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Characterization of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*)

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Abstract

The evolution of the digestive enzyme equipment in seabream from hatching to 30 days old larvae was studied; there was a progressive increase in the activity of protease, amylase and acid and alkaline phosphatase from day 15 onwards. The use of specific inhibitors, and SDS-PAGE provided evidence to suggest that most of the proteases belonged to the serine group. A high α-amylase activity was also denoted. Zymograms of larval extracts indicated that exogenous food has more a qualitative than a quantitative role in the secretion of digestive enzymes in this species.

Introduction

The successful mass rearing of fish larvae has a large economic importance in marine fish aquaculture. Therefore, a great part of current research in fish nutrition and feeding is devoted to the development of artificial diets for larvae of the more common marine species. It is clear that those studies must be based on a detailed knowledge of the digestive physiology of the early life stages of such fish. Different aspects, such as the temporary evolution of the digestive enzyme equipment, as well as a characterization of the rate of activity and location of enzymes, mut be known in order to determine the ability of larvae to utilize a given diet. Moreover, some proteolytic enzymes have been proposed as indicators of the nutritional condition of fish larvae, even in field investigations (Ueberschär 1993). A number of studies have been developed in some marine fish, such as the sea bass (*Dicentras labrax*), sole (*Solea solea*) and turbot (*Scophthalmus maximus*) (Lauff and Hofer 1984; Baragi and Lovell 1986; Cousin *et al.* 1987; Munilla-Morán *et al.*. 1990; Zambonino and Cahu 1994), but to date, with the exception of some data provided by Kolkowski *et al.* (1993) and Sarasquete *et al.* (1993) there is little information available about the digestive enzymes of gilthead seabream, (*Sparus aurata*), a very important species in Mediterranean aquaculture.

In this paper, we study the development of some digestive enzymes in larval stages of seabream, using biochemical and histochemical techniques in order to obtain a more complete perspective. Two different aspects were considered in the study: the quantification of different enzyme activities (proteases, amylase, phosphatases) during larval development, and a more specific characterization of protease activity.

Materials and methods

Fish

Seabream eggs were obtained from broodstock held at 19.5°C at the facilities of the I.C.M.A. (Instituto Ciencias Marinas Andalucía) in Cádiz (Spain); the broodstock was fed on minced cuttlefish. Eggs were incubated at the same temperature. After hatching, larvae were transfered to 300 l cylindro-conical tanks provided with a continous flow of seawater at 19°C and 33 g l^{-1} salinity. Constant illumination and slight aeration were pro-

vided and 25% of the seawater volume was replaced daily from day 4 on. Larval density was maintained at 60–100 larvae per l. Under these conditions, most of the larvae begin their exogenous feeding around day 4 after hatching, taking rotifers, *Brachionus plicatilis* (S-type and L-type). *Artemia* nauplii were added from day 15 (concentrations were adjusted to 10 rotifers per ml and 1 nauplius per ml). This feeding schedule ensured maximum survival rates which were 90% and 40% for larvae of 15 and 30 day, respectively. Larvae utilized in the starvation experiment were not fed and they died 9 or 10 days after hatching (day 9 is commonly identified as no return point in larval rearing of this species). At different intervals from 0 to 30 days, samples of fed larvae belonging to two different spawns and ranging from 40 to 250 individuals (in the case of lower sizes) were taken, pooled, washed and freeze-dried until analysis. Normal growth was controlled by weighing individually a subsample of 20–30 larvae belonging to each sample point. Only those larvae reaching the average size determined in such manner were utilized for preparation of extracts.

Analytical procedure

Preparation of samples

Samples of fish larvae were homogenized (35 mg ml^{-1}) in cold 50 mM Tris-HCl buffer, pH 7.5. The extracts obtained after centrifugation (12.500 × g; 30 min at 4°C) were filtered through a Sephadex G-25M column (1 × 10 cm) and then centrifuged (2000 × g, 2 min). The eluate was stored at –20°C and utilized for enzyme analysis. The concentration of soluble protein in pooled samples was determined by the method of Bradford (1976), using a microassay procedure. Bovine serum albumine (1 mg ml^{-1}) was used as standard.

Enzyme assays

Protease: Total protease activity was measured using the casein method of Kunitz (1947) as modified by Walter (1984), using substrate casein (1%) in 50 mM Tris/HCl buffer, pH 9 or Universal Buffer (Stauffer 1989). The standard method was as follows: samples (50 µl) of enzyme preparations were mixed with 0.5 ml of buffered sustrate at 25°C. Following incubation for 60 min the reaction was stopped by the addition of 0.5 ml 12% trichloroacetic acid (TCA), and after holding for 1h at 4°C, the reaction mixture was centrifuged in Eppendorf tubes for 5 min at 6500 × g. The supernatant was separated and the absorbance at 280 nm was recorded. Pure tyrosine was utilized as the standard and one unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 µg of tyrosine per min. Activity was calculated from the extinction coefficient for tyrosine = 0.008 ml μg^{-1} cm^{-1}. All measurements were performed in triplicate.

Two different approaches were used for the characterization of protease activity; a measure of changes of total protease activity in relation to pH variation at different stages of larval development, and the combination of specific inhibitors and electrophoresis. Variations of protease activity with pH were determined following the previously described method using Universal Buffer ranging from 3 to 11 in 0, 3, 5 and 18 days old larvae. Inhibition of protease activity was explored using soybean trypsin inhibitor (SBTI). Final inhibitor concentration were 12 µM in the buffer. SDS-PAGE of the protein fraction of extracts was carried out according to Laemmli (1970), using 12% acrylamide and 8 × 10 × 0.15 cm gels. Electrophoresis was performed at constant current of 15 mA per gel for 150 min at 5°C. Zymograms of proteinase activity of fractions separated by electrophoresis were done according to García-Garreño *et al.* (1993). After electrophoresis, the gels were washed and incubated in 50 mM TRIS/HCl buffer, pH 8.2, containing 2% casein for 30 min at 5°C, and then the temperature was raised to 30°C for 90 min without agitation. The gels were washed and stained with 0.1% Coomassie brillant blue (BBC R-250) in methanol-acetic acid-water solution (40:10:40) for 3h. Destaining was done with the same solution without BBC. The amount of protein utilized in samples is indicated in the figures.

The characterization of proteinases in SDS-PAGE zymograms were performed according to García-Garreño and Haard (1993). Enzyme preparations (40 µl) were mixed with 10 µl of different inhibitor solutions: TPCK (tosyl-phenylanine chloromethyl ketone); 10 mM in methanol, ZPCK (N-CBZ -L- phenylanine chloromethyl ketone); 10 mM in dioxane, TLCK (tosyl-lysine chloromethyl ketone); 10 mM in HCl 1 mM, PMSF (phenyl-

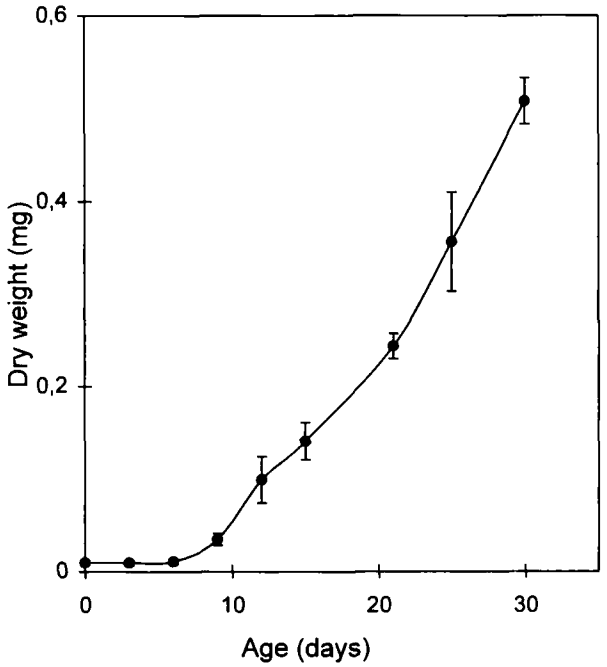

Fig. 1. Growth of larvae used in the experiments along 30 days of rearing. Each point is the mean of 20–30 individuals ± SD.

methylsulfonyl fluoride); 100 mM in 2-propanol and SBTI (soybean trypsin inhibitor); 250 mM in water. Pure trypsin and chymotrypsin, were used as controls. After incubation for 1h at 25°C, samples were mixed with buffer and loaded on SDS-PAGE plates. Electrophoresis and zymograms were done as previously. After electrophoresis, the excess inhibitor was washed in 50 mM TRIS/HCl buffer, pH 8.2, for 15 min at room temperature. Molecular weight (MW) of proteinases was measured using log MW vs migration plot and they were given in kDa.

Amylase: α-Amylase activity was quantified by incubating 0.1 ml of extract with 1% (w/w) soluble starch (0.25 ml) and pH 7.0 buffer (0.5 ml). After 1h of incubation, the reducing sugars were estimated using the Somogy-Nelson procedure described by Robyt and Whelan (1968). Maltose was used as standard. One unit is defined as the amount of enzyme able to produce one μg of maltose per min.

Acid and alkaline phosphatases: The phosphatase activity was essayed by incubating 0.1 ml extract with 2% (w/w) 4-nitrophenyl phosphate (0.1 ml) in acid citrate buffer (pH 4.8) or alkaline diethanolamine buffer (pH 9.8). After 30 min, 0.05N NaOH (10 ml) was added and absorbance at 405 nm recorded. One unit is defined as the amount of enzyme able to produce an increase of 0.01 units of absorbance.

Results

Evolution and basal levels of enzyme activities during larval development

Figure 1 shows the growth of seabream larvae, expressed in dry weight, during the period of study, and Figure 2A–B show changes in the specific enzyme activities of larvae, from hatching until an age of 30 days. Similar patterns were obtained for all the enzymes, showing a sharp peak in 9 d old larvae and a generalized decrease of activity in older larvae. Using the data of larvae masses, activities were expressed in mU per larva and the general trend was an increase of activity with age (Fig. 2C–D). Thus, it was possible to directly correlate dry weight of larvae to any of the enzyme activities, *e.g.*, alkaline protease (r^2=0.949; $p<.05$). Values of activity for protease, amylase, acid phosphatase and alkaline phosphatase existing in 30 days old larvae, were 15, 10, 20 and 16 times higher, respectively, than those measuredin larvae at the moment of mouth opening. In starved larvae, levels of alkaline protease activity decreased with age (from hatching to 9 days). No differences in specific activity were detected, but the protease equipment, expressed in mU per larva, represented only around 1 15% of that measured in fed larvae (Table 1). In the case of α-amylase the activity was also very low, being a 10% of the value determined in fed larvae.

SDS-PAGE revealed the existence of protease activity from day 4 onwards. A complete set of bands corresponding to the different proteases existing in 30 days old larvae was already detected at an age of 6 days. Increased activity in older larvae was manifested by progressively thicker bands (Fig. 3a). The appearance of additional bands in day 6 larvae were due to a higher concentrations of extract applied to the gel.

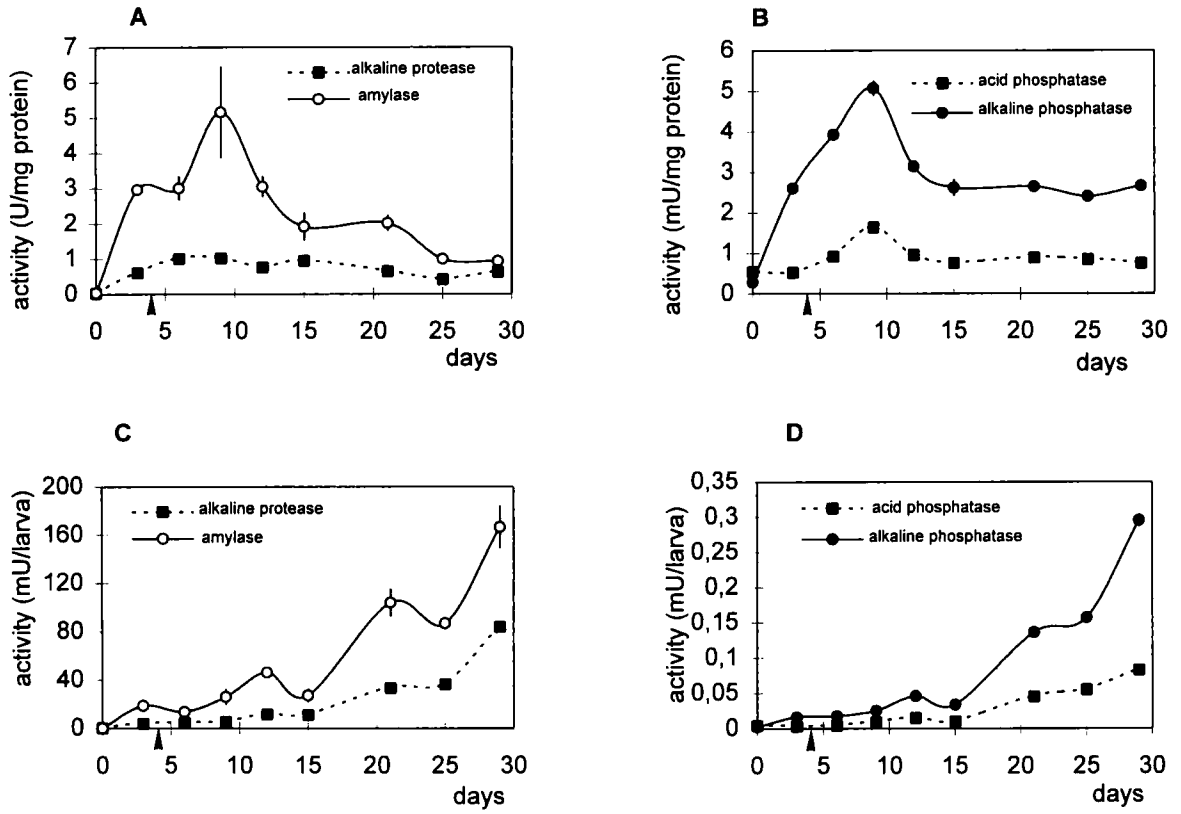

Fig. 2. Digestive enzyme activities in larval seabream along development expressed in U mg^{-1} soluble protein (A–B) or mU per larva (C–D). Each point represents the mean of triplicate measures. Bars representing SD are included only if necessary. Probable day of mouth opening is indicated by an arrow.

Characterization of the protease activity

Protease activity, measured under different pH conditions in larvae of 0, 3, 6 and 19 days, is shown in Figure 4. In newly-hatched larvae, 2 peaks of maximum activity could be detected at pH 6.0 and 9.0. In 3 d old larvae, the total activity decreased and peaks were slightly displaced to pH 7.0 and 10.0. In 6 d old larvae, protease activity was located at the alkaline zone, showing two peaks at pH 8.0 and 10.0. Finally, in 18 d old larvae, maximum protease activity was clearly established at pH 10.0.

Incubation of extracts with SBTI prior to the biochemical essay of protease activity allowed us to determine the relative amount of serine type proteases in the digestive equipment of the larvae. The proportion of such proteases remained almost constant from 0 to 30 days, accounting from 75 to 85% of total activity. The use of SBTI in samples of starved larvae did not show significant differences when compared to the results obtained in fed larvae.

SDS-PAGE separation applied to extracts of larvae of different ages revealed the progressive appearance of a total of 8 protease active bands, with molecular weights ranged from 21.5 to 95 kDa (Fig. 3a). The more significant bands corresponds to proteins of 40, 32 and 48 kDa. Characterization was completed by incubation of extracts prior to SDS-PAGE with specific inhibitors against trypsin-like (TLCK), chymotrypsin-like (TPCQ and ZPCK) and serine proteases (SBTI and PMSF) (Fig. 3b). Comparison of zymograms obtained with non-treated larval extracts allowed us to identify bands of 95, 52, 33.5, 32 y 23.5 kD as trypsin-like proteases, whereas bands of 40 and 21.5 kDa were classified as serine proteases and bands of 43.5 and 28 kDa corresponded to serine proteases with partial chymotrypsin-like activity (Table 2).

Table 1. Values of protease and amylase activities (mean of triplicates ± SD) in fed and starved larvae of the same age

		3 days (*)	6 days		9 days	
			Fed	Starved	Fed	Starved
Protease	U mg^{-1}	0.593±0.040	1.001±0.006	0.989±0.222	0.914±0.103	0.638±0.189
	mU ind^{-1}	3.759±0.004	4.550±0.028	0.723±0.136	4.816±0.303	0.410±0.121
Amylase	U mg P^{-1}	2.960±0.068	3.006±0.337	5.530±0.477	5.155±1.290	4.400±0.063
	mU ind^{-1}	13.650±1.530	18.770±0.434	7.093±0.341	25.880±6.500	2.831±0.040

(*) mouth opening takes place around day 4

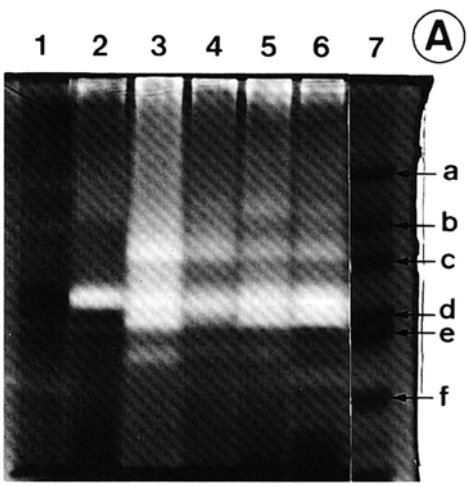

Fig. 3. A. – Substrate-SDS-PAGE of homogenates of *Sparus aurata* larvae at different ages. Gels were prepared and treated as described in Materials and Methods section. Molecular weight markers (MWM) used were: bovine serum albumin (66,000)(**a**), ovalbumin (45,000)(**b**), glyceraldehyde-3P-deshydrogenase (36,000)(**c**), carbonic anhydrase (29,000)(**d**), trypsinogen (24,000)(**e**) and soybean trypsin inhibitor (20,100)(**f**). Samples were diluted (1:1) in buffer without b-mercaptoethanol and not boiled. Column (C) 1=3 days (30 mg), C2=4 days (35 mg), C3=6 days (45 mg), C4=9 days (25 mg), C5=15 days (37 mg), C6=30 days old (40 mg) and C7=MWM (5 mg).

B. – Substrate-SDS-PAGE of a extract of 4 days old *Sparus aurata* larvae treated with different especific inhibitors. Gels, MWM and samples preparation is according to Materials and Methods and legend in Fig. 2a. Extracts were mixed with the inhibitors indicated in the column. Column (C) 1 and C10= MWM (5 mg), C2=TPCK, C3=ZPCK, C4=TLCK, C5=SBTI, C6=PMSF, C7=extract without inhibitors, C8=trypsin (2 mg) and C9=chymotrypsin (5 mg).

Discussion

The histology and histochemistry of the digestive tract, as well as alterations produced by starvation and the distribution pattern of some enzymes, have been recently studied in seabream larvae by Sarasquete *et al.* (1993) and Yúfera *et al.* (1993). When seabream, larvae hatch as in other species, the digestive tract after yolk resorption contains lipids, glycogen, proteins, lipoproteins as well as enzymes related to metabolism (digestion, absorption, transport) of such molecules (Tanaka 1972; Cousin *et al.* 1987; Segner *et al.* 1989; Sarasquete *et al.* 1993). In some species, the yolk syncytium contains non-specific esterases, but trypsin, aminopeptidases and alkaline phosphatases were not observed (Segner *et al.* 1989). In the digestive tract of the seabream, phosphatase, trypsin or ATPases activities were observed 3–4 days after hatching (Sarasquete *et al.* 1993). The aminopeptidases that enables the larvae a further catabolism of peptides obtained from trypsin digestion were already present in the yolk-sac during yolk resorption and were also observed from day 5–6 in the digestive tract. Biochemical assays performed in the present study confirmed those histochemical

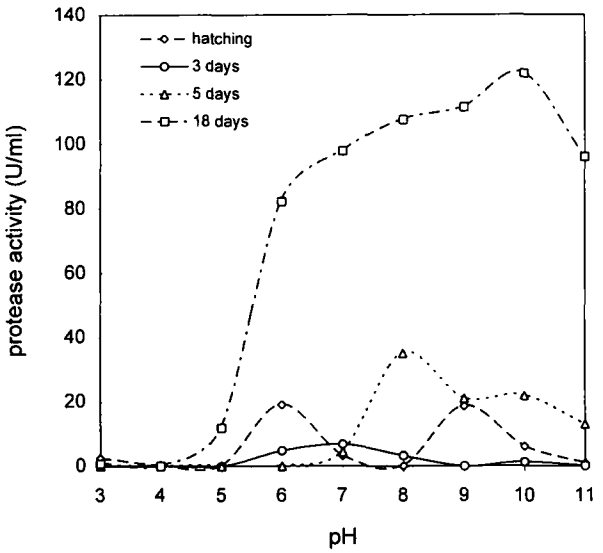

Fig. 4. Profiles of protease activity at different pH in larvae of 0, 3, 6 and 19 days old.

results since alkaline protease activity was detected early after hatching. The expression of such activity in relation to soluble protein showed no great variations during the first 30 days of life. Zymograms confirmed that some seabream alkaline proteases were already present in 4 days-old larvae and these progressively increased in number and type. In contrast, the activity measured in other species, such as turbot or sea bass, has been reported to decline markedly during the first days of development (Walford and Lam 1993; Zambonino and Cahu 1994). To explain this, these authors hypothesize either a denaturation of trypsin-like enzymes due to the early development of functional stomach (turbot) or a poor synthesis during a definite period (sea bass); this was also suggested by Pedersen *et al.* (1987) for herring (*Clupea harengus*) larvae. Nevertheless, a decrease in specific activity of proteases could be also explained in relation to an increased synthesis of enzymes and other soluble proteins during growth, thus causing the expression of proteolytic (and any other) activity in relation to soluble proteins to change progressively. When alkaline protease activity of seabream larvae is expressed in relation to individual, a progressive increase became apparent, which is in agreement with the expected improving in proteolytic equipment of a bigger fish. A similar correction has been recently introduced in other studies (Cahu and Zambonino 1994).

In relation to the characterization of proteases, the results obtained by measuring their optimal pH closely agreed to those reported in 3 d old turbot larvae (Munilla-Morán *et al.* (1990) or 24 d old sole (Clark *et al.* 1986). In the first case, a two peak profile was obtained with optimum points at pH 6.0 and 9.0 (7.0 and 10.0 in seabream). In the second case, a clear displacement to an optimum pH of 10.0, similar to that observed in seabream, was reported. The development of a functional stomach in seabream could be established from day 40 on, since a very low but detectable pepsin activity was measured at this age (data not included). The use of a specific inhibitor is an alternative approach to the utilization of specific substrates in the identification of proteases. Results obtained when assaying protease activity after incubation of extracts of gilthead seabream larave with SBTI, clearly showed the importance of serine proteases in the digestive equipment of such species; proteases represent about 80% of total proteases. The substrate inhibition assay revealed a more complete picture of the types of proteases; about 50% were trypsin-like proteases, 40% could be classified as serine proteases of several types and 10% were chymotrypsin-like. This result contrasts with those obtained in striped bass (*Morone saxatilis*) or sole, in which the specific of chymotrypsin represented 2 or 3 fold that of trypsin (Baragi and Lovell 1986; Clark *et al.* 1986), but is in agreement with results obtained is some freshwater fishes (Lauff and Hofer 1984), and also agrees with data of Ueberschär (1993). A high activity of trypsin in seabream larvae can be deduced from those data; nearly 250 mU mg protein^{-1}, is present in a 30 d old larva, whereas it is only about 40 mU mg protein^{-1} in sea bass larva of the same age. Consequently, considering both the increasing production of protease per individual and the fact that most of such protease activity is due to trypsin-like enzymes, the results obtained in seabream larvae are in agreement with those reported for striped bass (Baragi and Lovell 1987) or cod (Hjelmeland *et al.* 1984) and contrast with measures performed in sea bass (Walford and Lam 1993; Zambonino and Cahu 1994).

Taking into account both the major events in the development of different species of marine fish, as well as data provided by several authors cited in the text related to the first detection of protease activities, a comparative scheme was elaborated (Fig. 5). Data presented in this way allow us to

Table 2. Protein fractions with caseinolytic activity in a SDS-PAGE zymogram of gilthead seabream, their presumptive identification of class, and variation of this fractions and protease activity along larval development (MW = molecular weight)

MW	Inhibitor	Class of proteinases	Development days					
			3	4	6	9	15	30
95000	TLCK	Trypsin-like	–	+	+	+	+	+
52000	TLCK	Trypsin-like	–	–	+	+	+	+
43500	SBTI, TPCK	Serine, CHY-like	–	–	+	+	+	+
40000	SBTI, PMSF	Serine	–	–	+	+	+	+
33500	TLCK	Trypsin-like	–	+	+	+	+	+
32000	TLCK	Trypsin-like	–	+	+	+	+	+
28000	SBTI, TPCK	Serine, CHY-like	–	–	+	+	+	+
23500	TLCK	Trypsin-like	–	–	+	+	+	+
21500	SBTI, PMSF	Serine	–	–	–	–	–	+

Abbreviations: TLCK: tosyl-lysine chloromethylketone; SBTI: soybean trypsin inhibitor; TPCK: tosyl-phenylalanine chloromethyl-ketone; PMSF: phenyl-methylsulfonyl-fluoride

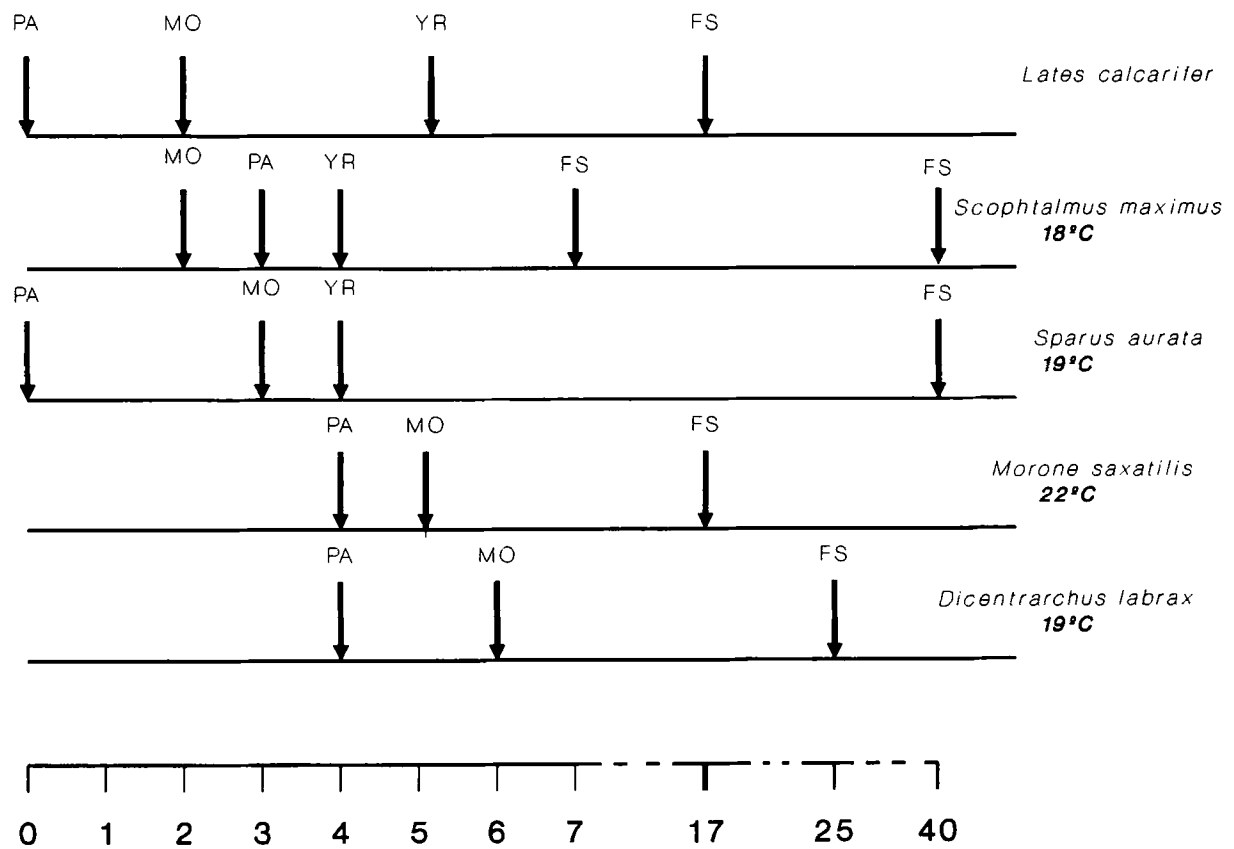

Fig. 5. Comparison of developmental stages in the digestive tract of several marine fish species. PA: protease activity; MO: mouth opening; YR: yolk resorption; FS: functional stomach (from different authors cited in the text).

evaluate the main differences that may determine the utilization of artificial food by the larvae. For example, in all cases (with the exception of turbot) alkaline protease activity was detected prior to mouth opening, which demonstrates that such phenomen are not promoted by food stimulation. On the other hand, although alkaline protease activity is detected very early in seabream, there is a longer period in this than in other species (again with the exception of turbot) between mouth open-

ing and the detection of pepsin activity, usually considered as indicator of a functional stomach. Nevertheless, in spite of the important role that pepsin plays in the course of protein digestion, a high alkaline protease activity could compensate for deficiencies during the first stages of development. This is supported by the fact that some fish species have no stomach at all, and is in agreement with the afore mentioned high activity of trypsin detected in seabream larvae. In this sense, investigations into the ability of seabream larvae to digest different proteins are currently in progress.

The activity of α-amylase was clearly detected from day 3 onwards. Early detection of such activity has been also reported for other marine fish larvae (Baragi and Lovell 1988; Cousin *et al.* 1987; Munilla-Morán *et al.* 1990) and in all cases, the activity increased with age. In the case of seabream, the high α-amylase activity measured during larval development was marked, especially considering the feeding habits of this species at this stage (predators on zooplankton). An important amylase equipment may be closely related to their ability to digest carbohydrates present in microalgae used as food for zooplankton. Although the activity of amylase increased proportionally less than that of the other enzymes measured (30 d old larvae had 10 times more amylase, but 20 times more protease than 6 d old larvae) other factors influencing its activity, such as nutrient utilization or digestibility, must be considered. This particular features should be taken into account when designing artificial diets for larval seabream; in fact, stimulation of amylase secretion has been observed in larvae fed on artificial diets containing significant amounts of starch (Cahu and Zambonino 1994).

According to Letelier *et al.* (1985), phosphatases are an important detoxifying system involved in nutritional and mineralization processes, phosphate transport and hydrolysis of phosphorylated proteins. The absence of pepsin in the mucosa of the digestive tract of seabream and other teleost fishes dring the early stages of life may be compensated by the micropinocytosis and intracellular digestion of proteins taking place in the posterior intestine. In these processes, acid phosphatases and cathepsin, as well as amino peptidases are involved (Stroband and Dabrowski 1979; Georgopoulou *et al.* 1985; Govoni *et al.* 1986; Sarasquete *et al.* 1993). The high activity of alkaline phosphatases in relation to acid phosphatases detected in seabream larvae point to a greater significance of the intestinal absorption of macromolecules in relation to pynocitic processes.

Several authors have studied the role of exogenous enzymes in larval digestion processes (Dabrowski and Glogowski 1977; Munilla-Morán *et al.* 1990). The latter authors established that exogenous enzymatic contribution to protease digestion in turbot larvae may represent about 43–60%, pointing to a great dependence of larval marine fish on exogenous enzymes for protein digestion. In the present study, two approaches were considered in order to evaluate the role and significance of exogenous contribution to the digestive equipment of larvae. Measurement of protease and amylase activities in starved larvae was used as a way of estimating basal levels of secretion that should allow us to calculate the exogenous contribution to the digestive equipment. However, the degeneration of secretory tissues in starved larvae of marine fish has been found histochemically to be due to decreases in proteases and peptidases (Cousin *et al.* 1987; Segner *et al.* 1989). In seabream, all enzymatic activities are decreased during starvation, as manifested by the absence of supranuclear vacuoles in the intestine (Yúfera *et al.* 1993) and the reduction in alkaline phosphatase in 8 d old larvae. Moreover, it must be taken into account that 9 d old starved larvae has not ingested amino acids to synthesize its own proteins, thus having a very poor enzymatic pool. Under there conditions measures of protease activity cannot be a good indicator of endogenous production and the results cannot be considered as indicative. On the contrary, SDS-PAGE may be an useful tool to reveal the presence in larval extracts of exogenous proteins, *e.g.*, protease. Characterization of rotifer proteases by Hara *et al.* (1984) revealed that alkaline proteases in this organism are extremely large (800 and 900 KD) when compared to those of mammals or marine animals. The use of SDS-PAGE to separate proteins of extracts of seabream larvae of different ages did not reveal bands of such molecular weight corresponding to the enzymes in the ingested rotifers (ingestion of rotifers was not directly observed but logically deduced from survival of fed larvae from day 9 on). In addition, other authors reported either the absence of alkaline proteases in the rotifer or the need of spe-

cial activators to develop their activity (Kleinow 1993). In the case of *Artemia* nauplii used to fed larvae from day 15 on, chymotrypsin is the more important alkaline protease existing in this organism (Bonnie *et al.* 1991), but the activity of such enzyme was not clearly detected biochemically or by electrophoresis in seabream larvae. Therefore, it seems that the intake of live food has a qualitative but not a quantitative effect in this species, more related to the stimulation of endogenous secretion of proteases than to a supply of non produced enzymes. A similar conclusion was made for seabass larvae (Zambonino and Cahu 1994) and the hypothesis is supported by the mechanisms of stimulation of trypsin secretion studied by Pedersen and Andersen (1992) in herring larvae. Those authors established that repletion of gut, even with an inert material exerts a major control over trypsinogen secretion, suggesting a neural mediated initiation of secretion. In this case, seabream larvae, which posses a well developed digestive enzyme equipment at early stages (Moyano and Sarasquete 1993) could be good candidates to be fed on microcapsules, once such artificial diets can be properly balanced. This fact is also supported by results reported by Corneille *et al.* (1989), who obtained good results feeding seabream larvae from day 25 onwards using a mixed diet including a 50% rotifer and a 50% artificial diet. More recently, Corneille and Delis (1992) bred successfully seabream larvae from day 15 onwards using a similar feeding schedule.

Acknowledgements

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