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Organogenesis and Enzymatic Functionality of Exocrine Pancreas in Cultured Gilthead Sea Bream (*Sparus aurata*) Larvae

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Abstract: The ontogeny of the exocrine pancreas was studied histological with the expression of digestive protease, trypsin, activity in gilthead sea bream, *Sparus aurata*, L. larvae from hatching to 30 days after Hatching (DAH). The pancreas was identified as a well developed differentiated cells located dorsal and slightly posterior to the liver at 3 DAH. Incipient pancreas with exocrine polyhedral cells could be observed and first zymogen granules detected at this period. Until the larval metamorphosis, the pancreas became diffuse, spreading throughout the mesentery enclosure the stomach, the upper intestine and the pyloric caeca. The specific activity of trypsin (48.35±4.3 mU/mg/protein) and chymotrypsin (205.38±46.2 mU/mg/protein) was determined as early as after hatching at 2.27±0.14 mm Total Length (TL) of larvae and increased immediately during the following days especially, after exogenous feeding. The highest trypsin and chymotrypsin activity was detected at 25 DAH as 118.26±10.23 mU/mg/protein and 1067.53±118.42 mU/mg/protein, respectively that concurrently detected with *Artemia* metanauplii introduction. It is concluded that exocrine pancreas organogenesis is the main critical step of the zymogen granules and specific activities digestive proteases, trypsin and chymotrypsin, were present as early as after hatching and continuously increasing with larval period of *Sparus aurata*.

Key words: *Sparus aurata*, exocrine pancreas, organogenesis, histology, enzymes, growth

INTRODUCTION

The gilthead sea bream, *Sparus aurata* (L.), is commonly cultivated in the Mediterranean countries. Also, it is a demersal Sparid fish inhabiting rocky bottoms and sea grass beds at depths up to 150 m and also, distributed along the Mediterranean Sea, rarely Black Sea, European and African coasts of the Atlantic Ocean, from Bay of Biscay to Sierra Leone, the Canary Islands and Cape Verde. Generally, it feeds on seaweeds, worms, mollusks and shrimps (Bauchot and Hureau, 1990).

The digestive tract of many marine fish larvae undergoes numerous morphological and functional changes during ontogeny that can substantially influence larval survival under culture conditions. Increasing the knowledge of the digestive capacity and nutritional requirements of the larvae of cultured species for aquaculture will aid in the development of optimal feeding protocols and greatly improve production under hatchery conditions. The organogenesis of exocrine pancreas is the main crucial stage for the establishment of exogenous feeding and digestion. With the exception of three studies (Beccaria *et al*., 1991; Kurokawa and Suzuki, 1996; Luizi *et al*., 1999), the majority of these studies have concentrated on the early embryonic development of the endocrine portion of this organ in warmer water species (Youson and Al-Mahrouki, 1999; Huang *et al*., 2001; Assouline *et al*., 2002). Additionally, pancreatic digestive enzymes such as trypsin and chymotrypsin for digestion are firstly secreted as zymogen granules or precursors. First zymogen granules of the exocrine part appear three days after hatch and become abundant at the onset of the trophic phase (Morrison, 1993). Physiology and nutrition studies of fish in the early stages of development, as well as the evolution of the digestive enzyme activity are valuable tools to better known the nutritional capabilities of young larvae and establish feeding protocols for optimizing larval mass rearing production (Diaz *et al*., 1997; Zambonino-Infante and Cahu, 2001).

As well described by several studies, digestive proteases, trypsin and chymotrypsin, are specific of pancreatic protein hydrolysis (Nolting *et al*., 1999; Zambonino-Infante and Cahu, 2001). Besides, the secretion of trypsin is known to occur in response to food ingestion and in larval pancreatic tissue most of the trypsin is present as an enzymatically inactive

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trypsinogen, whereas most of the trypsin in the intestinal tract is enzymatically active (Ueberschar, 1993, 1995). Several papers are devoted to either digestive tract ontogeny (Sarasquete *et al.* 1995; Elbal *et al.*, 2004) or digestive enzyme activities (Deguara *et al.*, 2003; Moutou *et al.*, 2004) in this species but no study was carried out especially, histological ontogeny exocrine pancreas, which is the main organ enzymatic secretion during larval development. Therefore, the objective of this study to describe the ontogenetic development of exocrine pancreas and concurrently digestive proteases, trypsin and chymotrypsin, activities of *S. aurata* larvae fed on live prey, until end of 30 (DAH).

MATERIALS AND METHODS

Larval rearing: Larval rearing was carried out in a closed sea water system between November-December 2008 at Kilic Sea Products Inc. Milas, Mugla, Turkey and larvae were reared in 1 m^3 cylinder-conical tanks. Water temperature was maintained between 17 and 23.0°C (temperature increased day by day from 17.0-19.0°C between 0 and 7 DAH, from 19.0-21°C between 8 and 20 DAH and from 21-23°C between 21 and 30 DAH). During larval culture period, oxygen, salinity and pH were maintained at >85%, 38.2% and 7.7, respectively. Ammonia and nitrite were kept constant below 0.01 mg L^{-1}. The water in the tank was static during the first 2 days of the rearing period. From day 3-12, the tank water was partially replaced (5-7% daily) by draining through a 200 μm mesh size. Water exchange rate was increased gradually with the age of the larvae. Photoperiod was set on a 24 h light cycle daily, until end of algal addition and then 16 h light and 8 h dark until end of the experiment. After the mouth opening, the larvae from 3-25 DAH were fed with rotifers (70% *Brachionus rotundiformis* and %30 *Brachionus plicatilis*) cultured with algae and enriched (DHA Protein Selco, Artemia Systems SA, Ghent, Belgium) at a density of 10-15 individuals mL^{-1} plus green-water composed of *Nannochloropsis* sp., *Chorella* sp. and *Isochrysis* sp. at a density of 2-3×10^5 cells mL^{-1}. From day 10-30 DAH with *Artemia* nauplii AF480, INVE Aquaculture) at 4-6 individuals mL^{-1} and from 25 DAH until end of the experiment, *Artemia* metanauplii at 2-4 individuals mL^{-1} both enriched with Protein Selco Larval feeding regime is schematized in Fig. 1.

Sampling: Growth rate was monitored weekly by sampling groups of larvae from each tank (30 larvae per sample $group^{-1}$) and at the last day of the experiment (30 DAH). Specific growth rate was calculated by formulae SGR = 100

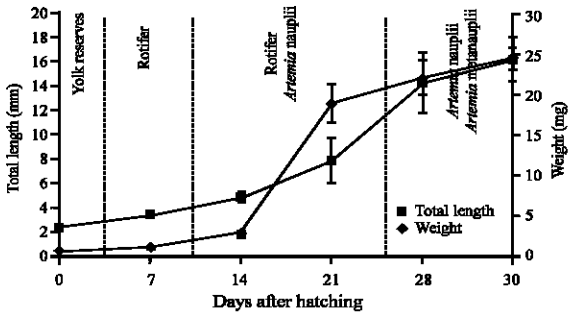

Fig. 1: Growth of *S. aurata* larvae: Each mean±SD is a pool of 30 larvae. The diet types at different stages are indicated between the dotted lines

(Ln FBW -Ln IBW)/Δt, with IBW, FBW: initial, final body weight of fish (mg), Δt: time interval (day). At the end of the experiment, larval survival was determined by counting larvae remaining in the tanks.

Histological analysis: For histological analyses, 10 larvae sampled from each tanks collected daily from 0-15 DAH and every third day from 15-30 DAH during post-hatching period. Samples were fixed in neutral formalin solution, dehydrated in different alcohol series, embedded in a parafin vax and cut in 3-5 μm-thick sagittal sections using a Leica RM 2125 rotary microtome. The Haematoxylin/Eosin (HE) stain was used for histological observations to describe the development of the exocrine pancreas and then slides with sections were mounted permanently. The sections of fish were randomly examined under an Olympus CX31 microscope. Photographs were taken with an Olympus DP20 digital photomicrographic attachment.

Enzymatic analysis: Pooled samples of larvae (50-250 individuals, depending on age and size) were collected for enzyme analysis. Sampling days for enzymatic analysis were carried out at 2, 3, 5, 7, 8, 10 DAH and 5 days intervals after this date until 30 DAH. Samples were collected and homogenized in 5 volumes v w^{-1} of ice-cold distilled water. Extracts utilized for enzyme assays were obtained after homogenization of larvae (35 mg mL^{-1}) in cold 50 mM Tris-HCl buffer, pH 8.0, followed by centrifugation (13.500×g; 30 min at 4°C). Trypsin activity was assayed spectrophotometrically using Ná-Benzoyl-DL-arginine-p-nitroanilide (BAPNA) as the substrate (Tseng *et al.*, 1982). Absorbance was measured at 253 nm for 5 min. One unit activity of trypsin was defined as 1 μmol of BAPNA hydrolyzed per min at 25°C. Chymotrypsin activity was analyzed spectrophotometrically using Benzoyl-L-Tyrosine Ethyl Ester (BTEE) as the

substrate (Worthington, 1982). Absorbance was measured at 256 nm for 5 min. One unit activity of chymotrypsin was defined as 1 μmol of BTEE hydrolyzed per minute at 25 sC. Enzymatic activities were expressed as specific activity (mU/mg/protein) and total activity (mU/larva). Protein was determined by the Bradford method (Bradford, 1976).

Statistical analysis: All measurements were carried out in triplicate. Results are given as mean±SD. The variance homogeneity of the data was performed using Levene's test. Survival was compared by Fischer's chi-square test and also, larval growth enzymatic activity data were compared by one-way ANOVA, followed by Newman-Keul's multiple range test and all significant differences were set at 0.05 level. Statistical analyses were performed by SPSS 15.0 software.

RESULTS

Growth: Growth of *S. aurata* larvae during the study is described in Fig. 1. At hatching, TL was measured as 2.27±0.4 mm and mouth and anus closed. Additionally alimentary tract appeared lying dorsally to the yolk sac. At 3 DAH, firstly anus and then mouth opened and TL of larvae were determined as 3.56±0.29 mm. At this stage exogenous feeding began and few rotifer and microalgae could be observed in digestive tube. Swimbladder inflation started and then it begun to elongate under the notochord on 7 DAH and 15 DAH, respectively. Specific growth rate averaged 7.63% day^{-1} and also, survival rate was calculated as 36.4% at the end of the study.

Organogenesis of pancreas: At hatching, the accessory digestive organs were unavailable and/or undifferentiated. By 2 DAH, the basophilic cytoplasm of the exocrine pancreas was homogeneous and zymogen granules and pancreatic ducts were not apparent yet (Fig. 2a). At 3 DAH the mouth is open and *S. aurata* larvae underwent rapid developmental changes, so that the pancreas at this stage exists as a distinct and compact organ situated just posterior to the liver (Fig. 2b). Zymogen granules appeared within the exocrine cells and the main pancreatic duct was visible, opening into the anterior intestine. The pancreatic duct, like the bile duct, was lined with a cuboidal ephitelium and opened into the ventral part of the anterior intestine just after the pyloric sphincter. In the pancreas, zymogen granules first appeared in the centre of acini from day 3-4 (Fig. 2c).

Zymogen granules increased after first feeding from 4 DAH (Fig. 3d) through the 15 DAH mainly depends on size of pancreas. The digestive system, as well as the liver and the pancreas, showed a normal structure and appearance with zymogen-granules in the exocrine pancreas in 15 DAH (Fig. 3e). The digestive tract becomes more complex; however, the post-esophageal swelling still does not exhibit evidence of gastric gland formation between 15 DAH and 25 DAH. By 20 DAH, the pancreas is still found in close association with the liver (Fig. 3f). At 30 DAH, the pancreas at this stage shows a diffuse distribution interspersed throughout the mesentery surrounding the intestine whereas the liver remains as a compact organ situated anterior and posterior to the digestive tract, the digestive system, with the exception of the pyloric caeca, is fully formed (Fig. 4g). Pancreatic acinar cells of the larvae were rich in zymogen granules and also blood vessels and islets were observed in the exocrine pancreas in 30 DAH (Fig. 4 h).

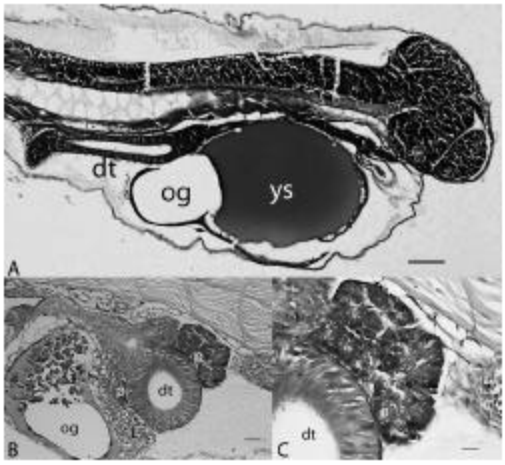

Fig. 2: Histological sections of *Sparus aurata* larvae. a) Larvae at hatching showing the yolksac, oil globules and undifferentiated digestive tract. b) Larvae at 3 DAH, progressive resorption of the yolksac and oil globules. A columnar epithelium is visible in the rest of digestive portions (future stomach and intestine). Differentiation of the liver and exocrine pancreas (less basophilic) is evident. C. In the pancreas, zymogen granules appeared in the centre of acini. Scale bar: 100 μm. dt: Digestive tract; L: Liver; ys: Yolksac; og: Oil globule; p: Pancreas; asterisks showing zymogen granules

Trypsin activity: The specific activity of trypsin was determined as early as after hatching (48.35±4.3 mU/mg/protein) at 2.27±0.4 mm TL of larvae and increased immediately during the following days especially after exogenous feeding. As expected, from 3 DAH (mouth opening) specific tryptic activity continuously increased depending on larval age and size until 25 DAH. After that

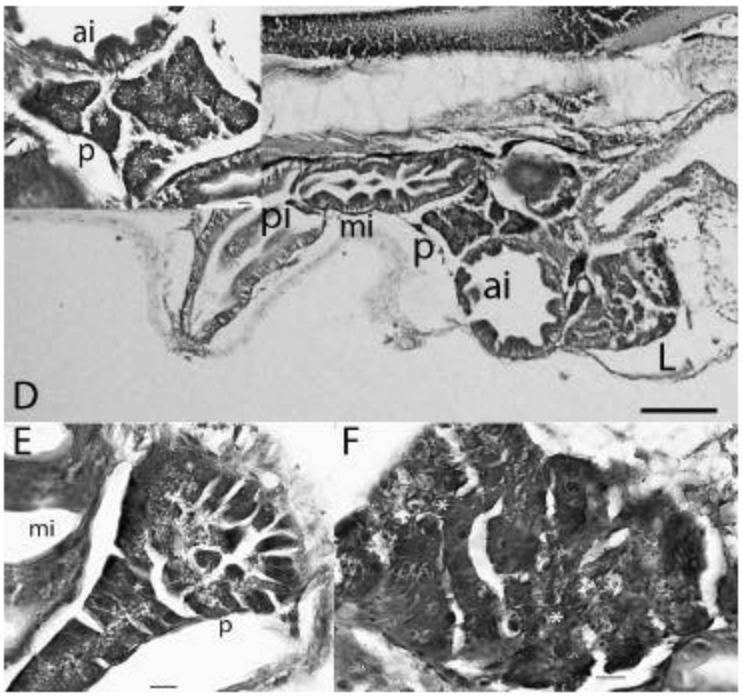

Fig. 3: Histological sections of *Sparus aurata* larvae between at 4-25 DAH. D. Larvae at 4 DAH, a differentiated liver with an evident vascular system, a functional exocrine pancreas with acidophilic zymogen. E-F: Zymogen granules increased from 15 DAH through the 25 DAH mainly depends on size of pancreas, respectively. Scale bar: 100 µm. L: Liver; p: Pancreas; ai: Anterior intestine; mi: Middle intestine; pi: Posterior intestine; asterisks showing zymogen granules

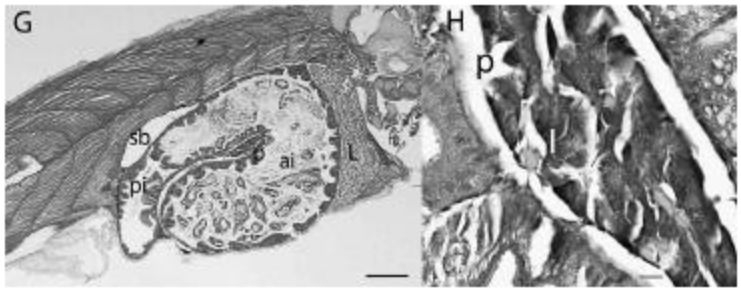

Fig. 4: Histological sections of *Sparus aurata* larvae at 30 DAH. G: The pancreas has spread through surrounding the digestive tract, especially in the region near the anterior intestine. H: Blood vessels and islets were observed in the exocrine pancreas. Scale bar: 100 µm. L: Liver; p: Pancreas; ai: Anterior intestine; pi: Posterior intestine; sb: Swim bladder; I: Islets; asterisks showing zymogen granules

approximately, 20% a sharp decline was measured in this activity until 30 DAH ($p<0.05$). Then, this decline were continued on specific activity of trypsin until end of experiment ($p>0.05$). The highest tryptic activity was detected at 25 DAH as 118.26±10.23 mU/mg/protein (Fig. 5).

Generally, the developmental pattern of the total activity of trypsin was similar to that of specific activity to 25 DAH as exponential increasing with larval age and development. After detection at hatching, it sharply increased and fluctuated up to 25 DAH ($p<0.05$). From this date, slight decreases were measured, until end of the experiment ($p>0.05$). The peak of total activity was measured at 25 DAH as 0.83±0.07 mU/larva (Fig. 5).

Chymotrypsin activity: As observed for trypsin, specific activity of chymotrypsin was detected after hatching (205.38±46.2 mU/mg/protein) and increased immediately

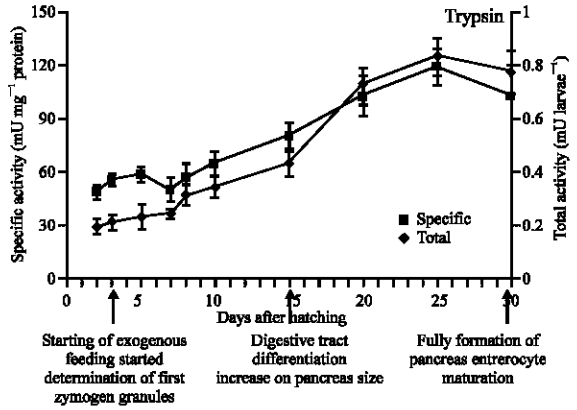

Fig. 5: Trypsin activity assayed in homogenates of whole sea bream larvae. Results are expressed as means±SD (n = 5). Major events taking place during the anatomical differentiation of digestive organs in larvae of this species reared (Sarasquete *et al*., 1995) are included as a reference (arrows)

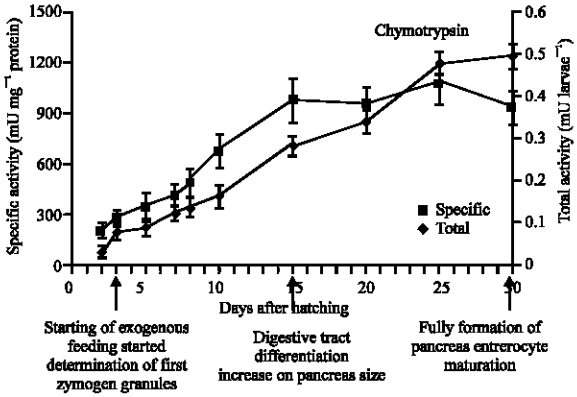

Fig. 6: Chymotrypsin activity assayed in homogenates of whole sea bream larvae. Results are expressed as means±SD (n = 5). Major events taking place during the anatomical differentiation of digestive organs in larvae of this species reared (Sarasquete *et al*., 1995) are included as a reference (arrows)

during the following days especially after exogenous feeding at 3 DAH. The specific enzyme activity was increased to day 25 and then decreased. Similarly, the peak of specific activity was assayed at 25 DAH as 1067.53±118.42 mU/mg/protein (Fig. 6).

As assayed for trypsin, similar developmental pattern for total activity of chymotrypsin was observed in seabream larvae. During the experiment, this activity showed regularly and linear increase depend on larval age and size ($p>0.05$). The highest of total activity was measured at 30 DAH as 0.49±0.03 mU/larva (Fig. 6).

DISCUSSION

In current study, the organogenesis of exocrine pancreas and also characterization of digestive proteases, trypsin and chymotrpsin, in *S. aurata* larvae were investigated from hatching to 30 DAH. Larva were showed exponentially growth and relatively similar with previous studies on this species. SGR was calculated as 10.74% day^{-1} on day 40 and also this average was parallel with one of the candidate Sparids, common pandora, *Pagellus erythrinus* L. as 16.03% day^{-1} (Suzer *et al*., 2006a).

The sea bream has a single pancreatic primordium like sea bass (Becceria *et al*., 1991) in contrasts pike observed two (Vortsman, 1978) and trout observed three (Weber, 1902). Until, the mouth opening, Becceria *et al*. (1991) reported that developmental sequence of cell differentiation and multiplication of exocrine pancreas did not depend on rearing conditions, apart from the temperature, which effected the length of prelarval stage. These results were paralleled with previously study recorded by Saraquete *et al*. (1995). Since, the researchers carried out their experiments in the same temperature at 19°C and also it is determined that zymogen granules were observed at 3 DAH as same as the study. According to Hjelmeland (1995), yolksac larvae give priority to synthesis and accumulation of digestive capacity in the form of pancreatic enzymes, suggesting a strategy of the larva to be ready to digest to the establishment of exogenous feeding.

The pancreas in *S. aurata* were observed for the first time between 2 and 3 DAH, lying dorsal to the intestine and close to the liver, but later in development becomes distributed through the mesentery surrounding the intestine in a diffuse manner. As a mentioned in Sarasquete *et al*. (1995), we observed that the pancreas was extra hepatic from hatching to first month of larval stage in *S. aurata*. Also, it was separated two parts, one attached to the liver and the other between the anterior and posterior of intestine. In this species, the pancreas became completely diffuse at around 30 DAH, in synchrony with the complete development of the gastric glands, this differentiation was detected at 30 DAH in t he red porgy larvae (Darias *et al*., 2005). Also, present histological observations are in agreement with previous quantitative studies described in the common dentex (*Dentex dentex*) larvae by Santamaria *et al*. (2004) showing that the volume of the whole larva, liver and pancreas increased dramatically from 20 DAH till the end of experimentation (30 DAH).

Exogenous feeding was onset with rotifers and microalgae after 3 DAH. At the time, the formation of digestive systems and accessory glands of *S. aurata* showed similar evolutions as described to other Sparids (Micale *et al*., 2006; Santamaria *et al*., 2004). In this time, morphological and enzymatic materials such as hepatocytes, enterocytes and pancreatic cells exist in larvae. Some researchers consider the pancreas functional when zymogen granules appear in the acinar cells. This occurs after first feeding in some species: *Diplodus sargus* (Ortiz-Delgado *et al*., 2006), *S. aurata* (Sarasquete *et al*., 1995), Senegale sole, *Solea senegalensis* (Sarasquete *et al*., 1995), Summer flounder, *Paralichthys dentatus* (Bisbal and Bengston, 1995) and from hatch in others: Haddock, *Melanogrammus aeglefinus* (Hamlin *et al*., 2000). On 3 DAH, the first zymogen granules observed into exocrine pancreas in this study. A comparison of the results obtained in this study with observations on the development of pancreatic cells in *Salmo gairdneri* (Vernier and Sire, 1976), Sea bass, *Dicentrarchus labrax* (Diaz and Connes, 1991) and Dover sole, *Solea solea* (Boulhic and Gabaudan, 1992) shows a similar sequence of the development and differentiation of the glands during the period of endo-exogenous feeding in the *S. aurata* larvae. It was suggested that activity of some pancreatic enzymes, i.e., protease, amylase could be defined related with morphological development of zymogen granules described by Caruso *et al.* (2001), Micale *et al.* (2006) and Suzer *et al.* (2007a). Additionally, it is reported that ontogenetic expression digestive proteolytic enzymes, trypsin and chymotrypsin and also amylase were detected concurrently with the formation of zymogen granules in some cultured species such as *P. erythrinus* (Suzer *et al*., 2006a) red porgy, *Pagrus pagrus*, L. (Suzer *et al*., 2007b) *S. senegalensis*, L. (Ribeiro *et al*., 1999) and yellowtail kingfish *Seriola lalandi* L. (Chen *et al*., 2006).

It is well known that the secretion of trypsin is known to occur in response to food ingestion and in larval pancreatic tissue most of the trypsin is present as an enzymatically inactive trypsinogen, whereas most of the trypsin in the intestinal tract is enzymatically active (Ueberschar, 1993, 1995). Also, it is well recorded that trypsin and chymotrypsin are specific of pancreatic protein hydrolysis (Nolting *et al*., 1999; Zambonino-Infante and Cahu, 2001). Furthermore, onset and changes of development patterns of trypsin and chymotrypsin in larval fish could be genetically controlled (Zambonino-Infante and Cahu, 2001). A similar pattern about activities of these proteases increase in early stages and then relatively reduction is reported in some Sparids such as *P. erythrinus* (Suzer *et al*., 2006a), *P. pagrus*, (Suzer *et al*., 2007b), blackspot seabream, *Pagellus bogaraveo*, (Riberio *et al*., 2008) and other fish larvae such as herring larvae, *Clupea harengus*, L. (Pedersen and Andersen, 1992) and tilapia, *Oreochromis niloticus*, L. larvae (Drossou *et al*., 2006). The above stated decline in specific enzyme activities of these digestive proteases during larval ontogeny of *S. aurata* could be basically explained by the normal increase of tissue proteins in growing larvae, which reflects anatomical and physiological changes in fish larvae and does not correspond to a lowering in the amount of digestive enzymes or dietary shifts (Zambonino-Infante and Cahu, 2001). On the other hand, it is well reported that abiotical factors such as temperature, illumination and pH are strongly effected digestive proteases in this species. In previous studies, it is hypothesized that different light intensities and salinity were influenced the specific activities trypsin and chymotrypsin in the both *S. aurata* and *P. erythrinus* larvae (Moutou *et al*., 2004; Suzer *et al*., 2006b). Besides, it is well recorded in *S. aurata* and *D. puntazzo* that pH variations along the digestive tract also, clearly affected activities of these proteases (Deguara *et al*., 2003; Aktulun *et al*., 2008).

It is reported that the fluctuations in specific enzyme activities covered the period of morphological differentiation in the digestive tract and associated glands (Zambonino-Infante and Cahu, 2001). After the formation of gastric glands, the digestive system became functional and the specific activities of these digestive enzymes remained constant, while the total enzyme activities increased gradually with age. As reported in some fish larvae, trypsin specific activity could be decreased with age and size after gastric gland formation (Cahu and Zambonino-Infante, 2001; Kolkovski, 2001; Zambonino-Infante and Cahu, 2001). Similar pattern for tryptic activity as important decrease were found in *P. erythrinus*, *P. pagrus*, *S. senegalanesis*, *S. aurata* and *D. labrax* larvae (Moyano *et al*., 1996; Ribeiro *et al*., 1999; Zambonino-Infante and Cahu, 2001; Suzer *et al*., 2006a, 2007a, b). The second phenomenon supported for this decline in tryptic activity could be considered that ontogenic development of brush border membrane and enterocyte mainly occurred in the same time. In the previuos study, it could be noted that specific activity of alkaline phosphatase and aminonpeptidase N were gradually increase with the decline of tryptic activity and formation of gastric glands (Suzer *et al*., 2007a).

CONCLUSION

It could be concluded that the ontogenic development of the digestive system of *S. aurata* larvae

followed the same general pattern that most Sparidae species described to date. Also, it is commonly known that exocrine pancreas organogenesis is the main critical stage of the trypsin and chymotrypsin expression concurrently with the onset of the larval feeding. This developmental pattern suggests that trypsin contributes to protein digestion in *S. aurata* larvae by synchronously compensating with trypsin for the absence of pepsin until formation of functional stomach. Moreover, these findings are of considerable importance for the evaluation of the digestive tract functionality and will be useful for establishing of optimal rearing techniques and artificial food for *S. aurata* larvae and the commercial production of this species.

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