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Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae

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Abstract

Variations in some enzyme activities during larval development of sea bass fed live prey were investigated from hatching to day 40. Fluctuations in the enzyme specific activities (except for trypsin) occurred in three phases: initially a sharp increase until day 12, followed by a plateau and subsequently a decrease around day 23. Then activities remained constant until day 40. Trypsin activity kept rising until day 23, then fell. Enzymatic adaptation to a change in diet was studied by feeding larvae with microparticulate diet from day 25. Adaptation to dietary change was observed for amylase, alkaline phosphatase and leucine aminopeptidase, assayed in whole larvae. In larvae fed microparticulate dry diet, the activities of these three enzymes tended to be higher than in those fed natural prey. Although poor growth was observed in larvae fed microparticles, the brush border enzyme activities purified from whole body homogenate, were not impaired.

Introduction

In most fish larvae, organogenesis is not completely achieved by the time of hatching and continues during larval development. In particular, the anatomy of the digestive system of the larvae is quite different from that of juvenile fish (Cousin and Baudin-Laurencin 1985; Govoni *et al.* 1986; Ferraris *et al.* 1987; Beccaria *et al.* 1991; Boulhic *et al.* 1992). Data on morphological changes in the digestive tract and on enzymatic activities during larval development have been extensively reviewed by Smith (1988). However, most of the results were related to freshwater fish since marine fish larvae have been less extensively studied. Clark *et al.* (1986) reported very low specific activities of digestive enzymes in *Solea solea* larvae compared to the values obtained in juveniles. Low activities have often been considered to be reponsible for poor growth and survival in larvae fed formulated diets (Lauff and Hofer 1984; Munilla Moran *et al.* 1990).

While pancreatic enzyme activity in fish larvae has been investigated (Pedersen *et al.* 1987; Munilla Moran and Stark 1989; Walford and Lam 1993), studies on intestinal enzyme activities are scarce (Clark *et al.* 1986) and little is known about brush border enzymes of enterocytes. The importance of this phase of digestion (hydrolytic and absorptive mechanisms) has been clearly proved, particularly in mammals (Matthews and Adibi 1975; Kim *et al.* 1979; Koretz and Meyer 1980) but also in juvenile fish (Bogé *et al.* 1982; Ferraris and Ahearn 1984; Avella *et al.* 1992).

The aims of this work were to study the development of some digestive enzyme activities during the early larval life of sea bass, and to determine

Table 1. Larval feeding scheme

Day	LP group	MP group
	Diet per day	
0–6	no food	
6–10	50–200 Br/larva	
10–13	200 Br + 30–60 Na/larva	
14–16	30 Na + 60–120 Al/larva	
16–26	150–200 Al/larva	
25–40	200–250 Al/larva	2–3 g compound diet/tank

Br: *Brachionus*, Na: *Artemia nauplii*, Al: one day old *Artemia*

whether these enzyme activities were modulated in response to a change of diet. Experiments were conducted with sea bass larvae (*Dicentrarchus labrax*) fed live prey or compound diet as microparticles.

Materials and methods

Animals and diets

Eggs of European sea bass (*D. labrax*) were obtained from Ifremer-Station de Palavas. Larvae were reared at Ifremer-Station de Brest for 40 days and during this period, they were considered as larvae according Balon's (1975, 1984) criteria, since the morphogenesis was not achieved before day 40 (Barnabé *et al.* 1976). Newly-hatched larvae were transferred from incubators to 24 conical fiber glass tanks (35 l) with black walls (initial stocking density: 80 larvae/l). They were supplied with running sea water which had been filtered through a sand filter, then passed successively through a tungsten heater and a degassing column packed with plastic rings. The temperature range was 18–19°C, and the salinity 34.5 ppt. The oxygen level was maintained above 6 ppm by setting the water exchange rate at 30%/h (flow rate: 0.18 l/min); light intensity was 9 Wm^{-2} maximum at the surface.

The live prey feeding scheme is summarized in Table 1: days 6–13, rotifers enriched according to Gatesoupe and Luquet (1981); days 10–16, newly hatched *Artemia salina* (Brazil strain); from day 14, one day old *Artemia* produced according the method described by Robin (1989) and enriched in the same way as rotifers. Prey was offered to the fish twice a day. Rotifers were given in excess in order to compensate their washing out of the tank through a 180 µm filter.

On day 25, larvae were divided into two groups (12 tanks/group), one was fed live prey (LP diet), the other a compound balanced diet mainlly composed of fish meal and containing 12% of precooked starch (MP diet). This microparticulate diet was manufactured as expanded pellets, crumbled and sieved (120–200 µm). Microparticles were offered to the fish with a direct switch from *Artemia*. Fish were fed continuously in large excess 18 h/day using a belt feeder. Table 2 shows the composition of the diets given from day 25: one day old *Artemia* (LP) and microparticles (MP).

Table 2. Lipid, protein and amino acid composition of diets, one day old *Artemia* (LP) and microparticles (MP), expressed as g/100 g of dry matter

	LP	MP*
Lipids	21.3	10.2
N × 6.25	57.0	57.1
Amino acid		
Aspartic acid	4.2	5.4
Threonine	2.1	2.4
Serine	2.4	5.6
Glutamic acid	5.5	8.0
Proline	2.7	2.4
Glycine	2.2	3.4
Alanine	2.5	2.8
Cysteine	0.7	1.0
Valine	2.4	2.8
Methionine	1.2	1.4
Isoleucine	2.3	2.7
Leucine	3.1	3.9
Tyrosine	2.2	2.3
Phenylalanine	2.2	2.5
Lysine	3.5	4.3
Histidine	1.4	1.7
Arginine	3.0	3.2

*Ingredients of MP diet (% incorporation): fish meal, 32.7; shrimp meal, 6; mussel meal, 6; squid meal, 6; fish protein concentrate, 10; wheat flour, 7; lactic yeast, 7; precooked starch, 12; cod liver oil, 7; lecithin, 1; vitamin mixture, 3.29; mineral mixture, 2; B.H.T., 0.01.

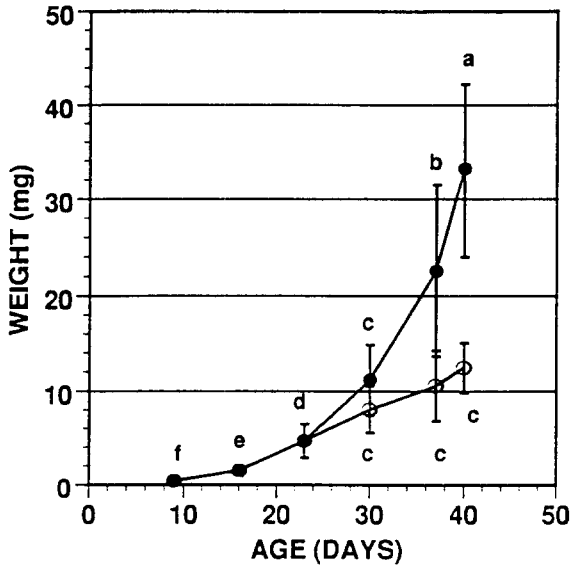

Fig. 1. Growth of sea bass larvae fed live prey (black circle) and compound diet (clear circle). Means (± SEM (n = 30)) with the same superscript letter are not significantly different ($p < 0.05$).

Sampling

In order to monitor growth, 4 samples (30 larvae/sample) were taken weekly from each group and preserved for one month in 10% formaldehyde sea water prior to weighing. Survival rates were determined by counting individually at the end of the experiment.

From days 3–6 (mouth opening), about 100 larvae were collected daily and pooled in order to detect the first enzymatic expression. Subsequently, each group of tanks was divided into 4 sub-groups (3 tanks per sub-group); twice a week, 30 larvae were pooled from each sub-group, and were immediately stored at −80°C until assayed. Samples were taken in the morning before food distribution; at this time, the gut of the fishes still contained food.

Analytical methods

Samples were homogenized in 5 volumes (v/w) of ice-cold distilled water. Trypsin and amylase activities were assayed according to Tseng *et al.* (1984) and Metais and Bieth (1968), respectively. Alkaline phosphatase (AP), leucine aminopeptidase N (AN) and γ-glutamyl transpeptidase (γGT) were assayed according to Bessey *et al.* (1946), Maroux *et al.* (1973) and Meister *et al.* (1981), respectively. Enzyme specific activities were expressed as μmoles of substrate hydrolysed min/mg protein (*i.e.*, U/mg protein) at 37°C for AP, AN and γGT and at 25°C for trypsin. Amylase activity was expressed as the equivalent enzyme activity which was required to hydrolyse 1 mg of starch in 30 min at 37°C. Brush border membrane (BBM) fraction from whole body homogenates were obtained according to a method settled for intestinal scrapping (Crane *et al.* 1979). Protein was determined by the Bradford procedure (Bradford 1976). The amino acid composition of the diets was analysed using a Waters HPLC system. Hydrolysis, derivatization and analysis were made according to the Pico-Tag method (Bidlingmeyer *et al.* 1984).

Results are given as mean ± SEM (n = 4). Data were compared by one way and two way analysis of variance followed by LSD multiple range test when significant differences were found at 0.05 level.

Results

Rates of survival at day 40 were not significantly different ($p < 0.05$) for the group fed live prey and the group fed microparticules, 25.0 ± 9.51% and 20.5 ± 6.96% respectively. The growth rates of the two groups are shown in Figure 1. Final weight was lower with MP although the microparticles were ingested: observation under the microscope showed that the digestive tract was full half an hour after food distribution.

The development of the activities of the two pancreatic enzymes studied is summarized in Figures 2A and 2B. Trypsin activity was detected on day 4 for the first time (Fig. 2A). A sharp increase in this activity coincided with mouth opening (day 6) and continued until day 9. After staying at the same level between day 9 and day 16, there was a further increase in activity up to day 23 followed by a sharp decrease. As in the case of trypsin, there was an increase in amylase specific activity at mouth opening (Fig. 2B). Subsequently, there was little change in

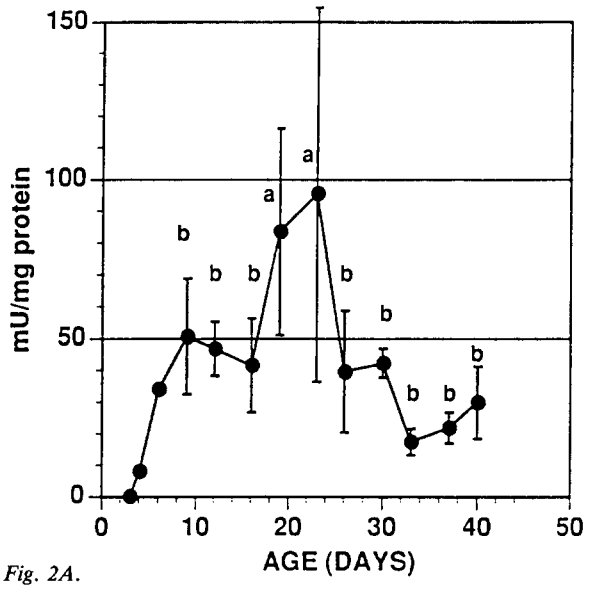

Fig. 2A.

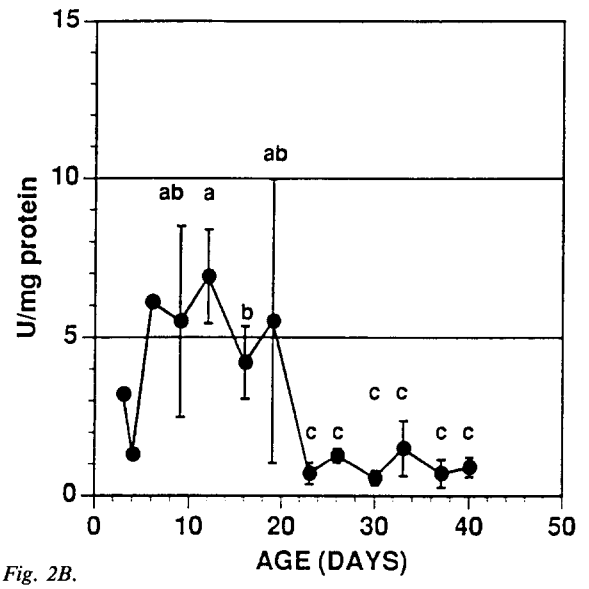

Fig. 2B.

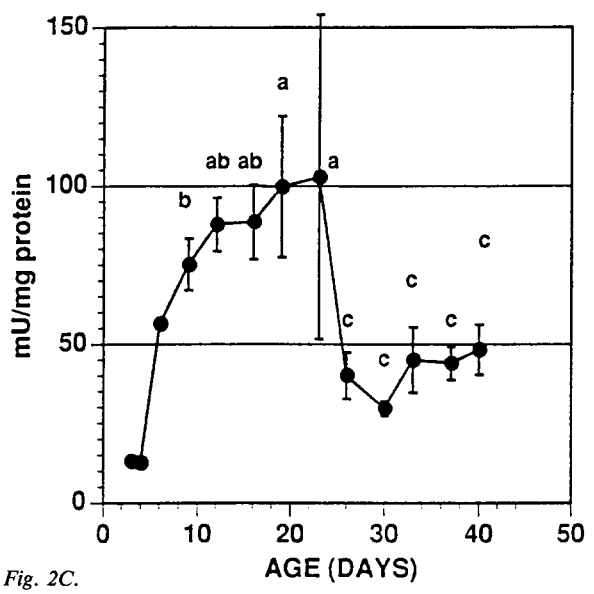

Fig. 2C.

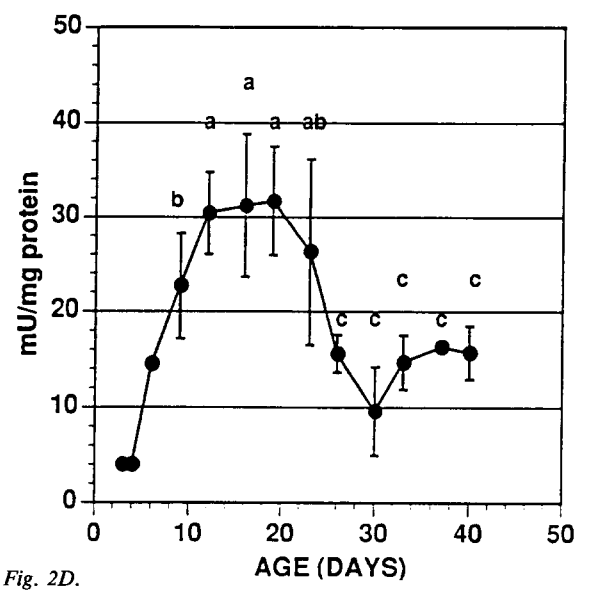

Fig. 2D.

Fig. 2. Specific activities (mU/mg prot. = 10^{-3}.U/mg prot.) of trypsin (A), α-amylase (B), alkaline phosphatase (C), leucine aminopeptidase (D) and γ-glutamyl transpeptidase (E) during larval development of sea bass fed live prey up to day 40. Means (± SEM (n = 8 until day 23, then n = 4)) with the same superscript letter are not significantly different ($p < 0.05$).

activity until day 19; at day 23 the amylase activity dropped and then remained constant until the end of the experiment.

Figures 2C, 2D and 2E show AP, AN and γGT activities, respectively. These enzymes had a similar pattern of expression: an increase in enzyme activity up to day 23, followed by a fall to a constant value until day 40.

Figures 3A to 3H show changes in the enzyme activities in response to a change of diet from day 25 to day 40. While no significant difference was noted in trypsin activities between the LP and the MP group (Fig. 3A), the group fed the MP diet had a higher amylase specific activity (Fig. 3B), although this difference was not significant at day 40.

The activities of the intestinal enzymes in the

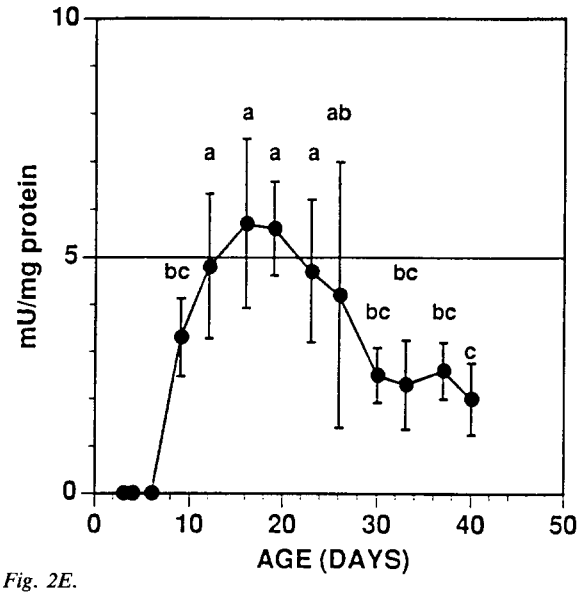

Fig. 2E.

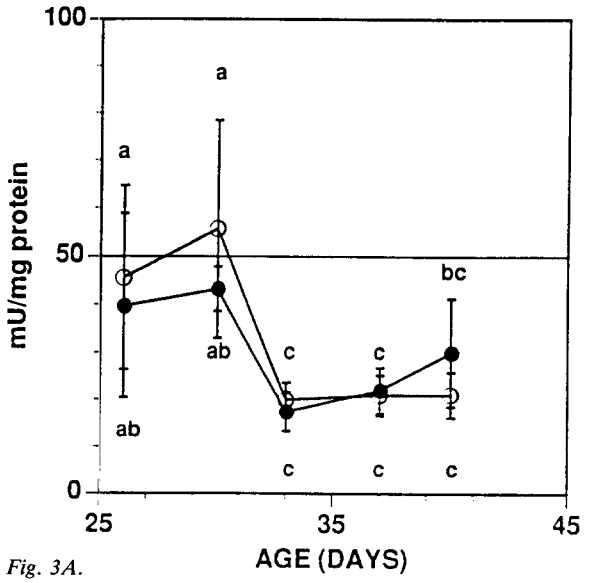

Fig. 3A.

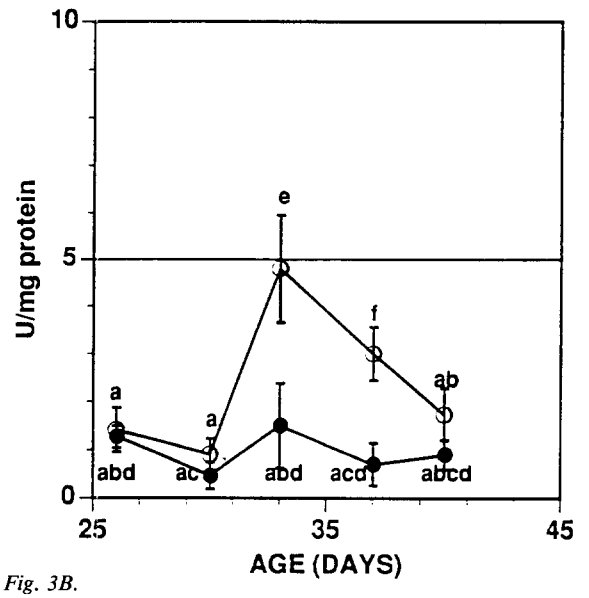

Fig. 3B.

Fig. 3. Specific activities (mU/mg prot. = 10^{-3}.U/mg prot.) of trypsin (A), α-amylase (B), alkaline phosphatase (C), brush border alkaline phosphatase (D), leucine aminopeptidase (E), brush border leucine aminopeptidase (F), γ-glutamyl transpeptidase (G) and brush border γ-glutamyl transpeptidase (H) in larvae fed live prey (black circle) or compound diet (clear circle). Means (± SEM (n = 4)) with the same superscript letter are not significantly different ($p < 0.05$).

crude extracts were also significantly higher for the group fed MP diet (Figs. 3C, 3E and 3G), with the exception of γGT activity in which there was no significant difference between the two groups. Figures 3D and 3F show specific activities for AP and AN respectively assayed in the brush border membrane fraction (BBM). The enzyme activities were generally higher for the MP group but, except at day 30 for AP, differences between the two groups were not significant. Specific activity for γGT in the BBM fraction was significantly higher for the MP group except at day 40, as shown in Figure 3H. It should be noted that the degree of purification of BBM fraction, taking AP and AN as markers of brush border membrane, were 7 and 6 fold respectively.

Discussion

Development of enzyme activities during larval development

For all the enzymes investigated except γGT, a 4 fold increase in activity was observed between day 4 and day 6. This enhancement coincides with mouth opening and is independent of food intake since larvae were not fed before day 6. Since the phenomenon was not promoted by food stimulation, it can be assumed that this increase is genetically programmed. Data on digestive enzymes exist in the literature for both fed and unfed marine fish

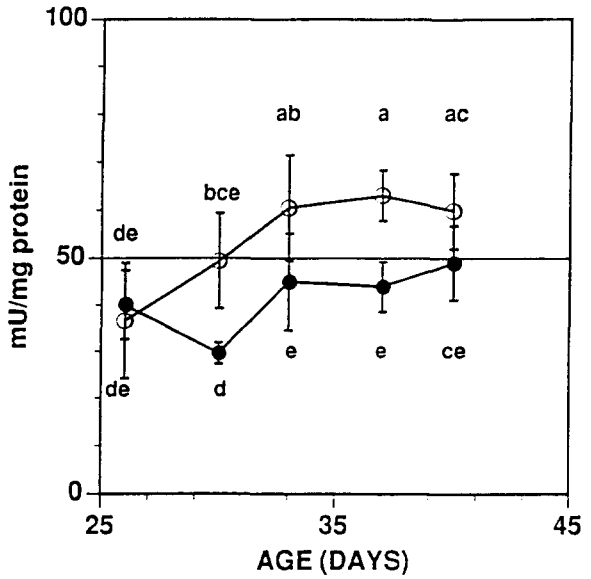

Fig. 3C.

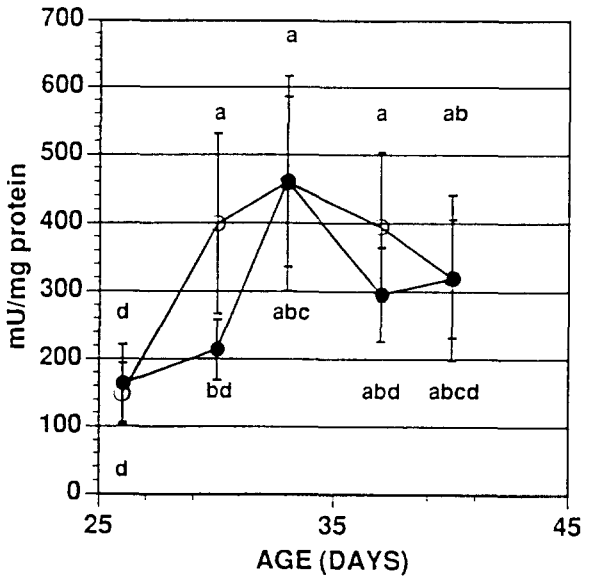

Fig. 3D.

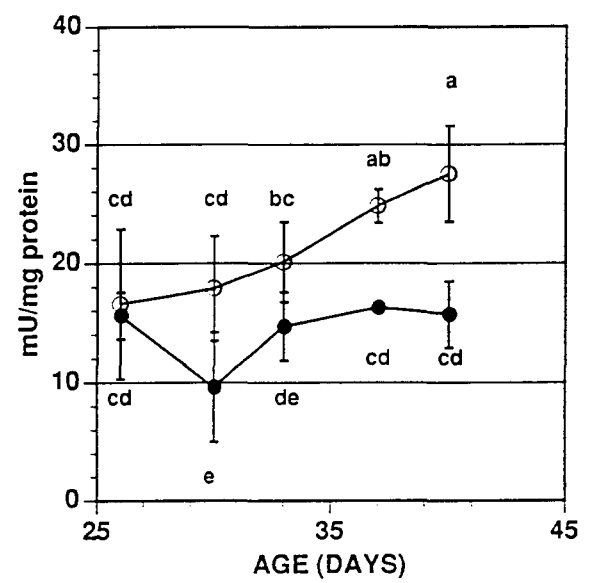

Fig. 3E.

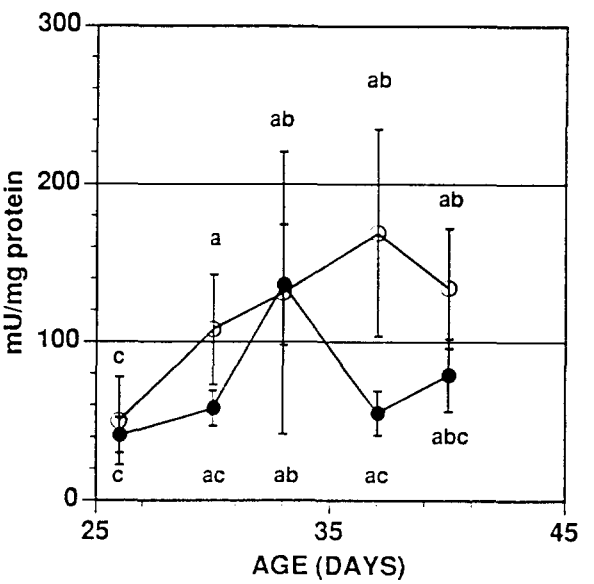

Fig. 3F.

larvae immediately at mouth opening (Vu 1983; Pedersen *et al.* 1987; Munilla Moran and Stark 1989; Walford and Lam 1993). In particular, an increase of trypsin activity between hatching and first feeding was reported by Pedersen *et al.* (1987) in herring larvae. In our experiment, γGT activity could not be detected between day 4 and day 6, because the assay was not sensitive enough for this larval stage.

On and after day 12, all the enzyme activities measured except trypsin showed a plateau following the complete resorption of the yolksac (day 10); a decrease of specific activities was observed around day 23, then levels were constant until the end of the experiment. This decline may result from a greater degradation or a lower synthesis of enzyme. A greater degradation of membrane enzymes would imply damage to intestinal villi. On the other

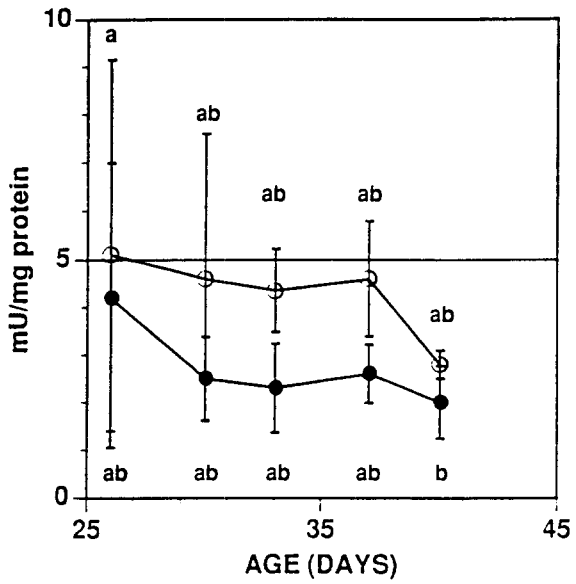

Fig. 3G.

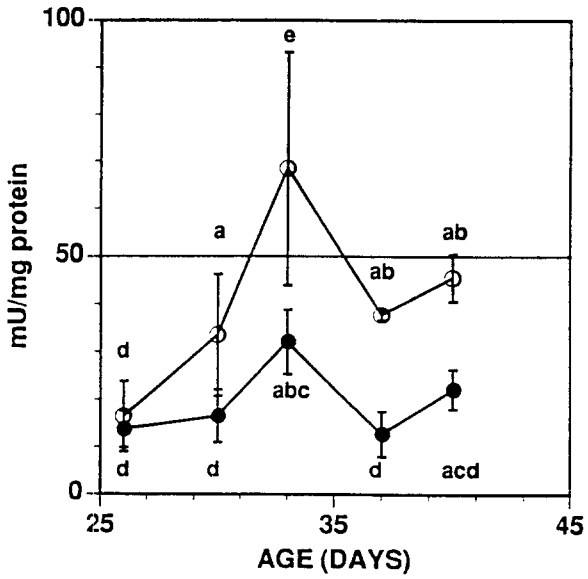

Fig. 3H.

hand, reduced enzyme synthesis for a period during early development in herring larvae has been suggested by Pederson *et al.* (1987). This lowering in certain enzymatic activities can be related to anatomical and physiological modifications in the larvae, constituting a step in the metamorphosis that will be achieved around day 40 (Person-Le Ruyet *et al.* 1993). Studies carried out on hormonal development and specially on thyroid hormones which are involved in morphogenesis reported an important increase in the triiodothyronine level (T_3) in sea bass around day 20, suggesting morphological changes at this moment (Leloup 1990). Furthermore, during this stage in *Lates calcarifer*, the digestive tract becomes fully functional with the differentiation of a stomach and the onset of pepsin (Walford and Lam 1993), and allows a better degradation of diet components. Substitution of formulated diet for live prey begins to be possible from this moment in several fish species (Tucker *et al.* 1988; Person-Le Ruyet *et al.* 1993).

Enzyme adaptation to diet

Enzyme activities measured after day 26 were dependent to some extent on the nature of the diets: in our experiment, feeding microparticulate diet resulted in higher enzymatic activities except in the case of trypsin. Larvae were able to alter their digestive capacity in response to a new diet, in order to provide their nutritional needs. Amylase activity increased in the MP group, as an adaptation to the starch content (12%) of the diet. Adaptation of pancreatic enzyme activities to diet composition has been extensively reported in mammals (Grendell and Rothman 1981; Wicker *et al.* 1983; Valette *et al.* 1992), fish (Kawai and Ikeda 1973) and even in the hepatopancreas of marine invertebrates (Samain *et al.* 1980).

Tseng *et al.* (1982) showed that trypsin levels are related to the protein content in the lumen; on the other hand, Johnson *et al.* (1977) and Roy and Schneeman (1981) reported that the effect of dietary protein on pancreatic enzyme activities is inversely proportional to its digestibility. As in the present study, the trypsin levels were similar in the two groups, two hypotheses have to be considered. First, that larvae of the two groups would have similar protein quantity in their digestive tract and digestibility of the two diets was similar; and second, that differences in protein quantity in the guts between the two groups would be balanced by different ingestion, transit and digestibility, inducing similar trypsin level.

In this study, there was no quantitative control of

ingestion. Nevertheless, food intake was evident in both groups, particularly in the microparticulate group. Since the animals were fed *ad libitum*, it can be supposed that the enzymatic changes observed were not a direct effect of the ration level. On the contrary, although there were no distinct differences in the primary composition of dietary proteins (Table 2), we cannot assume that digestibility was similar. Investigations into ingestion, transit and protein digestibility in larvae are required for further interpretations.

The trypsin results suggest that a contribution of exogenous enzymes from live prey is unlikely, despite the conclusions of several authors (Dabrowski and Glogowski 1977b; Lauff and Hoffer 1984; Munilla Moran *et al.* 1990). Nevertheless, this hypothesis was much debated and Dabrowski and Glogowski (1977a) did not observe any effect of enzyme addition in diet on growth of carp fry.

Membrane enzymes located in the brush border of the intestine often have both an hydrolytic and a transport function. AN activity is known to be influenced by the nature and level of dietary proteins (Nicholson *et al.* 1974; McCarthy *et al.* 1980). γGT, in addition to its hydrolytic function, is involved in peptide transport (Tate and Meister 1974; Griffith and Meister 1980). AP activity is induced by all the phosphorylated substrates, phospholipids and phosphoproteins (Shirazi *et al.* 1978; McCarthy *et al.* 1980) and is a sensitive indicator of malnutrition status (Kumar and Chase 1971; Pathak *et al.* 1982). Poorest growth was observed in the MP group and, yet, this group had higher specific activity for the three intestinal enzymes assayed in whole body homogenates. Ragyanszki (1980) reported higher enzymatic levels in fasted carp larvae than in animals. In our experiment, though the larvae ingested microparticles, their enzymatic response was similar to that of fasting animals. These data suggest an inadequacy of the diet.

Separation of the BBM fraction may allow a better assessment of intestinal digestive enzyme activities than by using whole body homogenates which provide information on the enzyme activities in the organism. The isolation of brush border membranes from intestinal scraping allowed a 9 fold purification of enzymes, taking AP as marker (Crane *et al.* 1979). Since this ratio was 7 in our experiment with whole body homogenates, the obtained fraction could be considered as a BBM fraction. The results obtained with the BBM fractions showed little differences in enzyme activities between the two groups: this is unexpected and inconsistent with the poor growth performances of the MP group. Differences in intestinal enzyme activities could be masked because the BBM fractions purified from whole body homogenate contained brush border membranes issuing from different organs such as kidney and liver. This assumption was confirmed by later purifications using dissected intestinal segment of larvae; these experiments will be exposed in a further paper.

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