Development of the Gastrointestinal Tract in Sharpsnout Sea Bream (*Diplodus puntazzo*) Larvae: Histological and Enzymatic Ontogeny

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Abstract: Digestive system ontogeny of the sharpsnout sea bream (*Diplodus puntazzo* L., 1758) was detected from hatching until 40 Days After Hatching (DAH) using histological techniques and enzymatic assays. At hatching, total length of larvae measured at 2.8±0.34 mm and it was determined on 40 DAH as 11.84±1.76 mm. At mouth opening (3 DAH), larvae almost absorbed their yolk-sac reserves. An incipient stomach could be distinguished at 2 DAH. The first evidence of gastric glands development was detected at 28 DAH, increasing in number and size by 33-40 DAH. In the fundic region, gastric gland determined as strogly and mucusal acinar cell accumulates at 30 DAH and the intestinal mucous cells appeared and developed between at 10-28 DAH. Then, functional stomach formation was detected for the first time on 32 DAH supported with sudden increase on activity of acid protease. In terms of digestive protease activity, alkaline protease was detected as early as hatching and also suddenly increased after starting of exogenous feeding. Developmental pattern for alkaline protease demonstrated constant enhancement, until metamorphosis at 25 DAH. After this date, this activity slowly decreased until end of the experiment. In contrast to this, acidic protease activity exhibited relatively lower activities during the early stages. Afterwards, concurrently with the formation of functional stomach at 32 DAH, acid protease activity suddenly >3-fold enhanced and continued to increase until end of the experiment. It is concluded that organogenesis of alimentary tract and ontogeny of digestive proteases in *D. puntazzo* larvae followed the same general pattern that most Sparidae species.

Key words: *D. puntazzo*, sharpsnout sea bream, digestive system, histology, digestive enzymes, ontogeny

INTRODUCTION

Among new possible species, the sharpsnout sea bream, *Diplodus puntazzo* has become one of the most attractive candidates for diversification of aquaculture (Marangos, 1995; Boglione *et al.*, 2003; Papandroulakis *et al.*, 2004). Also, *D. puntazzo* is a demersal Sparid teleost 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). In the Mediterranean Sea, spawning takes place during September-November, when water temperature ranges between 20 and 23°C (Marangos, 1995; Micale *et al.*, 1996).

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 new candidate species for aquaculture will aid in the development of optimal feeding protocols and greatly improve production under hatchery conditions. Successful development of the digestive system is crucial for the survival and growth in fish larvae because an efficient digestive system enables fish to capture, ingest, digest and absorb food (Kjorsvik *et al.*, 2004). Although, larval fish may be morphologically capable of capturing food items at first feeding (Segner *et al.*, 1994; Bisbal and Bengtson, 1995), the digestive system needs a series of developmental changes before being fully functional (Govoni *et al.*, 1986; Canino and Bailey, 1995). Knowledge on the structural

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development of the digestive system is essential to understand the digestive physiology and determine the appropriate timing to wean fish larvae (Watanabe and Kiron, 1994; Baglole *et al.*, 1997; Cahu and Zambonino Infante, 2001).

During the last decade, there is an increase on number of papers, which are devoted to the onset and development of digestive systems during larval growth of cultured fish species. Studies related to histological formation and enzymatic activity of fish during the early stages of development are valuable tools to better known the nutritional capabilities of young larvae and establish feeding protocols for optimizing larval mass rearing production (Ueberschar, 1993, 1995; Diaz *et al.*, 1997; Zambonino Infante and Cahu, 2001; Santamaria *et al.*, 2004). Moreover, ontogenesis of digestive tract, accessory glands and developmental features of the digestive system have been well documented in several cultured species, such as *D. labrax* (Zambonino-Infante and Cahu, 1994), *S. aurata* (Sarasquete *et al.*, 1995; Elbal *et al.*, 2004), summer flounder, *Paralichthys dentatus* (Bisbal and Bengtson, 1995), Senegal sole, *Solea senegalensis* (Ribeiro *et al.*, 1999), red banded sea bream, *Pagrus auriga*, (Sanchez-Amaya *et al.*, 2007) and common pandora, *Pagellus erythrinus*, (Micale *et al.*, 2006). Additionally, recent studies with *D. puntazzo* have been focused on macronutrient selection (Atienza *et al.*, 2004), neuromast and olfactory organ development (Boglione *et al.*, 2003), sensory evaluation (Hernández *et al.*, 2001), feeding rate, dietary and food intake (Vivas *et al.*, 2006) and polyculture (Favaloro *et al.*, 2002) in usual juvenile and/or adult fish.

Despite, the general similarity of the developmental stages, the duration of each developmental formation varies among fish species. Therefore, the understanding of ontogenetic development of the digestive system and also digestive enzyme activity is crucial for larval fish rearing in any economically important aquaculture species. The objective of this study was firstly examine the structural changes in the gastrointestinal system and also, development of digestive enzymes of hatchery-reared sharp snout sea bream larvae from hatching until metamorphosis, aiming to understand the sequence of organ development and provide fundamental knowledge on hatchery management for commercial aquaculture.

MATERIALS AND METHODS

Larval rearing: Larval rearing was carried out in a closed sea water system at Teknomar Sea Fish Broodstock Centre (Akuvatur Mediterranean Sea Foods, Izmir, Turkey) and larvae were reared in 1 m^3 cylinder-conical
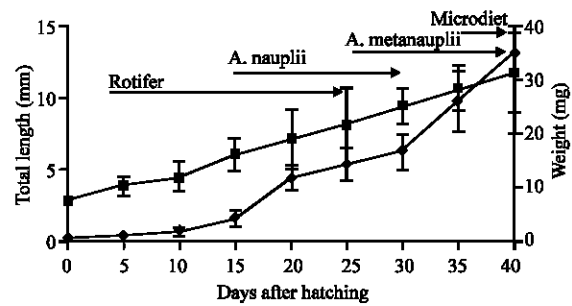

Fig. 1: Growth of *D. puntazzo* larvae: total length (solid line) and weight (dashed line). Each mean±SD represents a pool of 30 larvae. The diet types at different stages are indicated by arrows

tanks. Water temperature was maintained between 19.0 and 23.0°C (temperature increased day by day from 19.0-20°C between 0 and 7 DAH, 20 and to 21°C between 8 and 20 DAH, from 21-22°C between 21 and 30 DAH, 22-23°C between 31 and 35 DAH). During larval culture period, oxygen, salinity and pH were maintained at >85 %, 38.2% and 7.7, respectively. 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*, *Chorella* and *Isochrysis* sp. at a density of 2-3×10⁵ cells mL^{-1}. From day 15-30 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. Extruded microdiet (Proton, INVE Aquaculture, Ghent, Belgium) was introduced from 35 DAH until end of the experiment as 8-10% of biomass. Larval feeding regime is schematized in Fig. 1.

Sampling

Growth and survival: In order to monitor of growth, groups of larvae sampled from each tank 5 days interval (30 larvae sample group). Specific growth rate was calculated by formulae SGR = 100 (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 calculated by counting larvae remaining in the tanks.

Histological analysis: For histological analyses, 15 larvae sampled from each tanks every day's intervals during post-hatching period. Obtained samples preserved in buffered formaldehyde solution for fixation and then dehydrated in different alcohol series and embedded in paraffin. The fixed fish were individually embedded in paraffin blocks and sectioned in serial sagittal sections (5 μm thick, Leica RM 2125 rotary microtome). The Haematoxylin/Eosin (HE) and Periodic Acid-Schiff (PAS) stain were used to describe the development of the gastrointestinal system under a light microscope (Olympus CX31) for histological observations.

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). Alkaline and acid protease activities in the supernatantwere measured using the methods detailed by Alarcon *et al.* (1998) and Anson (1938), respectively. One unit of activity was defined as 1 μg of tyrosine released per min. Enzymatic activities were expressed as specific activity (mU/mg/protein). 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 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 AND DISCUSSION

Growth and survival: Growth of *D. puntazzo* larvae during the study is described in Fig. 1. At hatching, total length was measured as 2.87±0.34 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 total lengths of larvae were determined as 3.47±0.26 mm. At this stage exogeneous feeding began and few rotifer and microalgae could be observed in digestive tube.

During the early stages total length development gradually increased and sharp increase in this parameter was observed from 25 DAH. Also, weight gain presented two phases. After smooth increasing in early stages, first increase was observed from 15 DAH and then kept on gradually rising. Second increase was detected from 30 DAH coincided with MD introduction. Concisely, larvae represented >40 times increase from 5-40 DAH. Specific growth rate averaged 10.74% day^{-1} and also, survival rate was calculated as 21.7%.

Histological analysis
Stomach: The presumptive stomach appeared with a simple cuboidal epithelium as a protrusion at the end of the oesophagus on 3 DAH. A preliminary ploric sphincter formed and the mucose of stomach developed as a similar in oesophagus on 5 DAH (Fig. 2a). At 10 DAH, the stomach epithelium began to differentiate from a simple cuboidal epithelium to a columnar epithelium and then cardiac, fundic and pyloric sections could be occurred (Fig. 2b). When larvae began to feeding with Artemia nauplii, stomach developing started with presence of gastric glands. Gastric glands were first appeared on 28 DAH (Fig. 2c and d) especially in the cardiac and fundic region and increased with larval growth until the 40 DAH (Fig. 2e). In the fundic region, gastric gland determined as strogly and mucusal acinar cell accumulates at 32 DAH. Increasing number of gastric glands observed with larval development and fully developed glandular stomach could be defined at 38 DAH (Fig. 2f).

Intestine: On 2-3 DAH, incipient intestine was observed with columnar cells. When, the mouth and anus opened, beginning of structural differentiations observed into the incipient intestine on 3-4 DAH with the primordial mucosal folds starting (Fig. 3a). Non-staining vacuoles and PAS-positive brush borders could be seen in the midgut coinciding with larvae feeding on rotifers on 4 DAH. At 6 DAH, supranuclear vacuoles occurred in the enterocytes of posterior intestine. Incipient mucosal folds observed in both portions of the intestine and they developed rapidly between 10-28 DAH with increasing larval growth. With the beginning of *Artemia* nauplii feeding, increased number of lipid vacuoles determined at the entrocytes on 12 DAH (Fig. 3b). In anterior intestine, goblet cells were found in the entrocytes at 28 DAH (Fig. 3c). They were spreaded out exteremely increasing in enterocytes of both anterior and posterior intestine between 33 and 40 DAH (Fig. 3d and e).
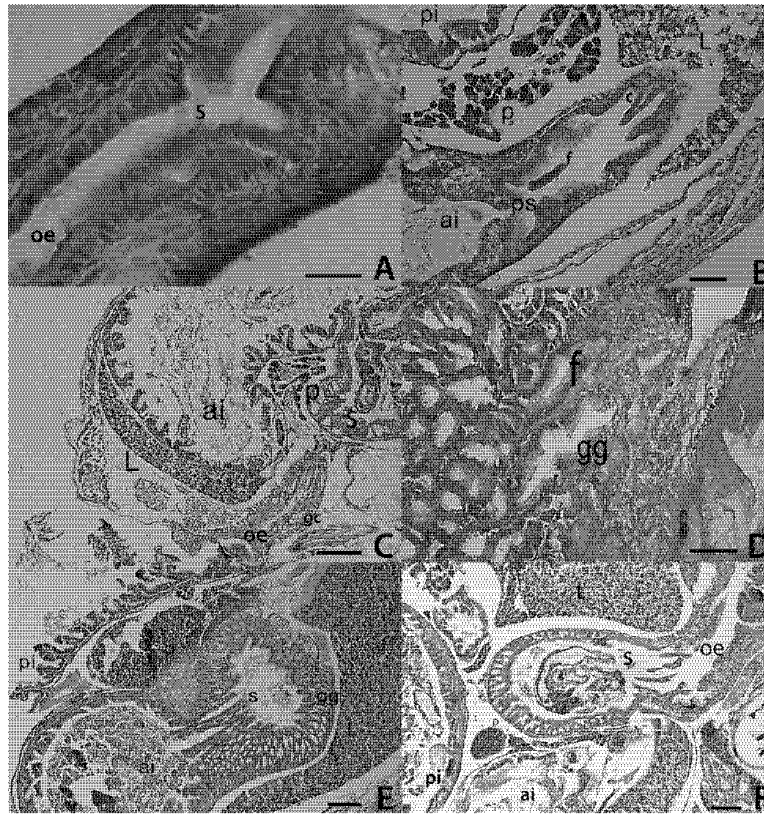
Fig. 2: Histological sections of *D. puntazzo* larvae between at 5-40 DAH. a) View of the stomach and oesophagus in a 5 DAH sharpsnout sea bream larva, b) General view of the gastrointestinal system in a 10 DAH larva, pyloric sphincter is visible, c) General view of the gastrointestinal system in a 28 DAH larva, d). Fundic portion of the stomach of a 28 DAH larva, showing first gastric glands developing, e) Fully developed glandular stomach in a 38 DAH larva, f) General view of the gastrointestinal system in a 40 DAH larva. Showing numerous gastric glands. Scale bar: 100 μm. L: Liver; p: Pancreas; s: Stomach; ai: Anterior intestine; f: Fundic stomach; c: Cardiac stomach; oe: Oesophagus; pi: Posterior intestine; ps: Pyloric sphincter; gg: Gastric gland

Enzymatic analysis

Alkaline protease: Alkaline protease activity was detected as early as hatching (288.65±26.5 mU/mg/protein) and increased immediately during the following days especially after exogenous feeding. As expected, from 3 DAH (mouth opening) tryptic activity gradually increased depend on larval development until 25 DAH. Especially, after appereance of functional stomach, the constant decline in this activity was assayed and continued until end of the experiment. The highest tryptic activity was determined at 25 DAH as 916.41±76.6 mU/mg/protein (Fig. 4).

Acid protease: Acid protease activity demonstrated very low activities, until metamorphosis at 25 DAH. After this date, this activity slowly increased to 30 DAH. Concurrently with the formation of functional stomach at 32 DAH, acid protease activity suddenly >3-fold

enhanced and continued to increase until end of the experiment. The peak of acid protease activity was measured at the end of the experiment (40 DAH) as 8.31±0.72 mg protein^{-1} (Fig. 5).

This study initiated that the ontogeny and formation stages of gastrointestinal systems in *D. puntazzo* larvae were investigated from hatching to 40 DAH with enzyme expressions. Larvae were showed exponentially growth and relatively similar with previous studies on this species. While, Boglione *et al.* (2003) estimated that total length of larvae reached about 12 mm, Papandroulakis *et al.* (2004) recorded that this parameter measured about 16 mm on day 40. Also, SGR was calculated as 10.74% days^{-1} on day 40 and also, this average was parallel with one of the candidate Sparids, *P. erythrinus* as 16.03% day^{-1} (Suzer *et al.*, 2006). Moreover, Papandroulakis *et al.* (2004) was calculated survival rates as 53.7±7.5%, which was relatively higher
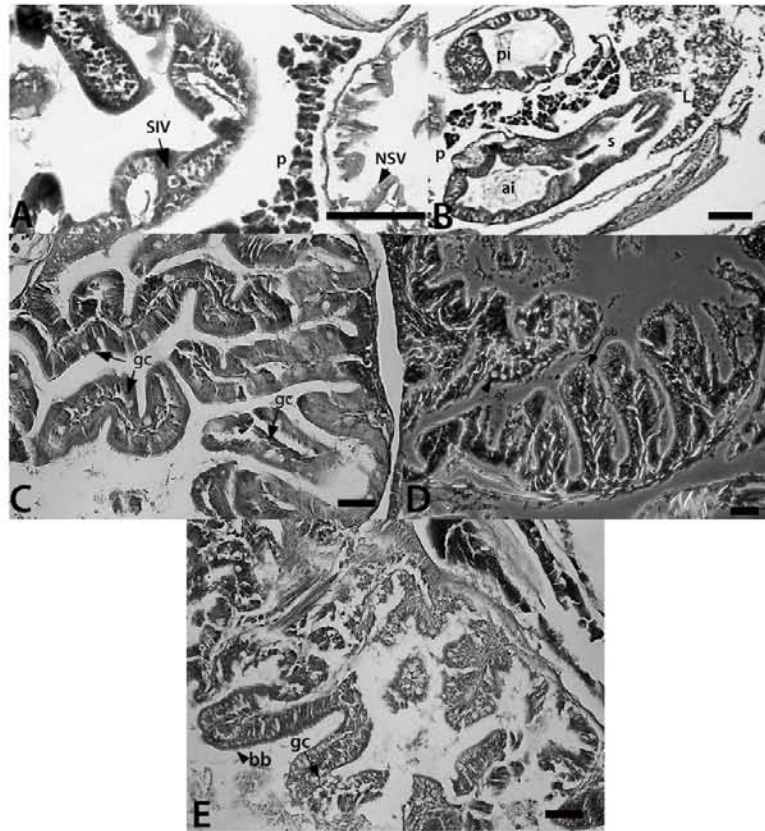
Fig. 3: Histological sections of *D. puntazzo* larvae between at 3-40 DAH. a) Sagittal section of the digestive tract of a larvae 3 DAH. Non-staining vacuoles and SIV appeared in the anterior and posterior intestine, respectively, b) General view of the gastrointestinal system in a 10 DAH larva, c) Detail of anterior intestinal epithelium with small vacuoles and goblet cells are visible in 28 DAH larva, d) Mucosal folds in anterior intestine at 33 DAH larva, showing first gastric glands developing, e) Numerous supranuclear inclusions and goblet cells are evident in the posterior intestine at 40 DAH larva. Scale bar: 100 μm. L: Liver; p: Pancreas; s: Stomach; ai: Anterior intestine; pi: Posterior intestine; gc: Goblet cells; bb: Brush border; SIV: Supranuclear Inclusion Vesicle; NSV: Non-Staining Vacuoles
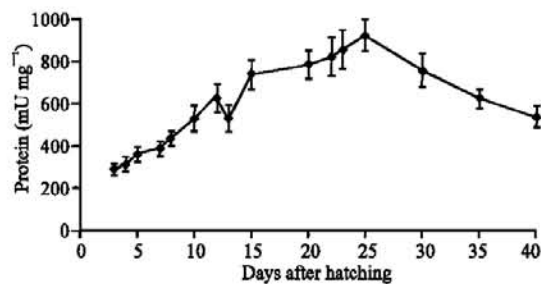

Fig. 4: Specific activity in mU mg^{-1} of alkaline protease in *D. putazzo* larvae, until 40 DAH. Results are expressed as means±SD (n = 5)
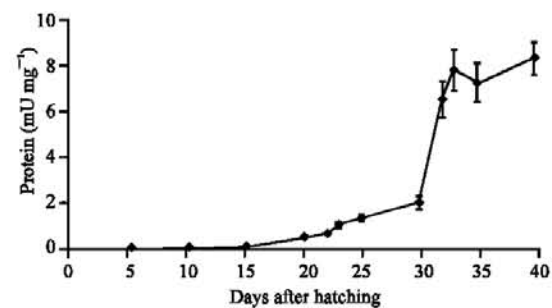

Fig. 5: Specific activity in U mg^{-1} of acid protease in *D. putazzo* larvae, until 40 DAH. Results are expressed as means±SD (n = 5)

than present study (21.7%). Besides, Franicevic (1989) was recorded larval survival ranged between 18 and 22% until day 60. As described to Sarasquete *et al.* (1995), the appearance of gastric glands indicated the developed functional stomach and this case is an indictor role of the transition from larval to juvenile stage. Although, gastric glands of *D. puntazzo* apperared in 28 DAH in this study, its occured different times according to their growing

features for example, on 36 DAH in yellowtail flounder (Baglole *et al.*, 1997), on 60 DAH in gilthead sea bream (Elbal *et al.*, 2004), on 15 DAH in yellowtail kingfish (Chen *et al.*, 2006), on 17 DAH in common dentex (Santamaria *et al.*, 2004), on 24-31 DAH in common pandora (Micale *et al.*, 2006). In other study, Micale *et al.* (2008) was reported that gastric glands appeared at 30 DAH in *D. puntazzo*. This result parallel with the data that acidic protease activity strongly increased after 32 DAH.

Gastric glands were observed prior to other Sparids species (Micale *et al.*, 2008), such as *D. sargus* (Ortiz-Delgado *et al.*, 2003), *D. dentex* (Santamaría *et al.*, 2004) and *P. pagrus* (Darias *et al.*, 2005), but considerably later in the gilthead seabream *S. aurata* (Elbal *et al.*, 2004). Similar findings were determined in the study. Also, Ortiz-Delgado *et al.* (2003) reported that, the location of gastric glands is species-specific and has been related to feeding habits. In *D. puntazzo*, gastric glands occurred only in the fundic region of the stomach (Micale *et al.*, 2008), as was also informed in the yellowtail flounder *Pleuronectes ferruginea* (Baglole *et al.*, 1997), the summer flounder *Paralichthys dentatus* (Bisbal and Bengtson, 1995) and the turbot *Scophthalmus maximus* (Segner *et al.*, 1994). On the other hand, gastric glands were determined in the cardiac region in *D. sargus* (Ortiz-Delgado *et al.*, 2003), shi drum and red porgy *P. Pagrus* (Darias *et al.*, 2005).

Exogenous feeding was onset with rotifers and microalgae after 3 DAH. At the time, the formation of digestive sytems and accessory glands of *D. puntazzo* showed similar evolutions as described to other Sparids (Micale *et al.*, 2006, 2008; Santamaria *et al.*, 2004; Sarasquete *et al.*, 1995). In this time, morphological and enzymatic materials such as hepatocytes, enterocytes and pancreatic cells exists in larvae on 3 DAH, PAS-positive brush borders detected into the intestine epithelial. *D. puntazzo* larvae are able to digest carbohydrates at 3 DAH and then at 5-6 DAH first lipid and protein absorbtion was discernible in vacuolar texture of anterior and posterior intestine. It was suggested that activity of some pancreatic enzymes, i.e., protease, amylase could be defined according to other researchers (Caruso *et al.*, 2001; Micale *et al.*, 2006; Suzer *et al.*, 2006, 2007).

The first goblet cells in the digestive tract of sharpsnout seabream were visible in the pharynx and oesophagus after the first exogenous feeding, the buccal goblet cells appearing thereafter, while goblet cells in the intestine appeared finally at 33 DAH (Micale *et al.*, 2008). In contrast to this, goblet cells were firstly detected at 28 DAH in this study. Additionally, in common pandora

(Micale *et al.*, 2006) and red porgy (Darias *et al.*, 2005), intestinal goblet cells has been reported later stages of larval growth. Also, the mucosal bubstance in goblet cells altered with the digestive region or development phase in *D. puntazzo* ontogeny (Micale *et al.*, 2008). In 5-6 DAH, nutrients absorbtion was detected like supranuclear-infranuclear unstained vacuoles and supranuclear stained vacuoles in the anterior and posterior intestine with the onset of exogenous feding, respectively as described in Micale *et al.* (2008) and Ortiz-Delgado *et al.* (2003). In addition, as clearly described by Sanchez-Amaya *et al.* (2007), digestive and absorptive processes continued developing with the appearance of the gut mucosa folds, acidophilic supranuclear inclusions in the enterocytes of the posterior intestine and lipid infranuclear vesicles in the anterior and middle intestine enterocytes between 5-15 DAH in *P. auriga* larvae.

The digestive physiology of many marine fish larvae undergoes numerous morphological and functional changes during ontogeny that can substantially influence larval survival under culture conditions. Therefore, the assessment of the presence and level of activity of digestive enzymes may be used as a comparative indicator of the rate of development of the fish larvae, food acceptance, digestive capacity, as well as of their further survival rate (Ueberschär, 1993). Moreover, the digestion of nutrients occurs in the gastrointestinal tract and is performed by the enzymes of the stomach, exocrine pancreas and intestine, but also includes the absorption/transport of nutrient by the intestinal cells (Zambonino-Infante and Cahu, 2001). The alkaline protease activity of *D. puntazzo* was much lower than that determined in sea bream, but when all the protease activity (acid+alkaline) was considered, the total activity was greatly increased, exceeding values determined in sea bream larvae, which do not possess acid activity at that age. Since, some recent experiments have demonstrated the possibility of rearing sea bream larvae on artificial diets (Yúfera *et al.*, 1999). It is reported that abiotoical factors are strongly affected the enzymatic acitivity in fish. For instance, acidic pH levels were dramatically decreased digestive protease, chymotrypsin, activity in the *D. puntazzo* larvae (Aktulun *et al.*, 2008). Additionally, different illumination levels influenced tryptic and chymotryptic activity in common pandora (*Pagellus erythrinus*) larvae (Suzer *et al.*, 2006). However, Moutou *et al.* (2004) reported that in sea bream juveniles, chymotrypsin and the total activity of alkaline proteases in the intestine were lower in fish exposed to the low salinity conditions. Nevertheless, chymotrypsin appeared to contribute considerably to the total alkaline activity

irrespective of salinity since, its activity was significantly and positively correlated with total alkaline activity at different salinities in sea bream juveniles. Major mortality observed at the beginning of the metamorphosis (25 DAH) related with organogenesis and at the beginning of the weaning (35 DAH) due to refusing of compound diet with live food. It is thought that this morphological event occurred at the same time for most of larvae in this experiment because a sharp increase in acidic protease activity was measured between 32 and 40 DAH. This phenomenon suggested that weaning of *D. puntazzo* larvae could be started synchronously with formation of functional stomach (32 DAH), but we have no information that how the survival rate was affected due to beginning of this treatment on this date. Additionally, as described by Zambonino Infante and Cahu (2001) acidic protease activity is mostly found in the stomach; this activity is due to the activation of pepsinogen in pepsin. Also, histological analysis evidenced that fully gastric glands and stomach formation was observed on day 32 in this species. After this date, digestion of compound microdiet by larvae could be effectively increased and also reflected relatively higher acidic protease activity and relatively better growth.

CONCLUSION

It is concluded that these observations are strongly important for evaluating of the digestive system development and for adjustment of acceptable rearing protocol of *D. puntazzo*. Especially, for the commercial production of this new candidate species, we introduced that transition time to artificial feeding was established related with previous digestive enzymes studies.

ACKNOWLEDGEMENTS

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