



Stage-Specific Ontogeny of Digestive Enzymes in the Cultured Common Dentex (*Dentex dentex*) Larvae

Cüneyt Suzer^{1*}, Deniz Çoban², Şükrü Yildirim¹, Müge Hekimoğlu¹, H. Okan Kamacı¹, Kürşat Fırat¹, Şahin Saka¹

¹ Ege University, Faculty of Fisheries, Aquaculture Department, 35100, Bornova, Izmir, Turkey.

² Adnan Menderes University, Faculty of Agriculture, Fisheries and Aquaculture Engineering Department, 09100, Aydin, Turkey.

* Corresponding Author: Tel.: +90.232 3115214; Fax: +90.232 3883685;
E-mail: cuneyt.suzer@ege.edu.tr

Received 17 January 2014
Accepted 20 August 2014

Abstract

Stage-specific ontogeny of main digestive enzymes in common dentex, *Dentex dentex*, were assayed during early life development according to notochord flexion studied from hatching to 45 days after hatching (DAH). The notochord was straight in yolk-sac larvae of *D. dentex* and preflexion stage was observed between 0 and 18 DAH as 2.75 and 6.27 mm at total length (TL). Then, flexion of the notochord was estimated between 19 and 30 DAH as 6.27 and 10.9 mm at TL. Important alterations in specific activities of all digestive enzymes measured during period of this study were mostly related with metamorphosis and co-feeding. Trypsin and chymotrypsin specific activities were firstly detected on day 3 and were related with mouth opening, whereas they slightly increased until 25 DAH respectively. Pepsin was firstly detected on day 28 and related with stomach formation and a sharp increase was observed until 30 DAH. Both amylase and lipase were measured on day 2 and 4, respectively and they also sharply increased during the first week of life. Additionally, except pepsin, most of the digestive enzyme activities were firstly secreted in the preflexion stage and also gastric secretion started and functional development digestive system were occurred in in flexion and postflexion stages, respectively.

Keywords: Digestive enzymes, stage-specific ontogeny, larval development, *Dentex dentex*, common dentex.

Kültürü Yapılan Sinarit (*Dentex dentex*) Larvalarında Sindirim Enzimlerinin Dönemsel Gelişimi

Özet

Bu çalışmada, sinarit (*Dentex dentex*) larvalarında açılımdan 45. güne kadar geçen erken dönem boyunca önemli sindirim enzimlerinin dönemsel ontogenileri notokorda bükülmesine göre incelenmiştir. Besin keseli sinarit larvalarında notokorda düz yapıda gözlenmiş ve bükülme öncesi dönem 0 ile 18. günler arasında 2,75 ile 6,27 mm total boy uzunlukları arasında izlenmiştir. Ardından, notokordanın bükülmesi ise 19 ile 30. günler arasında 6,27 ile 10,9 mm total boy uzunlukları arasında tespit edilmiştir. Çalışma boyunca izlenen sindirim enzimleri aktivitelerindeki önemli değişimlerin çoğunlukla metamorfoz ve toz yeme geçiş süreci ile ilişkili olduğu bulunmuştur. Tripsin ve kimotripsin spesifik aktiviteleri larvalarda ağız açılımı ile birlikte ilk olarak 3. günde tespit edilmiş ve 25. güne kadar yavaş bir artış göstermiştir. Pepsin aktivitesi ise mide oluşumu ile eş zamanlı olarak ilk kez 28. günde gözlenmiş ve 30. güne kadar ani bir artış sergilemiştir. Amilaz ve lipaz aktivitesi ise sırasıyla 2. ve 4. günlerde izlenmiş ve sonrasında larval dönemin ilk haftası boyunca hızlı şekilde yükselmiştir. Ayrıca, pepsin dışındaki tüm sindirim enzimleri aktivitesi ilk olarak bükülme öncesi dönemde izlenmiş ve gastrik salgının başlangıcı ve fonksiyonel sindirim sisteminin oluşması sırasıyla bükülme ve bükülme sonrası dönemde tespit edilmiştir.

Anahtar Kelimeler: Sindirim enzimleri, dönemsel ontogeni, larval gelişim, *Dentex dentex*, sinarit.

Introduction

Over the last decade, Mediterranean aquaculture saturation for farmed European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) resulted in the diversification of the marine fish production by promising new candidate species. Among new possible species, common

dentex, *Dentex dentex*, has become one of the most attractive candidates for diversification of aquaculture in Turkey.

On the other hand, some bottlenecks and handicaps have been still remaining for solution during the larval rearing of this species. These obstacles could be listed as nutritional disorders, skeletal deformities, cannibalism, external

manipulations, and diseases (Rueda and Martínez, 2001; Koumoundouros et al., 2004; Bermejo-Nogales et al., 2007). Therefore, more specific studies are needed to overcome and clarify some nutritional and culture requirements for the propagation of this species by higher survival rate under hatchery conditions.

During the last two decades, numerous studies have shown that early ontogeny of the digestive enzymes could be evaluated as effective tool for determination of larval quality, performance and nutritional status and physiological response for preferred diet during both larval and weaning stage under culture conditions (Ribeiro et al., 1999; Lazo et al., 2000; Cara et al., 2003; Suzer et al., 2006b; Suzer et al., 2007a,b; Ribeiro et al., 2008; Gisbert et al., 2009; Suzer et al., 2013). Moreover, there are some studies related with *D. dentex* focused on larval development (Rueda and Martínez, 2001), histological ontogeny of the digestive tract (Santamaria et al., 2004), early life history (Firat et al., 2005), and different culture techniques (Suzer et al., 2006a) of *D. dentex* however, some detailed investigations about digestive enzymes were conducted by SDS-PAGE (Alarcón et al., 1998) and spectrophotometric (Gisbert et al., 2009) methods and also effects of different dietary formulations (Pérez-Jiménez et al., 2009) in this species under culture conditions. Despite of these studies, stage-specific ontogenic development of main digestive enzymes according to notochord flexion in this species has not been examined till now.

It is well defined that the flexion of the notochord was synchronously developed by caudal complex and subsequently development of locomotion, feeding and shape of the whole body of larvae. After these critical changes, development of the vertebral column were formed during the early life development (Koumoundouros et al., 1999a,b; Russo et al., 2007). In addition to these, the caudal fin is often the first fin to show signs of differentiation when the urostyle, the final segment of the vertebral column, turns upward. The term “flexion” is frequently used to refer to this stage of development (Koumoundouros et al., 1999b, 2001; Russo et al., 2007). These osteological developments are usually occurred concurrently with ontogenesis of digestive system. Especially, pancreas, stomach and intestine are differentiated and also became more functional during the flexion and postflexion stage. (Koumoundouros et al., 1999b, 2001).

Therefore, the main goal of this study was to evaluate firstly the specific and total activities main pancreatic and gastric enzyme in common dentex larvae fed on live prey and compound microparticulate diet (MD) during the early developmental stages according to notochord flexion studied from hatching to 45 DAH under culture conditions.

Materials and Methods

Larval Rearing and Feeding

Larval rearing was conducted in a recirculated sea water system by using green water technique included *Nannochloropsis* sp., *Tetraselmis* sp., and *Isochrysis* sp. at a density of 150.000–200.000 cells.ml⁻¹. Larval rearing protocol and feeding regime was applied according to the culture procedures defined by Firat et al. (2005). Water temperature was gradually increased between 17.0 and 23.0°C depend on larval age and size. During larviculture, oxygen, salinity and pH were maintained at 6.6 mg L⁻¹, at natural sea water salinity (38.3‰) and 7.7, respectively. Ammonia and nitrite were kept constant always below 0.01 mg L⁻¹. Illumination was maintained between 30 and 100 lx and also. photoperiod was set on a 24 h light cycle daily until end of algal addition (18 DAH) and then 16-h light and 8-h dark until end of the experiment.

The feeding regime for larvae of *D. dentex* is summarised in Figure1. Yolk-sac reserves were nearly depleted during the first two days and also mouth opening was observed at 3 DAH. Exogenous feeding was started by two strains rotifers (*Brachionus plicatilis* but mainly with *Brachionus rotundiformis*) enriched with algae and enrichment media (DHA Protein Selco, Artemia Systems SA, Ghent, Belgium) and lasted until 18 DAH. In similar, *Artemia* (nauplii: 4–7 ind.ml⁻¹ between 12 and 25 DAH and metanauplii: 2–4 ind.ml⁻¹ from 25 to 45 DAH) and enriched with Protein Selco (Artemia Systems SA, Ghent, Belgium). Extruded compound MD (55% protein and 15% lipid content, Caviar, BernAqua, Belgium) was administered 5 times a day at 8–10% of biomass between 30 and 45 DAH. Larval feeding regime was summarized in Figure 1.

Sampling

Larviculture of *D. dentex* was carried out in triplicate. For measurements of growth (total length and weight), larvae were sampled by groups of larvae from each experimental tank 5 days interval (50 larvae sample group⁻¹). Specific growth rate (SGR) was calculated by the formula $SGR = 100 (\ln FBW - \ln IBW) / \Delta t$, with IBW, FBW: initial, final body weight of fish (mg), Δt : time interval (day). Larval stages were classified according to flexional ontogeny of the notochord (Russo et al., 2007; Çoban et al., 2009). At the end of the experiment, larval survival was determined by recording of daily mortality and counting of larvae remaining in the experimental tanks. Pooled samples of 100 larvae until 10 DAH, 75 larvae until 15 DAH and 50 larvae until 40 DAH were collected for enzymatic analysis. Whole body homogenates were used for enzymatic assays and samples were taken at the same hour, before food distribution.

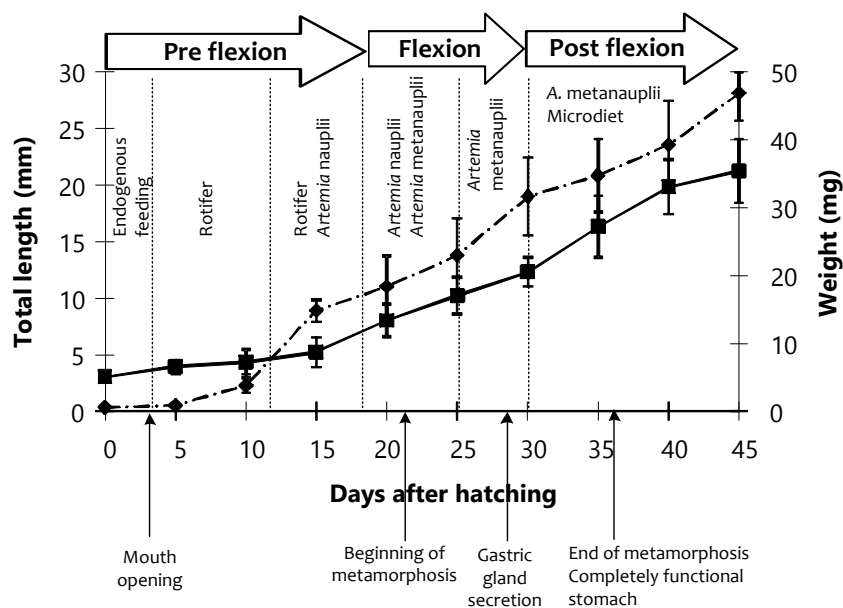


Figure 1. Stage-specific growth of *D. dentex* larvae: total length (solid line) and weight (dashed line). Each mean \pm SD represents a pool of 50 larvae. The diet types at different stages are indicated between the dotted lines. Major events taking place during the anatomical differentiation of digestive organs in larvae of this species reared (Santamaria *et al.*, 2004) are included as a reference (arrows).

Analytical Procedure

Samples were collected and homogenized in 5 volumes v/w 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 xg; 30 min at 4°C) and stored -20°C for enzymatic analysis. Both trypsin and chymotrypsin activity was measured at 25°C using N α -Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and Benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate by the method of Tseng *et al.* (1982) and Worthington (1982), respectively. Pepsin was analyzed by a modification of the method of Worthington (1982). In the test tubes, 100 ml of homogenate were incubated at 37°C with 500 ml of haemoglobin substrate 2% w/v haemoglobin in 0.06 HCl. The reaction was stopped after 10 min using 1 ml of trichloroacetic acid TCA 5%. In the blank tubes, the homogenate was added only after TCA. The precipitates were centrifuged (6 min at 4000 g) and the optical density of the test tube supernatant was read at 280 nm against the blank. Amylase activity was measured at 37°C using starch as the substrate by the method of Métais and Bieth (1968). Lipase activity was assayed with β -naphthyl caprylate as substrate by using the method of Mckellar and Cholette (1986) as modified by Versaw *et al.* (1989). One unit of lipase activity was defined as 1 mg of β -naphthol released per minute. Enzymatic activities were expressed as specific activities (mU/mg protein⁻¹) and total activity (mU/larva). Protein was determined by the Bradford method (Bradford, 1976).

Statistical Analysis

All measurements were conducted in triplicate and also data (except enzymatic) are presented as mean \pm SD. In order to determine of the variance homogeneity of the data Levene's test was used. Additionally, survival data and enzymatic activity data were compared by Fisher's chi-square and one-way ANOVA, respectively, followed by Newman-Keul's multiple range tests. The significance level was adjusted at P<0.05 level. Statistical analyses were performed by SPSS 15.0 software.

Results

Growth and Survival

The notochord was straight in the yolk-sac and early larval stages of *D. dentex* and also preflexion stage lasted between 0 and 18 DAH as 2.75 and 6.27 mm at TL. The mouth opening was observed at 3 DAH (3.52 \pm 0.26 mm total length) concurrently with starting of exogenous feeding. Yolk-sac and oil globule were fully absorbed at day 7 and 10, respectively. The flexion of the notochord was determined as 6.27 and 10.9 mm at TL (19 and 30 DAH). Development of the caudal complex was closely related to the flexion of the notochord. Fully ossification of caudal fin complex was measured between 31 and 45 DAH as 10.9 and 23.58 mm at TL. Growth rate of *D. dentex* larvae during the period of study is described in Figure 1. Also, larval growth increased more than of 80 times in weight from 5 DAH to 45 DAH (R²=0.97). The mean specific growth rate amounted to 7.28 \pm 1.2% d⁻¹ (min 6.8, max

9.3% d⁻¹) and survival rate was calculated as 16.2±4.7%.

Enzymatic Activity

Preflexion Stage (2.75 and 6.27 mm)

Both specific and total activities of trypsin and chymotrypsin were measured as early as hatching and they exponentially increased during this stage ($P<0.05$). Additionally, specific activity of amylase sharply increased during the first ten days ($P<0.05$) however, total activity was slower until 8 DAH ($P<0.05$). Then, while specific activity of amylase slowly decreased, total activity fluctuated until end of the preflexion stage ($P<0.05$). Both specific and total activity of lipase sharply increased, except 15 DAH for specific activity, until end of this stage ($P<0.05$). Pepsin activity was not detected at this stage.

Flexion Stage (6.27 and 10.9 mm)

During this stage, specific and total activities of digestive alkaline proteases, trypsin and chymotrypsin, demonstrated opposite developmental pattern. Specific activity of trypsin and chymotrypsin slowly decreased and fluctuated ($P<0.05$) however, total activity of these enzymes increased gradually to the end of flexion stage ($P<0.05$). In contrast to these, specific and total activities of amylase and lipase presented similar developmental pattern. In terms of lipase, both specific and total activities slightly increased to 25 DAH ($P>0.05$), and then they sharply decreased until end of this stage ($P<0.05$). Pepsin demonstrated two distinct phases during this stage. No activity was measured to until 28 DAH while pepsin was firstly detected at 28 DAH and also both specific and total activity of pepsin sharply increased until end of this stage ($P<0.05$).

Postflexion Stage (10.9 and 23.58 mm)

During the postflexion stage, both specific and total activities of trypsin and chymotrypsin showed in similar pattern characterized by a slowly decline until end of this study ($P<0.05$). Specific and total activity of lipase presented similar patterns during this stage while slight increase was measured to 35 DAH ($P<0.05$) and then sharply decreased ($P<0.05$). Additionally, there were opposite profile for developmental pattern of amylase. Specific activity of amylase declined while total activity slowly increased and then fluctuated ($P<0.05$). For pepsin, both specific and total activities were fluctuated, and then slightly increased to the end of this stage ($P<0.05$). (Figures 2, 3).

Discussion

During the past two decades, it is strongly

pointed out in some studies that digestive enzyme activities (e.g. trypsin, pepsin) could be accepted as comparative deterministic tool as well as a predictor not only for description of nutritional capabilities but also quality indicators of marine fish larvae during early ontogeny (Ribeiro *et al.*, 1999; Lazo *et al.*, 2000; Cara *et al.*, 2003; Suzer *et al.*, 2006b, 2007a,b, 2013; Ribeiro *et al.*, 2008; Gisbert *et al.*, 2009). Besides, knowledge about larval nutritional status and also ontogenetical changes of digestive enzymes in larvae of new candidate species will be supplied the development of processes of food digestion and assimilation. These information will be useful for establishment of feeding strategies of larvae for utilization of given diet (live food/compound diet) (Ueberschär, 1995; Martínez *et al.*, 1999; Zambonino Infante and Cahu, 2001; Zambonino Infante *et al.*, 2008). In present study, we distinctly investigated the developmental pattern for digestive enzymes (proteases, amylase, and lipase) mainly depending on flexional ontogeny in larvae of *D. dentex*.

Growth of *D. dentex* larvae was exponentially and somewhat faster than reported in previous studies on this species. Firat *et al.* (2005) recorded a total body length of approximately 12.1 mm after 30 DAH for *D. dentex* larvae. We reported here 12.32±1.3 mm at day 30. Also, Gisbert *et al.* (2001) reported 22.1 mm of standart body length after 50 DAH but in this study total length was measured as 21.24±2.8 mm on day 45. The slightly faster growth in the present study might be caused by somewhat higher temperatures and different larval feeding regime than in previous studies. On the other hand, as well reported by different authors, the end of the notochord flexion was characterized by the antero-posterior orientation of the caudal rays (Koumoundouros *et al.*, 1999b, 2001). It is determined that notochord flexion was completed on day 30 in common dentex larvae, this change was accomplished at 20 DAH in thick-lipped grey mullet *Chelon labrosus* in mesocosm conditions (Ben Khemis *et al.*, 2013). In addition, as stated by Koumoundouros, the size of notochord flexion is not only species-specific formation but also related with culture conditions and the authors estimated that caudal fins of common dentex and red sea bream developed relatively faster than the caudal fin of gilthead sea bream. Besides, they noted the starting of flexion stage at 5.0–5.5 mm and observed in 50% of the larvae at 5.5–6.0 mm, and also, the development of the caudal complex was observed concurrently with the hypuralia formation, which was closely related to the notochord flexion in *D. dentex* larvae (Koumoundouros *et al.*, 2001). Moreover, similar morphological changes were noted about 6.6±1.0 mm TL (20 DAH) in *Chelon labrosus* (Ben Khemis *et al.*, 2013) and 7.1±0.6 mm TL in *S. aurata* (Russo *et al.*, 2007). In this study, it was recorded that the similar findings about caudal fin development with respect to notochord flexion of common dentex larvae.

The digestive physiology of many marine fish

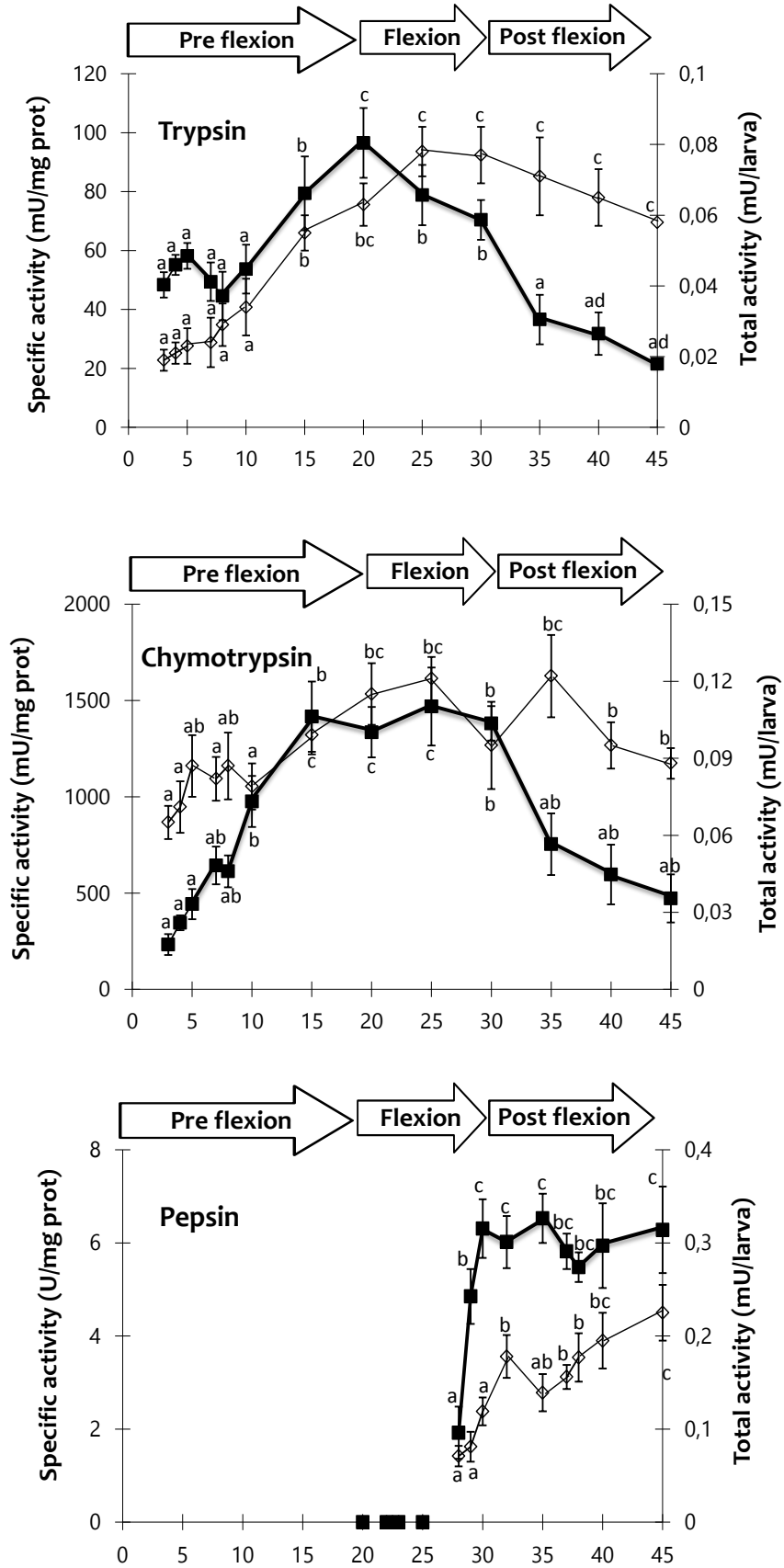


Figure 2. Specific activity in mU/mg (black square) and total activity in mU/larva (clear square) of proteases in *D. dentex* larvae until 45 DAH. Results are expressed as means±SE (n=3). Different superscripts indicate significant differences between means.

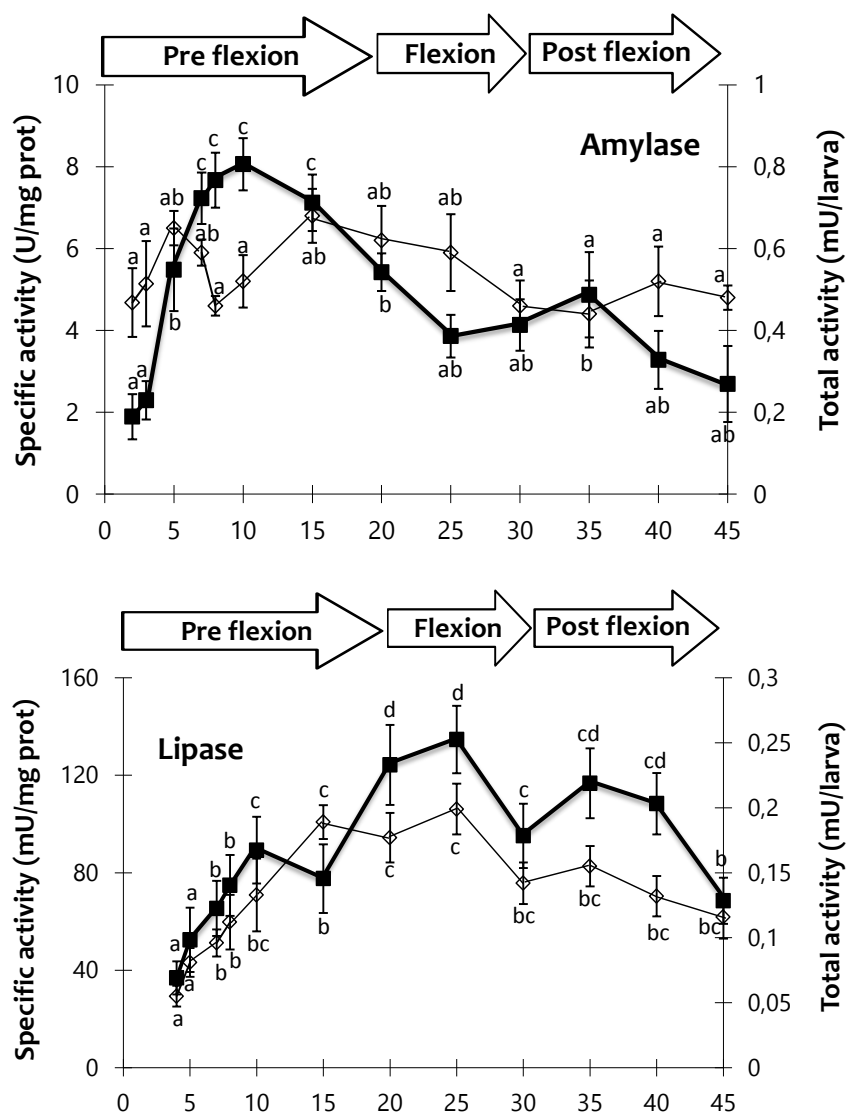


Figure 3. The specific activity in mU/mg (black square) and total activity in mU/larva (clear square) of amylase and lipase in *D. dentex* larvae until 45 DAH. Results are expressed as means \pm SE (n=3). Different superscripts indicate significant differences between means.

larvae undergoes numerous morphological and functional changes during early ontogeny that could substantially effect larval survival under culture conditions. Moreover, alkaline proteases, mainly trypsin and chymotrypsin and combination with intestinal cytosolic peptidase, play major role with for digestion process for protein during early ontogeny. It is well noted that some pancreatic enzymes such as trypsin could be detected before mouth opening and exponentially increased by exogenous feeding, larval age and size coinciding with secretion of zymogen granules (Zambonino Infante and Cahu, 2001; Zambonino Infante et al., 2008). In current study, trypsin was firstly detected as early as hatching (at 3.52 \pm 0.26 mm total length) and sharply increased from 3 DAH related to exogenous feeding in preflexion stage as observed for mostly cultivated Sparids. Also, Gisbert et al. (2009) recorded similar finding as first detection concurrently with hatching

for trypsin in the same species (Gisbert et al., 2009). For instance for the other cultured species, similar detection and developmental pattern was recorded in *S. aurata* at 3 DAH (Moyano et al., 1996), *P. auriga* at 3 DAH (Moyano et al., 2005), *P. pagrus* at 3 DAH (Suzer et al., 2007a), *P. erythrinus* at 3 DAH (Suzer et al., 2006a), *D. puntazzo* at 3 DAH (Suzer et al., 2007b) and meagre *Argyrosomus regius* at 3 DAH (Suzer et al., 2013).

On the other hand, as reported for both *D. dentex* and other cultured Sparids such as *S. aurata*, *D. sargus*, *D. puntazzo*, and *P. erythrinus*, trypsin specific activity was relatively higher during early stages and then sharply and/or slightly decline was observed about after 20 DAH correlated with alteration of feeding regime, development of gastric glands before acidic digestion in stomach (Moyano et al., 1996; Cara et al., 2003; Suzer et al., 2006, 2007a,b; Gisbert et al., 2009). These findings were

similar with herring larvae, *Clupea harengus*, (Pedersen and Andersen, 1992) and tilapia, *Oreochromis niloticus*, larvae (Drossou et al., 2006) which tryptic activity presented increased in early stages and then relatively lower profile under culture conditions.

It is well known that chymotrypsin is an endopeptidase selectively hydrolyzes peptide bonds on the carboxyl side of the aromatic side chains of tyrosine, tryptophan and phenylalanine and also it is usually activated by trypsin (Zambonino Infante and Cahu, 2001; Zambonino Infante et al., 2008). Like trypsin, it is presented similar profile during early developmental stage as well as the observed increase in specific activity and contributes to digestion of protein in larvae by partially compensating for the absence of acid proteases before the formation of a functional stomach (Applebaum et al., 2001; Zambonino Infante and Cahu, 2001). As observed for trypsin, chymotrypsin was first detected in preflexion stage on day 3 (concurrently with mouth opening) and its specific activity strongly correlated with tryptic activity of *D. dentex* larvae. Besides, chymotrypsin was first detected concurrently with trypsin at the day of mouth opening in the cultured marine fish *S. aurata* after 3 DAH (Moyano et al., 1996), *P. erythrinus* after 3 DAH (Suzer et al., 2006b), *P. pagrus* after 3 DAH (Suzer et al., 2007a), *S. senegalensis* after 2 DAH (Martinez et al., 1999), *S. ocellatus* after 3 DAH (Lazo et al., 2000), and *D. labrax* after 5 DAH (Zambonino Infante and Cahu, 1994). However, similarly to trypsin, chymotryptic activity exponentially increased by larval size and age during the first month and then gradually decreased until end of the study. This is the typical alkaline protease, trypsin and chymotrypsin, developmental profile during the early ontogeny for cultured Sparid larvae. Similar ontogenetic patterns were recorded for *D. dentex* (Gisbert et al., 2009) and also *P. erythrinus* (Suzer et al., 2006b), *P. pagrus* (Suzer et al., 2007a) and *D. puntazzo* (Suzer et al., 2007b) larvae under culture conditions.

The considerable rate (50%<) of mortality occurred at the beginning of the metamorphosis (20 DAH) related with organogenesis and also at the beginning of the co-feeding period (30 DAH) due to possibility of refusing of microdiet with live food. It is well evaluated that gastric secretion (pepsin and/or pepsin like activity) commonly accepted transition to acidic digestion from alkaline process, and also clear evident of formation of functional stomach. This phenomenon might indicate that co-feeding period of *D. dentex* larvae could be started synchronously with gastric secretion and functional stomach (28 DAH) in flexion stage but we have no information. Besides, Gisbert et al. (2009) reported that pepsin activity was not detected until 19 DAH and started to rise up after this day and also the total activity increased with larval age and reached to maximum value between 35 and 50 DAH in common dentex larvae (Gisbert et al.,

2009). The difference on starting of gastric secretion between these two studies might be related with relatively higher culture temperature (between 19 and 23°C) than our study (between 17 and 23°C) and different larval feeding regime and also this situation could be accelerated the organogenesis of digestive system. Additionally, it is recorded that histological analysis evidenced that primordial stomach and presence of first gastric glands was observed in flexion stage at 22 DAH and completed its definitive histological organization between 23 and 36 DAH in this species (Santamaría Rojas et al., 2004). Obtained results from our study supported this phenomenon which pepsin activity was firstly detected at 28 DAH and the exponentially increased until 35 DAH. Moreover, in other marine cultured fish, formation of functional stomach and pepsin activity was firstly assayed 15 DAH in *A. regius* (Suzer et al., 2013), 24 DAH in *D. labrax* (Zambonino Infante and Cahu, 1994), 25 DAH in *P. erythrinus* (Suzer et al., 2006b), 28 DAH in *P. pagrus* (Suzer et al., 2007a), 30 DAH in *P. auriga* (Moyano et al., 2005), 32 DAH in *D. puntazzo* (Suzer et al., 2007b), and 40 DAH in *S. aurata* (Moyano et al., 1996).

It is reported that amylase activity usually detected as early as hatching and also progressively increased by larval age during the first week life of marine fish larvae. After this date, it gradually decreased and fluctuated related with metamorphosis and ontogenic stages. This early species-specific developmental profile for amylase activity could be better explained as a result of programmed gene expression (Moyano et al., 1996; Zambonino Infante and Cahu, 2001; Zambonino Infante et al., 2008). This phenomenon is clearly stated that specific activity of amylase could be presented higher profile during larval stages and usually declines until starting of juvenile stage which is considered an indicator of pancreatic maturation in marine fish. Besides, shifting and biochemical composition of offered food and weaning could trigger amylase activity which is stimulated by glycolytic chains, glycogen, and starch content of food (Zambonino Infante and Cahu, 2001; Zambonino Infante et al., 2008; Rønnestad et al., 2013). Additionally, it is reported that carbohydrate content of live food (6-10%) and compound diet (<12%) used for feeding larvae which might have differentially stimulated the synthesis and secretion of amylase during early ontogeny of fish larvae (Ma et al., 2005; Suzer et al., 2006b, 2007a,b; Gisbert et al., 2009). Therefore, in current study, two developmental peaks were measured for amylase activity. Firstly, there was an initial sudden increase in preflexion stage until 10 DAH which might have been genetically programmed. Secondly, a sharp increase was measured at 35 DAH in postflexion stage, coinciding with extruded artificial diet introduction at 30 DAH. This may be connected with the starch content of about <12% of this diet. This phenomenon is clearly stated that specific activity of amylase could

be presented higher profile during larval stages and usually declines until starting of juvenile stage which is considered an indicator of pancreatic maturation in marine fish. Additionally, it could be related with high dietary level of glycogen in the live food which is stimulating the synthesis and secretion of this enzyme (Ma et al., 2005). Also, similar profile for amylase was recorded by Gisbert et al. (2009) that they reported similarly two peak steps which have been measured firstly around 2 DAH concurrently with hatching and starting of exogenous feeding and also secondly after 25 DAH for this species (Gisbert et al., 2009). Additionally, these information were parallel with results obtained from *D. labrax* (Zambonino Infante and Cahu, 1994), *S. ocellatus* (Lazo et al., 2000), large yellow croaker, *Pseudosciaena crocea*, (Ma et al., 2005), and *P. erythrinus* (Suzer et al., 2006b) larvae. Additionally, Pérez-Jiménez et al. estimated that dietary carbohydrate content seems to induce changes in protease, amylase and lipase activity even though the authors conducted feeding experiments in juvenile of *Dentex dentex* at 91.7 ± 1.4 g mean mass (Pérez-Jiménez et al., 2009).

It is well estimated that lipase catalyses the breakdown of emulsified esters of triacylglycerol first to diacylglycerol then to monoacylglycerol and also long chain fatty acids. Besides, lipolytic activities play a major role for adequate feeding for larvae during early life stages, since types of lipids including enrichment of live food by different media i.e. selco and also compound microdiet is comparative and deterministic marker for better utilization of lipids from these food sources (Zambonino Infante and Cahu, 2001; Kolkovski, 2001; Cahu and Zambonino Infante, 2001; Gisbert et al., 2009). In this study, two different developmental patterns were observed for lipase, which was firstly detected on day 4 and progressively increased by larval age and size to 20 DAH. After then, second increase was measured concurrently with artificial diet supplementation from 30 DAH. A quite similar developmental profile for lipolytic activity were reported in different cultured species such as, *Solea senegalensis* (Martínez et al., 1999), *S. ocellatus* (Lazo et al., 2000), *D. sargus* (Cara et al., 2003), *P. erythrinus* (Suzer et al., 2006b), and yellowtail kingfish, *Seriola lalandi* (Chen et al., 2006) larvae. Similar enzymatic profile in lipase activities were found in larvae of cultured species such as *Solea senegalensis* (Martínez et al., 1999), *S. ocellatus* (Lazo et al., 2000), *D. sargus* (Cara et al., 2003), *P. erythrinus* (Suzer et al., 2006b), *S. lalandi* (Chen et al., 2006), *P. pagrus* (Suzer et al., 2007a), and *D. puntazzo* (Suzer et al., 2007b). In spite of Gisbert et al. (2009) reported that lipase was detected at hatching and progressively increased with larval growth of *D. dentex* larvae, a contrast profile were noted after co-feeding period 30 DAH related with administration of microdiet. It is thought that these differences could be correlated with both dietary

concentration and characteristics (biochemical structure such as chain type) of offered food, nutritional requirements and culture conditions. Similar findings were recorded in *D. dentex* by Tibaldi et al. (1996) and Cardenete et al. (1997) that higher protein levels in diets is better utilized by juveniles than those with lower protein and higher lipid content.

It could be concluded that obtained results from this study complement by addition of notochord flexion to previous detailed information on ontogenic development of digestive enzymes in *D. dentex* during early ontogeny (Gisbert et al., 2009). Also the expressions of the digestive enzyme activities in this species followed the quite similar developmental profile generally determined for other cultured Sparid larvae examined to date. Both histological observations (Santamaría et al., 2004) and enzymatic analysis presented that most of the digestive organs and accessory glands in digestive tract are being incipiently formatted in the preflexion stage and also development, completion and transition to digestive system are occurred in the flexion and postflexion stages. Moreover, it could be determined that the expressions of digestive enzymes in *D. dentex* larvae indicate early functional development of this system and suggest that this species is a good candidate for Mediterranean aquaculture and also presented relatively faster growth than the other Sparid. However, further studies should be focused on establishment of early weaning protocols, development of adequate larval food effective growth from the first feeding a multidisciplinary approach for this species.

Acknowledgments

The authors wish to thank the staff of the Kılıç Sea Products Inc., Oren, Milas, Muğla, TURKEY where the experiments were conducted for their most efficient technical assistance.

References

- Alarcón, F.J., Díaz, M., Moyano, F.J. and Abellán, E. 1998. Characterization and functional properties of digestive proteases in two sparids; gilthead seabream (*Sparus aurata*) and common dentex (*Dentex dentex*). Fish Physiology and Biochemistry, 19: 257–267. doi: 10.1023/A:1007717708491
- Applebaum, S.L., Perez, R., Lazo, J.P. and Holt, G.J. 2001. Characterization of chymotrypsin activity during early ontogeny of larval red drum (*Sciaenops ocellatus*). Fish Physiology and Biochemistry, 25: 291–300. doi: 10.1016/j.aquaculture.2007.01.043
- Bauchot M.L. and Hureau J.C. 1986. Sparidae. In: P.J.P. Whitehead, M.L. Bauchot, J.C. Hureau, J. Nielsen, E. Tortonese (Eds.), Fishes of the North-Eastern Atlantic and Mediterranean. UNESCO, Paris: 883–907.
- Ben Khemis, I., Gisbert, E., Alcaraz, C., Zouiten, D., Besbes, R., Zouiten, A., Masmoudi, A.S. and Cahu, C. 2013. Allometric growth patterns and development in

- larvae and juveniles of thick-lipped grey mullet *Chelon labrosus* reared in mesocosm conditions. *Aquaculture Research*, 44: 1872–1888. doi: 10.1111/j.1365-2109.2012.03192.x
- Bermejo-Nogales, A., Saera-Vila, A., Calduch-Giner, J.A., Navarro, J.C., Sitjà-Bobadilla, A. and Pérez-Sánchez, J. 2007. Differential metabolic and gene expression profile of juvenile common dentex (*Dentex dentex* L.) and gilthead sea bream (*Sparus aurata* L.) in relation to redox homeostasis. *Aquaculture*, 267: 213–224. doi:10.1016/j.aquaculture.2007.01.024
- Bradford, M.M. 1976. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72: 248–254.
- Cahu C.L. and Zambonino Infante J.L. 2001. Substitution of live food by formulated diets in marine fish larvae. *Aquaculture*, 200: 161–180. doi: 10.1016/S0044-8486(01)00699-8
- Cara, J.B., Moyano, F.J., Cardenas, S., Fernandez-Diaz, C. and Yufera, M. 2003. Assessment of digestive enzyme activities during larval development of white bream. *Journal of Fish Biology*, 63: 48–58. doi: 10.1046/j.1095-8649.2003.00120.x
- Cardenete, G., Abellán, E., Hidalgo, M.C., Skalli, A. and Arizcun, M. 1997. Variation in the dietary level of protein and lipid. Effect on growth and nutritive utilization of the diet by the dentex (*Dentex dentex*). *Actas VI Congreso Nacional de Acuicultura*, Cartagena.
- Chen, B.N., Qin, J.G., Kumar, S.M., Hutchinson, W.G. and Clarke, S.M. 2006. Ontogenetic development of digestive enzymes in yellowtail kingfish *Seriola lalandi* larvae. *Aquaculture*, 260: 264–271. doi: 10.1016/j.aquaculture.2006.06.021
- Çoban, D., Suzer, C., Kamacı, H.O., Saka, Ş. and Firat, K. 2009. Early osteological development of the fins in the hatchery-reared red porgy, *Pagrus pagrus* (L. 1758). *Journal of Applied Ichthyology*, 25: 26–32. doi: 10.1111/j.1439-0426.2008.01165.x
- Drossou, A., Ueberschär, B., Rosenthal, H. and Herzig, K. 2006. Ontogenetic development of the proteolytic digestion activities in larvae of *Oreochromis niloticus* fed with different diets. *Aquaculture*, 256: 479–488. doi: 10.1016/j.aquaculture.2006.01.038
- Firat, K., Saka, Ş. and Çoban, D. 2005. Early life history of cultured common dentex (*Dentex dentex* L. 1758). *Turkish Journal of Veterinary and Animal Sciences*, 29: 735–741.
- Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y. and Estevéz, A. 2009. Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. *Aquaculture*, 287: 381–387. doi: 10.1016/j.aquaculture.2008.10.039
- Kolkovski S. 2001. Digestive enzymes in fish larvae and juveniles—implications and application to formulated diets. *Aquaculture*, 200: 181–201. doi: 10.1016/S0044-8486(01)00700-1
- Koumoundouros, G., Divanach, P. and Kentouri, M. 1999a. Ontogeny and allometric plasticity of *Dentex dentex* (Osteichthyes, Sparidae) in rearing conditions. *Marine Biology*, 135: 561–572. doi: 10.1007/s002270050657
- Koumoundouros, G., Divanach, P. and Kentouri, M. 1999b. Osteological development of the vertebral column and of the caudal complex in *Dentex dentex*. *Journal of Fish Biology*, 54: 424–436. doi: 10.1111/j.1095-8649.1999.tb00841.x
- Koumoundouros, G., Divanach, P. and Kentouri, M. 2001. Osteological development of *Dentex dentex* (Osteichthyes: Sparidae): dorsal, anal, paired fins and squamation. *Marine Biology*, 138: 399–406. doi: 10.1007/s002270000460
- Koumoundouros, G., Carrillo, J., Divanach, P. and Kentouri, M. 2004. The rearing of common dentex *Dentex dentex* (L.) During the hatchery and on-growing phases. *Aquaculture*, 240: 165–173. doi: 10.1016/j.aquaculture.2004.01.038
- Kurokawa, T. and Suzuki, T. 1996. Formation of the diffuse pancreas and the development of digestive enzyme synthesis in larvae of the Japanese flounder *Paralichthys olivaceus*. *Aquaculture*, 141: 267–276. doi: 10.1016/0044-8486(95)01237-0
- Lazo, J.P., Holt G.J. and Arnold, C.R. 2000. Ontogeny of pancreatic enzymes in larval red drum *Sciaenops ocellatus*. *Aquaculture Nutrition*, 6: 183–192. doi: 10.1046/j.1365-2095.2000.00124.x
- Ma, H., Cahu, C., Zambonino Infante, J.L., Yu, H., Duan, Q., Le Gall, M.-M. and Mai, K. 2005. Activities of selected digestive enzymes during larval development of large yellow croaker (*Pseudosciaena crocea*). *Aquaculture*, 245: 239–248. doi: 10.1016/j.aquaculture.2004.11.032
- Martinez I., Moyano F.J., Fernández-Diaz C. and Yufera, M. 1999. Digestive enzyme activity during larval development of the Senegal sole (*Solea senegalensis*). *Fish Physiol. Biochem.*, 21: 317–323. doi: 10.1023/A:1007802708459
- Mckellar, R.C. and Cholette, H. 1986. Determination of the extracellular lipases of *Pseudomonas fluorescens* spp. in skim milk with the beta-naphthyl caprylate assay. *J. Dairy Res.*, 53, 301.
- Métais, P. and Bieth, J. 1968. Détermination de l'α-amylase par une microtechnique. *Ann. Biol. Clin.*, 26, 133–142.
- Morales-Nin, B. and Moranta, J. 1997. Life history and fishery of the common dentex (*Dentex dentex*) in Mallorca (Balearic Islands in Western Mediterranean). *Fisheries Research*, 30: 67–76. doi: 10.1016/S0165-7836(96)00560-7
- Moyano F. J., Diaz M., Alarcon F.J. and Sarasquete, M.C. 1996. Characterisation of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*). *Fish Physiol. Biochem.*, 15: 121–130. doi: 10.1007/BF01875591
- Moyano F.J., Barros A.M., Prieto A., Cañavate, J.P. and Cárdenas S. 2005. Evaluación de la ontogenia de enzimas digestivas en larvas de hurta, *Pagrus auriga* (Pisces: Sparidae). *Revista AquaTIC*, 22: 39–47.
- Pedersen, B.H. and Andersen, K.P. 1992. Induction of trypsinogen secretion in herring larvae (*Clupea harengus*). *Marine Biology*, 112: 559–565. doi: 10.1007/BF00346173
- Péres A., Zambonino Infante J.L. and Cahu, C.L. 1998. Dietary regulation of activities and mRNA levels of trypsin and amylase in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiology and Biochemistry*, 19: 145–152. doi: 10.1023/A:1007775501340
- Pérez-Jiménez, A., Cardenete, G., Morales, A.E., García-Alcázar, A., Abellán, E. and Hidalgo, C.M. 2009. Digestive enzymatic profile of *Dentex dentex* and response to different dietary formulations. *Comparative Biochemistry and Physiology*, 154A: 157–164. doi: 10.1016/j.cbpa.2009.05.126
- Ribeiro, L., Zambonino-Infante, J.L., Cahu, C. and Dinis,

- M.T. 1999. Development of digestive enzymes in larvae of *Solea senegalensis*, Kaup 1858. *Aquaculture*, 179: 465–473. doi: 10.1016/S0044-8486(99)00180-5
- Ribeiro, L., Couto, A., Olmedo, M., Álvarez-Blázquez, B., Linares, F. and Valente, L.M.P. 2008. Digestive enzyme activity at different developmental stages of blackspot seabream, *Pagellus bogaraveo* (Brunnich 1768). *Aquacult. Res.*, 39: 339–346. doi: 10.1111/j.1365-2109.2007.01684.x
- Rueda, F.M. and Martínez, F.J. 2001. A review on the biology and potential aquaculture of *Dentex dentex*. *Reviews in Fish Biology and Fisheries*, 11: 57–70. doi: 10.1023/A:1014276700138
- Russo, T., Costa, C. and Cataudella, S. 2007. Correspondence between shape and feeding habit changes throughout ontogeny of gilthead sea bream *Sparus aurata* L., 1758. *Journal of Fish Biology*, 71: 629–656. doi: 10.1111/j.1095-8649.2007.01528.x
- Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø. and Boglione, C. 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge and gaps and bottlenecks in research. *Reviews in Aquaculture*, 5: 559–598. doi: 10.1111/raq.12010
- Santamaría, C.A., Marin de Mateo, M., Traveset, R., Sala, R., Grau, A., Pastor, E., Sarasquete, C. and Crespo, S. 2004. Larval organogenesis in common dentex *Dentex dentex* L. (Sparidae): histological and histochemical aspects. *Aquaculture*, 237: 207–228. doi: 10.1016/j.aquaculture.2004.03.020
- Suzer, C., Çoban, D., Kamacı, H.O., Saka, Ş. and Fırat, K. 2006a. Culture techniques used in larviculture of common dentex (*Dentex dentex*, L.). *Ege University, Journal of Fisheries and Aquatic Sciences*, 23: 31–35.
- Suzer, C., Fırat, K. and Saka, Ş. 2006b. Ontogenic development of the digestive enzymes in common pandora, *Pagellus erythrinus*, L. larvae. *Aquacult. Res.*, 37: 1565–1571. doi: 10.1111/j.1365-2109.2006.01598.x
- Suzer, C., Kamacı, H.O., Çoban, D., Saka, Ş., Fırat, K., Ozkara, B. and Ozkara, A. 2007a. Digestive enzyme activity of the red porgy (*Pagrus pagrus*, L.) during larval development under culture conditions. *Aquacult. Res.*, 38: 1178–1785. doi: 10.1111/j.1365-2109.2007.01841.x
- Suzer, C., Aktülün, S., Çoban, D., Kamacı, H.O., Saka, Ş., Fırat, K. and Albaz, A. 2007b. Digestive enzyme activities in sharpnose seabream (*Diplodus puntazzo*) larvae. *Comp. Biochem. Physiol.*, 148(A): 470–477. doi: 10.1016/j.cbpa.2007.06.418
- Suzer, C., Kamacı, H.O., Çoban, D., Yıldırım, Ş., Fırat, K. and Saka, Ş. 2013. Functional changes in digestive enzyme activities of meagre (*Argyrosomus regius*; Asso, 1801) during early ontogeny. *Fish Physiol. Biochem.*, 39: 967–977. doi: 10.1007/s10695-012-9755-5.
- Tibaldi, E., Beraldo, P., Volpelli, L.A. and Pinosa, M. 1996. Growth response of juvenile dentex (*Dentex dentex* L.) to varying protein level and protein to lipid ratio in practical diets. *Aquaculture*, 139: 91–99. doi: 10.1016/0044-8486(95)01150-1
- Tseng, H.C., Grendell, J.H. and Rothman, S.S. 1982. Food, deodenal extracts, and enzyme secretion by the pancreas. *Am. J. Physiol.*, 243: G304–G312.
- Ueberschär, B. 1995. The use of tryptic enzyme activity measurement as a nutritional condition index: laboratory calibration data and field application. *ICES Mar. Sci. Symp.*, 201: 119–129.
- Versaw, W.K., Cuppett, S.L., Winters, D.D. and Williams L.E. 1989. An improved colorimetric assay for bacterial lipase in nonfat dry milk. *J. Food Sci.*, 54: 1557–1558.
- Walford, J. and Lam, T.J. 1993. Development of the digestive tract and proteolytic enzyme activity in seabass (*Lates calcarifer*) larvae and juveniles. *Aquaculture*, 109: 187–205. doi: 10.1016/0044-8486(93)90215-K
- Worthington, T.M. 1982. Enzymes and related biochemicals. Biochemical Products Division. Worthington Diagnostic System Freehold, NJ.
- Zambonino Infante, J.L. and Cahu, C.L. 1994. Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiol. Biochem.*, 12: 399–408. doi: 10.1007/BF00004304
- Zambonino Infante, J.L. and Cahu, C.L. 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. *Comp. Biochem. Physiol.*, 130(C): 477–487. doi: 10.1016/S1532-0456(01)00274-5
- Zambonino Infante, J.L., Gisbert, E., Sarasquete, C., Navarro, I., Gutiérrez, J. and Cahu, C.L. 2009. Ontogeny and physiology of the digestive system of marine fish larvae. In: J.E.P. Cyrino, D. Bureau and B.G. Kapoor (Eds.), *Feeding and Digestive Functions of Fish*. Science Publishers, Inc., Enfield, USA: 277–344.