Partial Substitution of Di- and Tripeptides for Native Proteins in Sea Bass Diet Improves *Dicentrarchus labrax* Larval Development\(^1\)

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**ABSTRACT** To determine whether incorporation of peptides into diets can improve larval development, sea bass (*Dicentrarchus labrax*) larvae were fed for 21 d one of three isonitrogenous, isoenergetic semipurified diets in which enzymatic hydrolysate (75% di- and tripeptides) of fish meal proteins was substituted for 0, 20 or 40% of native fish meal proteins. Growth and survival were significantly greater (*P* < 0.05) in larvae fed peptide diets compared to those fed only native protein, with the best performance exhibited by those fed the 20% level of peptides. Chymotrypsin activity was much higher in groups fed peptide diets compared to that fed all native protein (*P* < 0.001), indicating a greater proteolytic capacity of the pancreas. At the intestinal level, activities of the brush border enzymes, aminopeptidase, maltase and γ-glutamyl transpeptidase, increased with age while the cytosolic enzyme, leu-ala peptidase, decreased with age (*P* < 0.001). These changes in enzymatic activities correspond to the normal development of intestinal digestion. This development occurred earlier in the group fed 20% peptide-substituted diet than in the two other groups. The better larval performances observed in groups fed diets containing peptides can be related to the enhanced proteolytic capacity of the pancreas and the earlier development of intestinal digestion. J. Nutr. 127: 608--614, 1997.

**KEY WORDS:** *Dicentrarchus labrax* • dietary peptides • pancreatic proteases • intestinal enzymes • development • aquaculture

The high cost of larvae production in hatcheries limits the development of marine fish aquaculture. Because there is no formulated diet suitable for marine fish larvae, they are fed live prey that are expensive to rear. Indeed, there is no formulated diet available that can be substituted for live prey during larval stages. Over the last two decades, several studies have been conducted to determine the nutritional requirements of marine fish larvae (for review see Watanabe and Kiron 1994). Determining only the optimal level and the nature of dietary lipids and proteins has proved insufficient for formulating a compound diet which is as effective as live prey for rearing larvae. Protein is the major diet component, and the amino acid requirement of fish larvae is met by diets containing fish meal (Kanazawa et al. 1989). However, the molecular size of the dietary protein fraction could play a major role in larval development. Indeed, the incorporation of casein hydrolysate in the diet led to increased survival of *Carassius auratus* (Szlamincka et al. 1991) and *Dicentrarchus labrax* (Cahu and Zambonino Infante 1995a), but no effect on growth was reported. On the other hand, Berge et al. (1993) observed only a slight growth improvement of salmon fry fed Concentré de Protéines Solubles de Poissons (CPSP). Even if no clear effect on larval growth has been reported in the literature, protein hydrolysate has long been supposed to be advantageous for larvae (Gabaudan et al. 1980). This product is incorporated into most larval diets, both for improving physical properties (Pigott et al. 1982) and nutritional value (Carvalho et al. 1995) of the diet. Recent data demonstrating that the increased survival of larvae fed hydrolysate was paralleled by the enhanced development of certain digestive functions (Cahu and Zambonino Infante 1995b) have aroused a new interest in this field of investigation. In particular, substitution of casein hydrolysate for part of the fish meal induced an earlier and greater rise in enzyme activity of brush border membranes, though native casein is of lower nutritional value than native fish meal. It was concluded that the presence of hydrolysate in the diet is essential for larval development. Moreover, hydrolysate containing short peptides has been shown to be effective in stimulating enzyme activity in brush border membranes and in facilitating nutritional rehabilitation in mammals (Sasaki et al. 1989, Scheppach et al. 1994). These short peptides, particularly di- and tripeptides, are absorbed quickly and efficiently by the intestine without any prior pancreatic digestion. Taking these data into account, we hypothesized that the incorporation in larval diet of a hydrolysate processed from a high quality protein and containing a high proportion of short peptides may be beneficial. The aim of this study was to test the effect of a fish meal hydrolysate characterized by 75% di- and tripeptides on the growth and survival of sea bass larvae, and to verify whether di- and tripeptides influenced the activity of pancreatic proteases and the development of intestinal enzymes.

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dietary peptides improve fish larvae development

DIETARY PEPTIDES IMPROVE FISH LARVAE DEVELOPMENT

**MATERIALS AND METHODS**

**Animals and diets.** Eggs of European sea bass (*Dicentrarchus labrax*) were obtained from the ferme marine du Douhet. Larval rearing was conducted at the Ifremer-Station de Brest and lasted 40 days. Newly hatched larvae were transferred from incubators to 25 conical fiberglass tanks (35 L) with black walls at an initial stocking density of 80 larvae L⁻¹. 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. Throughout the experiment, the water temperature and salinity were 18–19°C and 35 g·L⁻¹, respectively. The oxygen level was maintained above 6 mg·L⁻¹ by setting the water exchange up to 30% per hour (flow rate = 0.18 L·min⁻¹). The light intensity was 9 W·m⁻² maximum at the surface. All animal procedures and handling were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (NRC 1995).

The larvae were fed live prey from mouth-opening until d 19 in large excess to ensure a constant and high level of suspended microparticles in the water column. Food was distributed 18 h·d⁻¹ using a belt feeder. Food ingestion was monitored by observing digestive tracts of larvae under a binocular microscope.

**Sampling and dissection.** To monitor growth, 6 larvae per tank (*n* = 5 tanks/dietary group) were taken twice per week from each group and kept in 40 mL formaldehyde/L sea water for 1 mo prior to weighing. This procedure preserved the larvae until weighing, larval weight being stabilized at 80% of the initial weight after 3 wk in formaldehyde (Lockwood 1973). At the end of the experiment, larval survival rates were determined by counting individuals, and the rates of spinal malformation, i.e., scoliosis, lordosis and coiled vertebral column, were determined by examining 60 larvae per tank (*n* = 5 tanks/dietary group) under a binocular microscope.

On d 26 and d 40, before morning food distribution, 50 larvae were collected from each tank. They were immediately stored at −80°C pending dissection and assays. Dissection under microscope was conducted on a glass maintained at −2°C. Individuals were cut into four parts as described by Cahu and Zambonino Infante (1994); head, pancreatic segment, intestinal segment and tail, in order to limit the assay of enzymes to specific segments. This dissection inevitably produced a crude mixture of organs in each segment. The pancreatic segment, besides pancreas, contained liver, heart, muscle and spine. Intestinal segment contained intestine, muscle and spine.

**Analytical methods.** The pancreatic segments were homogenized in 5 volumes (w/v) of ice-cold distilled water. Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) activities were assayed according to Holm et al. (1988) and Worthington (1982), respectively. Purified brush border membranes from the intestinal segment homogenate were obtained according to a method developed for intestinal scraping (Crane et al. 1979). The degree of purification of brush border membrane, taking alkaline phosphatase and aminopeptidase N as markers of cell membrane fraction, was close to that reported by Crane et al. (1979) i.e., 13.5- and 10-fold, respectively. Enzymes of the brush border membrane, alkaline phosphatase (EC 3.1.3.1), aminopeptidase N (EC 3.4.11.2), γ-glutamyl transpeptidase (γ-GT; EC 2.3.2.2) and maltase (EC 3.2.1.20), were assayed according to Bessey et al. (1946), Maroux et al. (1973), Meister et al. (1981) and Dahlqvist (1970), respectively. Assay of a cytosolic peptidase, leucine-alanine (leu-ala) peptidase was performed using the method of Nicholson and Kim (1975). Enzyme activities were expressed as specific activities, μmol·mg⁻¹ protein⁻¹. Ratios of enzyme activities of brush border membrane related to leu-ala peptidase activity were calculated using the following sequence, expressed per larva per day: d 6 to d 9, 50±20; d 10 to d 12, 200; d 13 to d 19, 90±20; d 20 to d 26, 190±40 (Table 1). The fish meal hydrolysate was obtained from and processed by the Institut Univeritaire de Technologie de Limoges, using an alkaline bacterial serine proteinase, according to Bressollier et al. (1988). Resulting peptides were continuously extracted using a membrane ultrafiltration process (molecular weight cut off: 1000). Then, each peptide class, characterized by its size, was quantified on the basis of its amino acid content after separation by ligand exchange chromatography using Cu(II)-modified silica gel. The relative distribution was (mol/100 mol): single amino acids, 5; di- and tripeptides, 75; peptides with chain length < 6 residues, 20. The size of the microparticulate diets was 200–400 μm. Fish were continuously fed in large excess to ensure a constant and high level of suspended microparticles in the water column. Food was distributed 18 h·d⁻¹ using a belt feeder. Food ingestion was monitored by observing digestive tracts of larvae under a binocular microscope.

**RESULTS**

Observation of the digestive tract under the binocular microscope revealed an effective ingestion of the microparticulated

**TABLE 1**

Composition of the experimental diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>P0</th>
<th>P20</th>
<th>P40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal (77% protein, 14% lipid, 9% ash)</td>
<td>650</td>
<td>520</td>
<td>390</td>
</tr>
<tr>
<td>Short peptides (84.4% protein, 1.1% lipid, 14.4% ash)</td>
<td>0</td>
<td>119</td>
<td>237</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>39</td>
<td>51</td>
<td>63</td>
</tr>
<tr>
<td>Yeast</td>
<td>20</td>
<td>26</td>
<td>32</td>
</tr>
<tr>
<td>Maltose</td>
<td>56</td>
<td>56</td>
<td>56</td>
</tr>
<tr>
<td>Precooked potato starch</td>
<td>112</td>
<td>112</td>
<td>112</td>
</tr>
<tr>
<td>Cellulose</td>
<td>70</td>
<td>63</td>
<td>57</td>
</tr>
<tr>
<td>Vitamin mixture¹</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mineral mixture³</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Choline</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Inositol</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Protein (N × 6.25)</td>
<td>510</td>
<td>520</td>
<td>520</td>
</tr>
<tr>
<td>Lipid</td>
<td>151</td>
<td>153</td>
<td>151</td>
</tr>
<tr>
<td>Ash</td>
<td>97</td>
<td>87</td>
<td>91</td>
</tr>
<tr>
<td>Energy (J·kg⁻¹·L⁻¹)</td>
<td>17,105</td>
<td>17,258</td>
<td>17,182</td>
</tr>
</tbody>
</table>

¹ Dietary ingredients, except short peptides, were commercially obtained. Fish meal and cod liver oil were from La Lorientaise (Lorient, France). The soy lecithin was from Ets Louis Francois (St. Maur des Fossés, France). The potato precooked starch (Nutralys) was from Roquette (Lille, France). The Vitamin C (Stab C) was from F. Hoffmann-La Roche (Basel, Switzerland). Maltose (M2250) was purchased from Sigma Chemical (St Louis, MO).

² Per kg of vitamin mix: retinyl acetate, 340 mg; cholecalciferol, 2.5 mg; all-rac-a-tocopherol acetate, 4 g; menadione, 0.1 g; thiamin, 1 g; riboflavin, 2.5 g; α-calcium pantothenate, 5 g; pyridoxine HCl, 1 g; cyanocobalamin, 0.006 g; niacin, 10 mg; folic acid, 0.5 g; biotine, 0.1 g; meso-inositol, 100 g.

³ Per kg of mineral mix: KCl, 90 g; KI, 40 mg; CaHPO₄·2H₂O, 500 g; NaCl, 40 g; CuSO₄·5H₂O, 3 g; ZnSO₄·7H₂O, 4 g; CoSO₄·7H₂O, 20 mg; FeSO₄·7H₂O, 20 g; MnSO₄·H₂O, 3 g; CaCO₃, 215 g; MgSO₄·7H₂O, 124 g; NaF, 1 g.

⁴ Calculated as: total carbohydrate × 16.7 J/kg; fat × 37.7 J/kg; protein × 16.7 J/kg.
Survival rates of sea bass larvae fed isonitrogenous diets in which fish meal hydrolysate was substituted for 0, 20 or 40% of native fish meal proteins (designated P0, P20 and P40, respectively). Means ± SEM (n = 5) with different superscript letters are significantly different (P < 0.05).

FIGURE 2  Survival rates of sea bass larvae fed isonitrogenous diets in which fish meal hydrolysate was substituted for 0, 20 or 40% of native fish meal proteins (designated P0, P20 and P40, respectively). Means ± SEM (n = 5) with different superscript letters are significantly different (P < 0.05).

Specific activity of aminopeptidase increased slightly but significantly with age (Table 2); a threefold enhancement was observed in specific activities of maltase between d 26 and d 40. The specific activity of alkaline phosphatase did not change with age (Table 2). The activity of γ-glutamyl transpeptidase was not detectable at d 26 (Fig. 6). At d 40, the one-way ANOVA showed that this enzymatic activity was higher in the P20 group than in the group fed native proteins, and did not significantly differ in the 2 groups fed short peptides.

Diet composition slightly affected the activity of leu-ala dipeptidase (Table 2; Fig. 7). Leu-ala activity exhibited a sharp decrease between d 26 and d 40 (P < 0.001). The activity level of this enzyme was modulated much more by the age of larvae than by the dietary peptide level.

Segmental activity ratios of the four brush border enzymes vs. leu-ala are reported in Table 3. The ratios calculated for d 26 were higher in the P20 group than in the other two groups for aminopeptidase, alkaline phosphatase and maltase.

FIGURE 3 Malformation rates of sea bass larvae fed isonitrogenous diets in which fish meal hydrolysate was substituted for 0, 20 or 40% of native fish meal proteins (designated P0, P20 and P40, respectively). Means ± SEM (n = 5) with different superscript letters are significantly different (P < 0.05).
TABLE 2

Summary of two-way ANOVA of specific activities of some pancreatic and intestinal enzymes

<table>
<thead>
<tr>
<th></th>
<th>Diet Age (1, 24)</th>
<th>Diet Peptides (1, 24)</th>
<th>Diet Dose (1, 24)</th>
<th>Age × Diet Peptides (1, 24)</th>
<th>Age × Diet Dose (1, 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pancreatic enzymes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>315.77 0.001</td>
<td>22.50 0.001</td>
<td>13.10 0.005</td>
<td>31.60 0.001</td>
<td>0.33 NS</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>6.62 0.050</td>
<td>37.22 0.001</td>
<td>7.73 0.050</td>
<td>1.94 NS</td>
<td>5.81 0.050</td>
</tr>
<tr>
<td>Brush border enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aminopeptidase N</td>
<td>5.49 0.050</td>
<td>11.27 0.005</td>
<td>48.05 0.001</td>
<td>3.80 NS</td>
<td>3.02 NS</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>0.97 NS</td>
<td>0.41 NS</td>
<td>21.09 0.001</td>
<td>8.97 0.010</td>
<td>16.57 0.001</td>
</tr>
<tr>
<td>Maltase</td>
<td>106.99 0.001</td>
<td>1.72 NS</td>
<td>19.45 0.001</td>
<td>1.01 NS</td>
<td>3.02 NS</td>
</tr>
<tr>
<td><strong>Cytosolic enzyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu-ala peptidase</td>
<td>259.10 0.001</td>
<td>1.81 NS</td>
<td>8.47 0.050</td>
<td>2.16 NS</td>
<td>18.74 0.001</td>
</tr>
</tbody>
</table>

1 Degrees of freedom are given in parentheses.
2 Not significant, P > 0.05.
3 Data were log-transformed.

On the other hand, lowest ratios were observed in the P40 group for aminopeptidase and alkaline phosphatase. No difference was shown in the maltase to leu-ala peptidase ratio between the P40 and P0 groups. At d 40, the ratios of the three enzymes to leu-ala peptidase were not significantly different among the dietary groups. The γGT to leu-ala peptidase ratio was significantly higher in P40 group than in the other two dietary groups.

DISCUSSION

Formulated diets have recently received significant attention in the study of nutritional requirements of marine fish larvae. Previously these studies were conducted only using live prey, which restricted investigations. The few data obtained concerning the survival and growth of larvae fed formulated diets were compiled by Person-Le Ruyet et al. (1993); maximal survival rate and weight reported for 40-d-old larvae fed formulated diet for 3 wk was around 30% and 9 mg, respectively. In this experiment, a partial substitution of native protein by di- and tripeptides in the compound diet appeared to be beneficial for sea bass larvae. At first, a substantial weight gain was obtained in the groups fed short peptides. As far as we know, no beneficial effect of hydrolysate has been described in juvenile fish, although different kinds of enzymatic protein hydrolysates have been tested, such as hydrolysates of casein, wheat germ, greaves, feather and fish meal. It appears that the efficiency of a protein hydrolysate in sustaining fish growth depends on the quality of the native protein (Langar et al. 1993).

Secondly, di- and tripeptide diets enhanced larval survival; this effect was maximum for a moderate dose of peptides, i.e. 12 g/100 g of total ingredients. It is noteworthy that peptide chains of two or three amino acids induce improvement in survival as do the commercial casein hydrolysates (Szlaminska et al. 1991) which are mainly composed of peptide chains of 10 to 20 amino acids. On the other hand, free amino acids incorporated in diets failed to enhance larval survival (Cahu and Zambonino Infante 1995a).

Finally, the impressive reduction in skeletal malformation observed in groups fed peptides was an unexpected finding. Indeed, it is known, although rarely reported, that marine fish larvae fed formulated diets are affected by some malformations. This study showed, for the first time, that the molecular form of dietary nitrogen fraction can influence the malformation rate.

We are faced with the question as to why protein hydrolysates appear to be beneficial for fish larvae but do not affect or in some cases depress juvenile growth. The differences in digestive physiology between larvae and juveniles could partially explain this paradox. Indeed, the relative length of the

FIGURE 4 Trypsin (A) and chymotrypsin (B) specific activities in the pancreatic segments of 26- and 40-d-old sea bass larvae fed isonitrogenous diets in which fish meal hydrolysate was substituted for 0, 20 or 40% of native fish meal proteins (designated P0, P20 and P40, respectively). Results are given as means ± SEM (n = 5); for statistical analysis see Table 2.
two enzymes were modulated by the dietary protein content. Trypsin activity was enhanced by the native protein, whereas chymotrypsin activity was enhanced by the diets containing di- and tripeptides. Similarly, chymotrypsin activity in rat was enhanced to a greater extent than trypsin by a modification of the dietary protein, as reported by Lhoste et al. (1994). We assume that the total proteolytic capacity of pancreas of young larvae was enhanced by the incorporation of short peptides in diet. The better growth rate observed in groups fed P20 and P40 could be in part the result of a greater proteolytic capacity of the pancreas. The relationship between elevated proteolytic activity of pancreas and improved growth of pigs has already been suggested by Owsley et al. (1986).

During our experiment, the decrease in the activity of leu-alan peptidase, an enzyme mainly located in the cytosol, was observed concurrent with the increase (or onset) of some brush border enzymes of the enterocytes, aminopeptidase, maltase or.

intestine in larvae is short compared to juveniles (Segner et al. 1994). This can be compared to partially intesetuctimized mammals, for which short peptides represent the best nitrogen supply (Cosnes et al. 1992), while these peptides adversely affect the nitrogen balance in healthy subjects (Grimble et al. 1987). Moreover, digestion in marine fish larvae shows some specificities compared to juveniles: the synthesis of some pancreatic enzymes during larval stages is quite different from that observed in juveniles (Dabrowski 1984), and in intestine, larvae exhibited poorly differentiated brush border membranes and a high level of cytosolic digestion (Gawlicka et al. 1995).

In our experiment, we showed that the activities of two pancreatic enzymes, trypsin and chymotrypsin, were strongly linked to the age of larvae. Moreover, the activities of the enzymes were modulated by the dietary protein content. Trypsin activity was enhanced by the native protein, whereas chymotrypsin activity was enhanced by the diets containing di- and tripeptides. Similarly, chymotrypsin activity in rat was enhanced to a greater extent than trypsin by a modification of the dietary protein, as reported by Lhoste et al. (1994). We assume that the total proteolytic capacity of pancreas of young larvae was enhanced by the incorporation of short peptides in diet. The better growth rate observed in groups fed P20 and P40 could be in part the result of a greater proteolytic capacity of the pancreas. The relationship between elevated proteolytic activity of pancreas and improved growth of pigs has already been suggested by Owsley et al. (1986).

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In our experiment, we showed that the activities of two pancreatic enzymes, trypsin and chymotrypsin, were strongly linked to the age of larvae. Moreover, the activities of the
enzyme. At d 40, ratios revealed a similar intestinal maturation resulted from a sharp increase in activities of brush border enzymes vs. leu-ala peptidase, which reflects the relative importance of the brush border membrane digestion compared to intracellular digestion (Cahu and Zambonino Infante 1995a). At d 26, the highest ratios were obtained for all the enzymes in the P20 group, revealing an earlier maturation of the enterocytes in this group compared to the others. This high ratio resulted from a sharp increase in activities of brush border enzyme. At d 40, ratios revealed a similar intestinal maturation for the three dietary groups. The high of γGT-leu-ala peptidase ratio in the P40 group did not reveal a better intestinal maturation, but was the consequence of a stimulation of γGT by short peptides as previously discussed.

In conclusion, this study shows that the incorporation of short peptides in the diet improves the development and the nutritional status of sea bass larvae, by stimulating the acquisition of the adult mode of digestion.

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LITERATURE CITED


