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Expression and activity of trypsin and pepsin during larval development of the spotted rose snapper *Lutjanus guttatus*

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ABSTRACT

The present study aimed to describe and understand the development of the digestive system in spotted rose snapper (*Lutjanus guttatus*) larvae from hatching to 40 days post-hatch (dph). The mouth opened between 2 and 3 dph, at that moment the digestive tract was barely differentiated into the anterior and posterior intestine, although the liver and pancreas were already present. Gastric glands were observed until 20 dph, followed by the differentiation of the stomach between 20 and 25 dph. Trypsinogen expression and trypsin activity were detected at hatching, increasing concomitantly to larval development and the change in the type of food. Maximum levels of trypsinogen expression were observed at 25 dph, when animals were fed with *Artemia* nauplii, and maximum trypsin activity was detected at 35 dph, when larvae were fed with an artificial diet. On the other hand, pepsinogen gene expression was detected at 18 dph, two days before pepsin enzymatic activity and appearance of gastric glands. Maximum pepsin activity was also observed at 35 dph. These results suggest that in this species weaning could be initiated at an earlier age than is currently practiced (between 28 and 30 dph), since larvae of spotted rose snapper develop a functional stomach between days 20 and 25 post-hatch.

1. Introduction

The study of digestive enzyme activity during the early development of the digestive system in marine fish is a valuable tool to better understand the digestive physiology of larvae, since several enzymes can be used as indicators of their nutritional status. In addition, this information can be used to establish feeding protocols to optimize larval mass rearing production by synchronizing the feeding sequence with the physiological developmental stage of the larvae (Ueberschär, 1993; Gisbert et al., 2008; Zambonino-Infante et al., 2008. The ontogeny of the digestive system has been well documented for a large number of marine and freshwater fish species (Verreth et al., 1992; Segner et al., 1994; Mai et al., 2005; Rønnestad and Morais, 2008; Zambonino-Infante et al., 2008), however only few studies have combined molecular and biochemical procedures for describing the relationship between the transcription of the gene of a particular digestive enzyme and its activity, as reported in spotted sand bass *Paralabrax maculatofasciatus* (Peña et al., 2003; Alvarez-González et al., 2008, 2010), winter flounder *Pleuronectes americanus* (Douglas et al., 1999; Murray et al., 2004, 2006), bullseye puffer *Sphoeroides annulatus* (García-Gasca et al., 2006) and Atlantic salmon *Salmo salar* (Rungruangsak-Torrissen et al., 2006). Finally, studies integrating the development of the digestive system with expression and activation of digestive enzymes are scarce (Péres et al., 1998; Cahu et al., 2004; García-Gasca et al., 2006).

The spotted rose snapper (*Lutjanus guttatus* Steindachner, 1869) is a tropical marine fish species with a high potential for intensive culture in some Latin American countries. This species belongs to a group of several snapper species that are highly appreciated in local and international markets (Davis et al., 2000). *L. guttatus* is distributed along the Pacific coast of the American continent from Mexico to Peru (Grimes, 1987). In Mexico and Costa Rica, wild juveniles are captured and stocked in floating cages for on-growing purposes until they reach the market size (450 g). Because the supply of larvae and juveniles cannot rely on the availability of wild fish, efforts are heading towards controlling the artificial reproduction of this snapper species and the development of larviculture techniques to mass-produce the species in fish hatcheries (García-Ortega, 2009). Therefore, the present study aimed to provide insight into the larval digestive physiology of spotted rose snapper by measuring gene transcription and enzymatic activity of two major digestive proteases, trypsin and pepsin, and relate them with the development of the digestive tract and the larval feeding sequence, in order to understand the regulatory mechanisms triggering enzyme production and feed digestion.

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2. Materials and methods

2.1. Eggs and larval fish rearing

Fertilized eggs of spotted rose snapper were obtained from a captive broodstock held in the marine finfish hatchery of the Centro de Investigación en Alimentación y Desarrollo, located in the city of Mazatlan, Northwest Mexico. Females ($n=6$, individual mass = 0.85 kg) were induced to spawn according to Ibarra-Castro and Álvarez-Lajonchère (2009) using GnRHa ([D-Ala^6 Pro^9 NEt]-GnRH), placed together with mature males ($n=18$, individual mass = 1.5 kg) in a 18 m^3 circular fiberglass tank. Fertilized eggs were incubated in six 600 L circular black fiberglass tanks filled with sea water filtered through a sand filter, followed by 20 μm cartridges, and UV light. During egg incubation, water temperature ranged between 29 and 30 °C and salinity remained constant at 34 g L^{-1}. No water exchange was conducted during egg incubation. Illumination and air flow were maintained constant. After 20 h of incubation, the eggs started to hatch and newly hatched larvae were kept under static water conditions for three days. After then, 50% of the water volume was daily exchanged increasing to 200% at 10 days post hatch (dph) and 600% from 28 dph onwards.

Larval feeding was performed with some modifications from a previously described protocol for the species (García-Ortega et al., 2005). A mixture of microalgae (*Nannochloropsis oculata* and *Isochysis* sp) at a density of 100,000 cells mL^{-1} was added to the larval rearing tanks at 1 dph and maintained until 14 dph. Rotifers (*Brachionus rotundiformis*) were added to the culture tanks at 2 dph, at a density of 10 rotifers mL^{-1} and were reduced to 6 rotifers mL^{-1} at 20 dph. Enriched *Artemia* nauplii (A1 Selco™ INVE, Belgium) were offered to fish larvae twice a day from 18 dph. *Artemia* enrichment was performed in two steps, one of 16 h and the second of 8 h following the manufacturer's instructions. Initial density of *Artemia* nauplii was 0.5 nauplii mL^{-1} and was gradually increased to 1 nauplii mL^{-1} until 35 dph. Weaning started at 28 dph using a microbound diet NRD 4/6 (400–600 μm), 5/8 (500–800 μm), 12/20 (>800 μm) (INVE Aquaculture, Inc., Utah, USA) and finished at 35 dph, when the concentration of *Artemia* nauplii was completely replaced by the artificial diet (59% protein and 16% lipid). Larvae were fed with the microbound diet from 35 dph to the end of the study at 40 dph.

2.2. Sampling

Spotted rose snapper larvae ($n=20$ to 50 depending on their size) were randomly sampled from the rearing tanks using a 200 μm dip net. Sampling was conducted 1 h after feed was offered to larvae in order to evaluate the effects of the diet in the production of digestive enzymes. Active feeding behavior and visual verification confirmed that fish had food in the gut during sampling. Samples were collected daily from hatching to 6 dph, then the samples were collected every two days until 20 dph, and every five days thereafter until the end of the study at 40 dph. After sampling, larvae were sacrificed with an overdose of anesthetic (tricaine methanesulfonate-MS 222), rinsed with distilled water in order to remove the excess of salts and stored at −70 °C. Additional samples ($n=30$ larvae) were daily collected for measuring larval size in total length and body wet weight. Average total length (mm) was calculated for each sampling day by measuring 10 larvae under a dissecting microscope using a digital camera and the PAXcam2 (Pax-it version 6, Mis Inc., USA) software. The individual wet mass (mg, BW) of larvae was calculated by weighing three groups of larvae ($n=20$ larvae) using an analytical balance (Sartorius, Gottingen, Germany; precision of 0.1 mg), and considering the number of larvae contained in each group.

2.3. Histology

Larvae used for histological purposes were fixed in 2% paraformaldehyde for 24 h at 4 °C. Larvae were then washed, dehydrated in graded series of ethanol, cleared, and embedded in paraffin. Sagittal sections (5 μm) were obtained with a conventional microtome (Leica-RM 2125 RT), re-hydrated and stained with hematoxilin-eosin (H&E). Histological sections were viewed under a light microscope and photographed with an Infinity digital camera and the PAXcam2 software (Pax-it version 6).

2.4. Enzymatic activity determination

The biochemical quantification of digestive proteases by means of spectrophotometric methods was conducted using three different pools of larvae per sampling point. Pools of larvae were composed by 50 specimens from hatching to 10 dph, 30 larvae from 12 to 20 dph and 10 larvae from 25 to 40 dph. Because of the difficulties associated with dissecting and removing the digestive tract of small larvae, whole body homogenates were used in larvae younger than 16 dph. After this age, the digestive system was dissected on a glass slide supported on a frozen mini-table. Each sample was homogenized with a tissue grinder into 1 mL of ice-cold distilled water (4 °C), centrifuged using a Biofuge primo R Heraeus at 14,000 *g* for 30 min at 4 °C according to Zambonino-Infante and Cahu (1994), and the supernatants were stored at −70 °C until further analyses.

The level of soluble protein in enzyme crude extracts was determined according to the Bradford (1976) method, using bovine serum albumin as a standard. Trypsin (EC 3.4.21.4) activity was determined according to Erlanger et al. (1961), using BAPNA (N-α-benzoyl.DL-arginine *p*-nitroanilide) as substrate. The mixtures were incubated at 37 °C and the absorbance of the reaction products was measured at 410 nm. The reaction was stopped by adding 30% acetic acid. Acid proteinase (pepsin; EC 3.4.23.1) activity was determined as described by Sarath et al. (1989), using 2% hemoglobin as substrate. The enzyme crude extracts and the substrate were incubated at 37 °C and the absorbance of the reaction products measured at 280 nm. One unit of enzyme activity was defined as 1 μg tyrosine released per minute, using the molar extinction coefficient of 0.005. The specific activity in crude extracts was determined using the following equations:

$$\textit{Total activity}\left(\text{Units mL}^{-1}\right) = [\Delta \textit{abs reaction final volume}\,(\text{mL})]/[\textit{MEC}\cdot\textit{time}\,(\text{min})\;\textit{extract volume}\,(\text{mL})] \quad (1)$$

$$\textit{Specific activity}\left(\text{Units mg protein}^{-1}\right) = \textit{Total activity}/\textit{soluble protein}\,(\text{mg}) \quad (2)$$

Δabs represents the increase in absorbance at a determined wavelength and MEC represents the molar extinction coefficient for the product of the reaction (mL/μg/cm). All assays were carried out in triplicate.

2.5. Gene expression

Quantification of gene expression of the selected digestive enzymes in fish samples was performed according to García-Gasca et al. (2006). Briefly, total RNA was isolated using Trizol reagent (Invitrogen) followed by DNAse I treatment. cDNA synthesis was performed at 45 °C with 5 μg of total RNA, M-MLV reverse transcriptase (Promega), and random primers. Initial PCR amplifications were completed using degenerated primers for trypsin and pepsin precursors obtained by the alignment of available sequences from several marine fish species. Purified PCR products were ligated into a pGEM-T cloning vector (Promega). *E. coli* DH5α competent cells (Invitrogen) were transformed by heat shock and plasmid extraction was performed by

alkaline lysis. Bidirectional sequencing was carried out using labeled T7/SP6 universal primers and a LICOR IR^2 DNA sequencer. Sequence analysis was performed using the NCBI (National Center for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) program. The sequences obtained were submitted to GenBank (Table 1).

Trypsinogen and pepsinogen specific primers were designed using the Primer3 software to perform end-point and quantitative PCR analyses (Table 1). Expected PCR products for trypsinogen and pepsinogen genes were 156 and 184 bp respectively. Primers for *L. guttatus* 18sRNA gene were designed to render a product of 165 bp and this gene was used as internal control for qPCR analysis (Table 1), since it proved to be a stable control gene in previous studies (García-Gasca et al., 2006; unpublished data). Quantitative PCR was performed with a SmartCycler (Cepheid) using SYBR GREEN® under the following PCR conditions: 95 °C for 2.5 min, and 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. Dilution series of cDNA amplified with trypsin, pepsin and 18S rRNA primers were used to construct a standard curve for each gene. Standard curves were calculated by linear regression analysis using threshold cycle (C_T) values and log copy numbers (log Co) obtained from the serial dilution analysis. The copy numbers (Co) of unknown samples were calculated as follows: $a+[b*C_T]$ where $a=y$ intercept and $b=$slope of the standard curves. The normalized Co of trypsinogen and pepsinogen for each sample was determined by dividing the Co of each gene by the Co of 18S rRNA, and each normalized sample was divided by the internal calibrator at 1 dph.

2.6. Statistics

Enzymatic activity and gene expression data were analyzed using one-way ANOVA and Tukey tests for multiple comparisons with a significance level of $P<0.05$. All statistics were conducted using SigmaStat 11.0 for Windows (Sigma-Plot® 11.0, USA).

3. Results

Spotted rose snapper larvae showed an exponential growth in total length and wet weight from hatching until the end of the study at 40 dph (Fig. 1).

3.1. Histological development of the digestive system

At hatching (1.4 ± 0.34 mg BW), the digestive system was undifferentiated and appeared as a straight tube lined by a simple columnar epithelium laying dorsally to a large yolk-sac. The buccopharynx and anus were not yet differentiated and the digestive tract was closed to the exterior (Fig. 2A). During the yolk-sac absorption (2–3 dph, 2.0 ± 0.25 mg BW), the rudimentary digestive system started to differentiate into the buccopharynx, a short esophagus, the intestine and accessory digestive glands (liver and pancreas). Between 3 and 4 dph (2.1 ± 0.17 mg BW), the mouth opened and the posterior intestine was separated from its anterior section by the intestinal valve (Fig. 2B). No histological differences were observed between

Table 1
Gene-specific primers for qPCR analysis and GenBank accession numbers for spotted rose snapper trypsin and pepsin partial nucleotide sequences.

Gene	Primer name	Primer sequence	GenBank accession number
Trypsin	LgTryp-F	5′ ATGAGCTCCACTGCTGACAGAAAC 3′	HM754476
	LgTryp-R	5′ AGAGTCACCCTGGCAAGAGTCCTT 3′	
Pepsin	LgPepsin-F	5′ TGTCTTCGACAACATGATCAAGCA 3′	HM754478
	LgPepsin-R	5′ GCTGTCCATCTTGATCTGCCAGTA 3′	
18S rRNA	Lg18S-F	5′ CTGAACTGGGGCCATGATTAAGAG 3′	HM754479
	Lg18S-R	5′ GGTATCTGATCGTCGTCGAACCTC 3′	

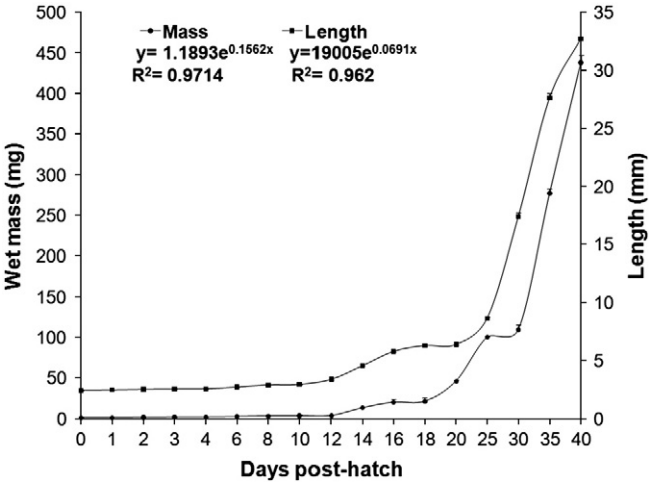

Fig. 1. Mean wet mass (mg ± SD, $n=30$, ●), and total length (cm ± SD, $n=10$, ■) of spotted rose snapper larvae cultured under experimental conditions.

the pre- and postvalvular intestine; both regions were lined by a simple columnar epithelium with basal nuclei, slightly basophilic cytoplasm, and prominent eosinophilic microvilli. During this period, the liver continued to differentiate and appeared as a mass of compact tissue of polyhedral hepatocytes with centrally located nuclei and reduced eosinophilic cytoplasm. In addition, several small clusters of basophilic pancreatic cells (exocrine pancreas) containing acidphilic zymogen granules were already visible. From the onset of exogenous feeding to 10 dph (3.6 ± 1.25 mg BW), the digestive system grew in size and histological complexity (Fig. 3A). In particular, the esophagus clearly differentiated into a simple cubical epithelium with very few scattered goblet cells and grew in length connecting the pharynx with the anterior intestine. In addition, the intestine coiled to accommodate the increasing length of the digestive tract inside the reduced abdominal cavity, and the intestinal mucosa folded. Folding of the intestinal mucosa was more prominent in the prevalvular than the postvalvular intestine. Regarding accessory digestive glands, hepatocytes were arranged around hepatic sinusoids and the bile duct lined with a simple cuboidal epithelium was clearly distinguishable. The exocrine pancreas was clearly visible as an extrahepatic basophilic tissue formed by pancreatic cells arranged in prominent acini and containing large inclusion of zymogen granules. No lipid inclusions were observed in the liver or the intestine.

At 18 dph (22.5 ± 4.24 mg BW), the most relevant feature concerning the development of the digestive system in spotted rose snapper was the appearance of the future stomach as a bulge of basophilic cubical cells at the end of the esophagus (Fig. 3B). Additional feature characteristics of this age were the longitudinal folding of the esophageal mucosa and the large increase in the number of intestinal goblet cells, as well as the increase in size and number of mucosal folds in the intestine.

At 20 dph (46.3 ± 1.52 mg BW), gastric glands were clearly distinguishable (Fig. 3C and D), whereas at 25 dph (100.5 ± 0.70 mg BW) the stomach was morphologically differentiated and three different regions could be distinguished: the *cardias*, *fundus* and *pylorus*. The cardiac region showed a reduced number of flattened mucosal folds and their lumen was lined by a simple short ciliated columnar epithelium with basal nuclei. This region of the stomach was surrounded by a thin layer of smooth circular musculature. The fundic region occupied most of the stomach and it was lined by a simple tall ciliated columnar epithelium and a large number of gastric glands. The pyloric region of the stomach was short and lined by a columnar epithelium devoid of gastric glands. The stomach was separated from the intestine by the pyloric sphincter. Another histological distinctive feature of spotted rose snapper larvae at this age is the increase of lipid deposits in

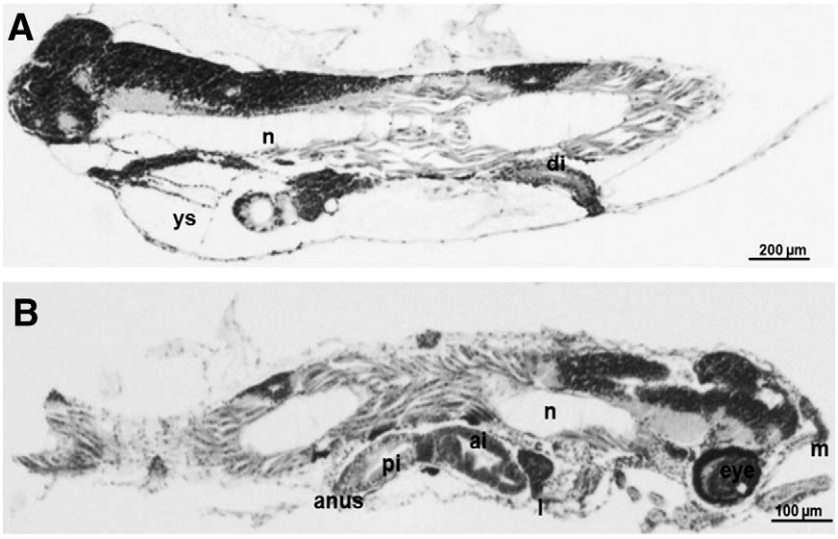

Fig. 2. Sagittal sections of larvae at days 0 (A), and 2 (B) post hatch. Abbreviations; ai, anterior intestine; b, buccopharynx; dt, digestive tube; ey, eye; l, liver; m, mouth; n, notochord; ep, exocrine pancreas; pi, posterior intestine; and ys, yolk sac. Hematoxylin and Eosin staining.

hepatocytes that was not observed at younger ages (Fig. 4A). At 30 dph (109.7 ± 5.74 mg BW), rudimentary pyloric caeca were observed around the stomach (Fig. 4B) and the level of lipid and glycogen deposits in the liver continued to increase until the end of the study at 40 dph when larvae measured 438.2 ± 9.28 mg in BW (Fig. 4C).

3.2. Trypsin

Trypsinogen mRNA expression was barely detected at 1 dph, increasing at the end of the yolk sac absorption phase at 3 dph which also coincided with the onset of exogenous feeding on rotifers and copepods (Fig. 5A). Expression levels increased again ($P = 0.035$) at 18 dph when the feeding changed to *Artemia* nauplii; at this time organs such as pancreas, intestine, liver and swim bladder were fully developed. Maximum expression levels were detected at 25 dph ($P < 0.001$), when the gastric glands and the stomach were fully developed and larvae were fed with *Artemia* nauplii. At 30 dph, a significant decrease ($P = 0.006$) in gene expression was observed, coinciding with the onset of weaning process (Fig. 5A).

Trypsin specific activity was detected as early as 1 dph (0.54 ± 0.07 mU mg protein^{-1}), and remained low during the following days; however, a significant increase in specific activity was observed at 18 dph (1.70 ± 0.29 mU mg protein^{-1}; $P < 0.001$) coinciding with a shifting from the rotifer-copepod mixed feeding period to enriched *Artemia* nauplii. After then, trypsin specific activity decreased by a 35% at 20 dph (1.11 ± 0.06 mU mg protein^{-1}), increasing again at 25 dph (2.04 ± 0.44 mU mg protein^{-1}, $P < 0.001$); maximum specific activity of trypsin was observed at 35 dph (6.36 ± 0.02 mU mg protein^{-1}) and since then, activities remained constant until the end of the experiment ($P > 0.05$) (Fig. 5B).

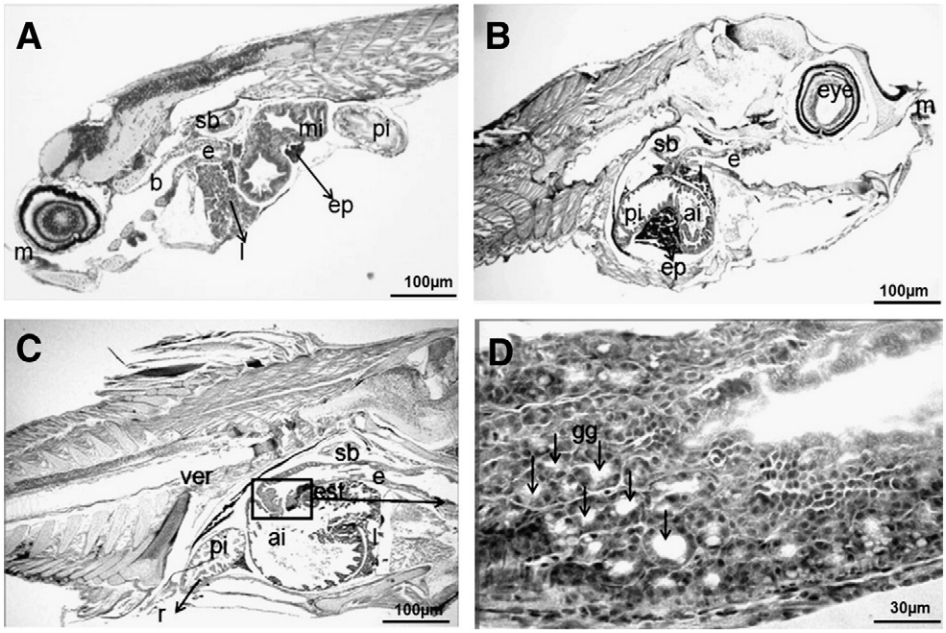

Fig. 3. Sagittal sections of larvae at days 10 (A), 18 (B), and 20 (C) and (D) post hatch. Abbreviations: a, anus; ai, anterior intestine; b, buccopharynx; cns, central nervous system; e, esophagus; e, eye; f, food; ga, gill arch; gg, gastric glands; l, liver; mi, medium intestine; m, mouth; pi, posterior intestine; r, rectum; sb, swim bladder; s, stomach; ud, urinary ducts; v, vertebra. Hematoxylin and Eosin staining.

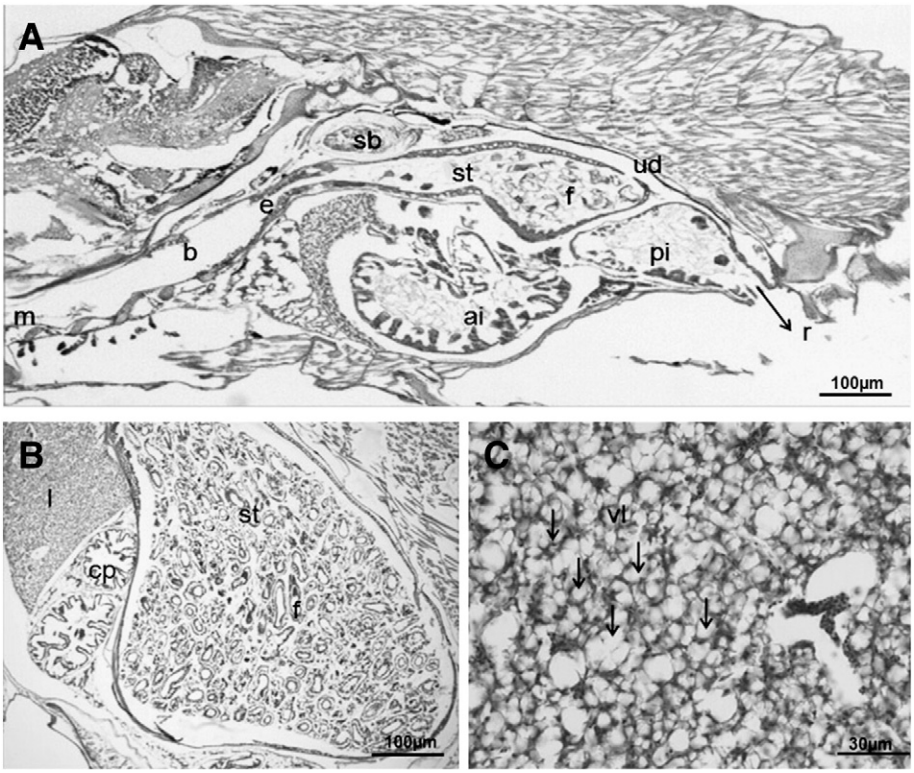

Fig. 4. Sagittal section of larvae at days 25 (A), 30 (B) and 40 (C). Abbreviations: cp, pyloric caeca; f, food; gg, gastric glands; l, liver; lv, lipid vacuoles; s, stomach. Hematoxylin and Eosin staining.

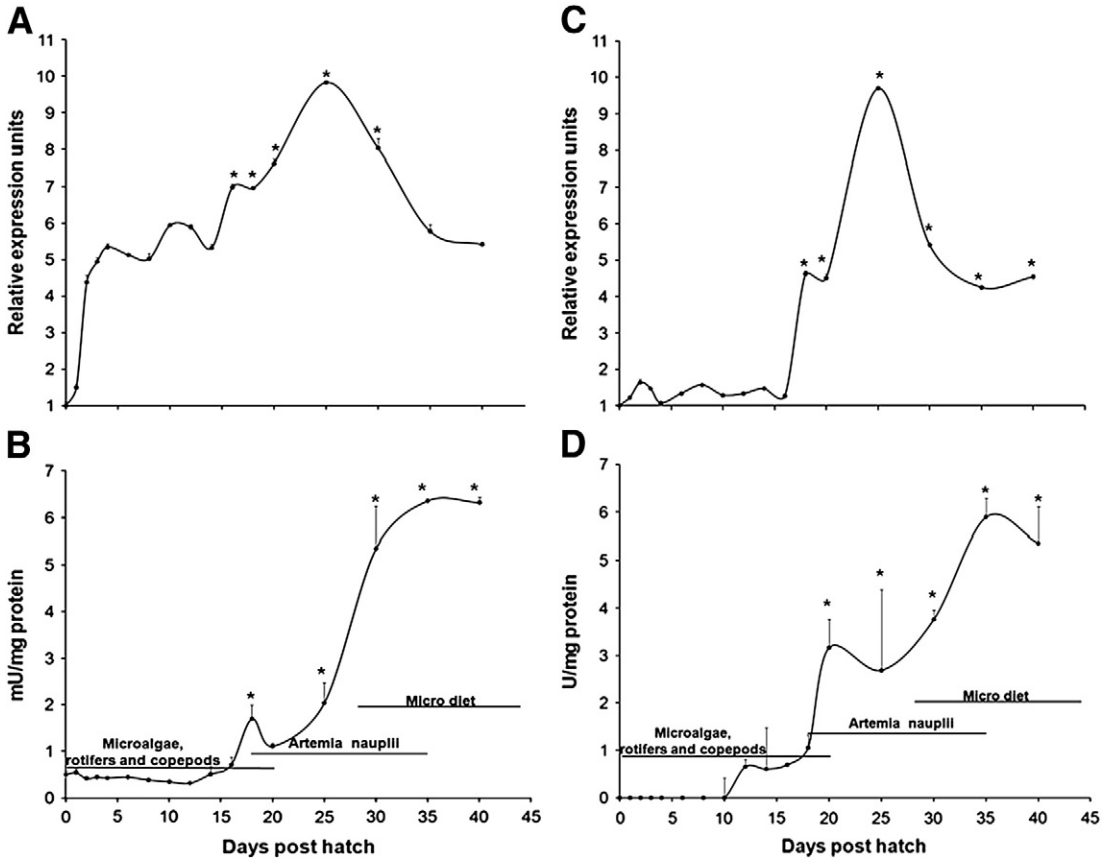

Fig. 5. Gene expression and enzymatic activity of trypsin and pepsin during spotted rose snapper larval development (mean ± SD, $n = 3$). (A) Trypsinogen mRNA expression. (B) Trypsin specific activity. (C) Pepsinogen mRNA expression. (D) Pepsin specific activity. Asterisks indicate significant differences in expression or activity levels of the two digestive enzymes.

3.3. Pepsin

Pepsinogen gene expression was significantly detected at 18 dph ($P<0.001$), two days before the histological differentiation of the gastric glands (Fig. 5C). Maximum expression levels were detected at day 25 ($P<0.001$), coinciding with the full development of the stomach, after then, expression levels decreased at 30 dph ($P<0.05$) and remained constant until the end of the experiment at 40 dph ($P>0.05$) (Fig. 5C).

Pepsin specific activity was not detected before 12 dph (0.65 ± 0.16 U mg protein^{-1}). A stepwise significant increase in specific activity of the acid protease was observed until the end of the experiment. The first significant peak in specific activity was observed at 20 dph (3.16 ± 0.60 U mg protein^{-1}, $P=0.038$) coinciding with the differentiation of the gastric glands. Since then, pepsin activity remained constant until 30 dph (3.7 ± 0.2 U mg protein^{-1}), and increased again at 35 dph (5.89 ± 0.4 U mg protein^{-1}). From this age to the end of the study, pepsin specific activity remained constant ($P>0.05$) (Fig. 5D).

4. Discussion

Protein digestion in fish larvae occurs mainly by the action of alkaline proteases (such as trypsin and chymotrypsin) and cytosolic peptidases (Zambonino-Infante and Cahu, 2001). During larval stages, these enzymes have limited capacity for digesting macromolecules that are absorbed by the pinocytotic activity of the enterocytes in the posterior intestine. In species with a stomach, the adult stage of food processing implies an acid digestion and consequently a more efficient extracellular digestion of proteins (Segner et al., 1994).

Gastric glands can be detected a few days or weeks after hatching and their number increase progressively, partially or completely covering the stomach epithelium depending on the species (Ribeiro et al., 1999; Ortiz-Delgado et al., 2003; Elbal et al., 2004). Accordingly, the secretions of these glands, pepsinogen and hydrochloric acid, induce a progressively lower pH environment in the lumen of the stomach and the conversion of pepsinogen into pepsin (Yúfera et al., 2004; Yúfera and Darias, 2007). The time sequence of gastric glands appearance and the maturation of the stomach vary among families and species (Falk-Petersen, 2005). In addition to the onset of acid digestion, the increase of aminopeptidase N activity in intestinal brush border membranes, and the progressive decrease of cytosolic peptidases characterize the progress of enterocyte maturation indicating a functional intestine (Zambonino-Infante and Cahu, 2001).

The development of the digestive system in tropical spotted rose snapper larvae presented similarities with other marine fish species such as gilthead sea bream *Sparus aurata* (Sarasquete et al., 1995), California halibut *Paralichthys californicus* (Gisbert et al., 2004), bullseye puffer fish *S. annulatus* (García-Gasca et al., 2006), and European sea bass *Dicentrarchux labrax* (Giffard-Mena et al., 2006), among other tropical and temperate marine fish species. In most species, the alimentary canal (buccopharynx, esophagus, intestine, and rectum) is differentiated at 3 dph, coinciding with the opening of the mouth and the onset of exogenous feeding. Similar results were observed in our study, since spotted rose snapper larvae experienced major changes in the development of the digestive system between 3 and 4 dph, including the differentiation of enterocytes, the folding of the intestinal mucosa, and the development of the liver, buccopharynx and esophagus.

According to Segner et al. (1994), the cytological differentiation of enterocytes indicates that the intestine is suitable for absorption of nutrients at the beginning of the exogenous feeding. In the spotted rose snapper, this differentiation coincided with the folding of the digestive tract and its subsequent division into anterior and posterior intestine by the intestinal valve. Gastric glands were differentiated at 20 dph, increasing in number and size during the next days until the end of the larval period. According Baglole et al. (1997), the development of gastric glands is considered the last major event in digestive tract development, and some authors suggest that their presence is a characteristic feature of the end of the larval period and the onset of the juvenile stage (Tanaka, 1972; Segner et al., 1994; Bisbal and Bengtson, 1995; Sarasquete et al., 1995; Peña et al., 2003).

The complete development of the functional stomach may increase the digestive capacity of the gastrointestinal system due to the production of pepsin and hydrochloric acid by gastric glands, which involve the establishment of acid digestion and, consequently, a more efficient extracellular digestion of proteins (Yúfera and Darias, 2007), enhancing the intracellular digestion in the posterior intestine (Govoni et al., 1986). In addition, the full development of the stomach plays an important role in the digestive capacity when larval feeding is changed from live food to an inert diet (Lee and Litvak, 1996; Falk-Petersen and Hansen, 2001).

Expression and activity of trypsin in *L. guttatus* larvae were detected at hatch at very low levels, increasing concomitantly with larval development. These results agree with other reports in larvae of several fish species (Baragi and Lovell, 1986; Pedersen, 1993; Walford and Lam, 1993; Zambonino-Infante and Cahu, 1994; Oozeki and Bailey, 1995; Moyano et al., 1996; Ribeiro et al., 1999; Lazo et al., 2000; Srivastava et al., 2002; García-Gasca et al., 2006; Murray et al., 2006; Alvarez-González et al., 2010). Interestingly, Zambonino-Infante and Cahu (1994) detected enzymatic activity of trypsin and other digestive enzymes in *D. labrax* larvae at 4 dph, while the first feeding occurred at 6 dph. The enzymatic activity of trypsin prior to the first feeding suggests that the activity of this enzyme is derived from genetically preprogrammed expression and not by the first exogenous feeding (See Péres et al., 1998; Ribeiro et al., 1999; Lazo et al., 2000; Zambonino-Infante and Cahu, 2001; Alvarez-González et al., 2006, 2008).

Zambonino-Infante and Cahu (2001) mentioned that the secretion of pancreatic enzymes in the intestinal lumen increases during the first three weeks of larval life (at least in temperate species); this is a normal process due to the maturation of the exocrine pancreas and the production of zymogen granules. According to García-Gasca et al. (2006), the time for trypsin synthesis and enzymatic activity is species-specific and would depend on the larval stage of development and the type of exogenous feeding.

In *L. guttatus* larvae, peaks of trypsin expression preceded those of specific activity, and generally coincided with changes in food supply. Gene expression increased with larval development starting at 3 dph when the larvae opened the mouth and were fed live food (rotifers and copepods), showing maximum expression levels at 25 dph, while enzymatic activity showed maximum levels 10 days later (35 dph), suggesting that the pancreas was fully functional after 25 dph. The efficient synthesis and secretion of pancreatic zymogen granules play an important role in the hydrolysis of food proteins and the activation of other enzymes (Hjelmeland and Jørgensen, 1985).

In *L. guttatus*, the stomach was fully developed at 25 dph, five days after the appearance of gastric glands. A peak in pepsinogen expression was first detected at 18 dph whereas pepsin enzymatic activity was not detected until 20 dph, coinciding with the appearance of gastric glands. Then a second, major peak of pepsinogen gene expression was detected at 25 dph, while pepsin activity was detected at 35 dph, however, the change of feeding from live food to microdiet was not performed until 28 dph. These results indicate that the stomach was fully functional after 25 dph. Several authors have reported for different fish species (e.g. *Pagellus erythrinus*, Suzer et al., 2006; *Mystus nemurus*, Kamarudin et al., 2011; *Atractoscion nobilis*, Galaviz et al., 2011, among others) a decrease in trypsin activity when the stomach becomes functional and pepsin activity takes over the digestion process. In other words, trypsin activity decreases when pepsin activity increases. We did not observe this pattern in trypsin and pepsin activities, since activity of both enzymes remained high at the end of the

experiment, whereas gene expression decreased in both cases. This means that both enzymes were synthesized in parallel, and when the amount of each enzyme was enough to perform digestion, transcription dropped and activity increased. Since fish are a diverse group (from the physiological point of view), the inverse behavior between trypsin and pepsin activities has not always been observed. For instance, Doi (2005) detected parallel increments in trypsin and pepsin activities in mandarin fish (*Siniperca chuatsi*) larvae and juveniles, although there was a sharp increase in pepsin activity at 3 dph, when gastric glands first appeared, increasing even further as gastric glands continued to develop. Nevertheless, trypsin activity (even if lower than pepsin) also increased until the end of the sampling period at 47 dph. In the dourado (*Salminus brasiliensis*) pepsin activity increased at 6 dph while trypsin activity remained almost constant until the end of the sampling period at 7 dph (Vega-Orellana et al., 2006). It is possible that in some fish species pancreatic enzymes (such as trypsin) are still required even if acid digestion starts taking place.

Also, differences between the time of development of gastric glands and the time of pepsin secretion have been observed in some marine fish species such as *D. labrax* (Vu, 1983), *S. senegalensis* (Ribeiro et al., 1999), and *P. californicus* (Alvarez-González et al., 2006). Although Zambonino-Infante and Cahu (2007) demonstrated that gastric gland differentiation was not necessary to feed larvae with artificial diets (since they successfully reared European seabass larvae with artificial diets at the time of first feeding), according to our results and experience, we would not advise to wean *L. guttatus* before development of gastric glands; we suggest that weaning could be performed between 20 and 25 dph instead of the current weaning protocol of 28–30 dph.

5. Conclusions

In spotted rose snapper larvae, the digestive system is fully developed and functional between 20 and 25 dph, as demonstrated by histological observations and the molecular and biochemical quantification of two major proteases, trypsin and pepsin. At this time, larvae should be able to digest inert food and absorb nutrients. Based on these results, the onset of the weaning period (currently performed between 28 and 30 dph) could be performed earlier, almost certainly between 20 and 25 dph.

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