestion in rainbow trout. Ultrastructural and biochemical study. Biol. Cell 53, 269–282.

Gjellesvik, D.R., Lombardo, D., Walther, B.T., 1992. Pancreatic bile salt dependent lipase from cod (*Gadus morhua*): purification and properties. Biochim. Biophys. Acta 1124, 123–134.

Govoni, J.J., Boehlert, G.W., Watanabe, Y., 1986. The physiology of digestion in fish larvae. Environ. Biol. Fisches 16, 59–77.

Hjelmeland, K., Raa, J., 1982. Characteristics of two trypsin like isozymes isolated from the arctic fish capelin (*Mallotus vollosus*). Comp. Biochem. Physiol. 71B, 557–562.

Hjelmeland, K., Huse, I., Jorgensen, T., Molovik, G., Raa, J., 1984. Trypsin and trypsinogen as indices of growth and survival potential of cod (*Gadus morhua*) larvae. In: Dahl, E., Danielsen, D., Moksness, E., Solemdal, P. (Eds.), The Propagation of Cod (*Gadus morhua*). Flodevigen Rapportseries, Norway, pp. 189–202. Arendalm, Norway.

Hoehnereitan, K., 2001. Development of bile salt-dependent lipase in larval turbot. J. Fish Biol. 58, 737.

Holt, G.J., 1993. Feeding larval red drum on microparticulate diets in closed recirculating water system. J. World Aquac. Soc. 42, 225–240.

Khablyuk, V.V., Proskuryakov, M.T., 1983. Purification and some properties of leucine aminopeptidase from carp hepatopancreas. Prikl. Biohim. Mikrobiol. 19, 427–430.

Kolkovski, S., 2001. Digestive enzymes in fish larvae and juveniles — implications and applications to formulated diets. Aquaculture 200 (1–2), 181–201.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lazo, J.P., 1999. Development of the digestive system in Red Drum (*Sciaenops ocellatus*) larvae. PhD Dissertation. The University of Texas at Austin. USA.

Lazo, J.P., Holt, G.J., Arnold, C.R., 2000a. Ontogeny of pancreatic enzymes in larval red drum (*Sciaenops ocellatus*). Aquac. Nutr. 6 (3), 183–192.

Lazo, J.P., Dinnis, M.T., Faulk, C., Holt, G.J., Arnold, C.R., 2000b. Co-feeding microparticulate diets with algae: toward eliminating the need for zooplankton at first feeding in larval red drum. Aquaculture 188, 339–351.

Moyano, F.J., Díaz, M., Alarcón, F.J., Sarasquete, M.C., 1996. Characterization of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*). Fish Physiol. Biochem. 15, 121–130.

Murray, H.M., Gallant, J.W., Perez-Casanova, J.C., 2003. Ontogeny of lipase expression in winter flounder. J. Fish Biol. 62 (4), 816–833.

Nicholson, J.A., Kim, Y.S., 1975. A one-step L-amino acid oxidase assay for intestinal peptide hydrolase activity. Anal. Biochem. 63, 110–117.

Oozeki, Y., Bailey, K.M., 1995. Ontogenetic development of digestive enzyme activities in larval walleye pollock, (*Theragra chalcogramma*). Mar. Biol. 122, 177–186.

Overnell, J., 1973. Digestive enzymes of the pyloric ceca and of their associated mesentery in the cod (*Gadus morrhua*). Comp. Biochem. Physiol. 46, 519–531.

Pedersen, B.H., 1993. Protein digestion in herring (*Clupea harengus*) larvae: trypsinogen secretion and effects of a transitory food restriction on mortality, growth and digestive enzyme content. In: Walther, B.T., Jorgen-Fyhn, H. (Eds.), Physiological and Biochemical Aspects of Fish Development. University of Bergen, Norway, pp. 220–225.

Perez-Casanova, J.C., Murray, H.M., Gallant, J.W., 2006. Development of the digestive capacity in larvae of haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*). Aquaculture 251 (2–4), 377–401.

Person-Le Ruyet, J., 1990. Early weaning of fish larvae onto microdiets: constraints and perspectives. Advances in Tropical Aquaculture. Aquacop. Infremer, Actes de Colloque, vol. 9, pp. 625–642.

Rauscher, E., Bulow, S., Hagele, E.O., Shaich, U.E., 1986. Ethylidene protected substrate for the assay of human amylase. Fresenius Z. Anal. Chem. 324, 304–305.

Rust, M.B., 2002. Nutritional Physiology in Fish Nutrition. In: Halver, J.E., Hardy, R.W. (Eds.), Academic Press, California, U.S.A., pp. 367–452.

Sarath, G., de la Monte, R.S., Wagner, F.W., 1989. Protease assay methods. In: Beynon, R.J., Bond, J.S. (Eds.), Proteolytic Enzymes: A Practical Approach. Oxford University Press, New York, pp. 25–56.

Segner, H., Rosch, R., Verreth, J., Witt, U., 1993. Larval nutritional physiology: Studies with *Clarias gariepinus*, *Coregonus lavaretus* and *Scophthalmus maximus*. J. World Aquac. Soc. 24, 121–134.

Shi, Q., Jackowski, G., 1998. One-dimensional polyacylamide gel electrophoresis. In: Hames, B.D. (Ed.), Gel Electrophoresis of Proteins: A Practical Approach. Oxford University Press, New York, pp. 1–52.

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. J. Nutr. 129 (6), 1186–1194.

Smith, L., 1989. Digestive functions in teleost fishes. In: Halver, J. (Ed.), Fish Nutrition. Academic Press, Inc., London, pp. 332–422.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, M.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Sveinsdottir, H., Thorarensen, H., Gudmundsdottir, A., 2006. Involvement of trypsin and chymotrypsin activities in Atlantic cod (*Gadus morhua*) embryogenesis. Aquaculture 260 (1–4), 307–314.

Tanaka, M., 1972. Studies on the structure and function of the digestive system in teleost larvae. Part 5: Epithelial changes in the posterior gut and protein ingestion. Jpn. J. Ichthyol. 19, 172–180.

Thomas, P., Arnold, C.R., Holt, G., 1995. Red Drum and Other Scianids. In: Broomerage, N.R., Roberts, R.J. (Eds.), Broodstock Management and Egg and Larval Quality. Blackwell Science, United Kingdom, pp. 118–137.

Tucker, J., 1998. Marine Fish Culture. Kluwer Academic Press, USA. 750 pp.

Ueberschar, B., 1988. Determination of the nutritional condition of individual marine fish larvae by analyzing their proteolytic enzyme activities with a highly sensitive technique. Meeresforschung 32, 144–154.

Vonk, H.J., Western, J.R.H., 1984. Comparative Biochemistry and Physiology of Enzymatic Digestion. Academic Press, London.

Walford, J., Lam, T.J., 1993. Development of the digestive tract and proteolytic enzyme activity in seabass (*Lates calcarifer*) larvae and juveniles. Aquaculture 109, 187–205.

Watanabe, Y., 1984. An ultrastructural study of intracellular digestion of horseradish peroxidase by rectal epithelium cells in larvae of freshwater cottid fish *Cottus nozawae*. Bull. Jpn. Soc. Sci. Fish. 50, 409–416.

Zambonino Infante, J.L., Cahu, C., 1994. Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae. Fish Physiol. Biochem. 12, 399–408.

Zambonino Infante, J.L., Cahu, C., 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comp. Biochem. Physiol. 130 (4), 477–487.