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Ontogeny of Fertilized Eggs and Yolk Sac Larvae of Sea Bass (*Dicentrarchus labrax*)

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Abstract

The biochemical compositions of fertilized eggs, yolk sac larvae, and larvae at the beginning of exogenous feeding of sea bass (*Dicentrarchus labrax*) were determined. Eggs and yolk sac larvae contain more monounsaturated and polyunsaturated fatty acids than saturated fatty acids. Essential and non-essential amino acid contents tended to drop at hatching, then sharply increase by the end of endogenous feeding. Amylase and trypsin activity was detected in fertilized eggs. Trypsin activity peaked at the beginning of exogenous feeding. The alkaline phosphatase activity was lowest in fertilized eggs, higher at hatching, and highest at the end of the experiment. Leucine alanine peptidase activity was higher than other enzymatic activity in all three stages. Aminopeptidase N activity increased until hatching, then stabilized until the beginning of exogenous feeding.

Introduction

Early life history of marine larvae is a complex phenomenon of growth and differentiation. Larvae of marine fish begin exogenous feeding when their digestive system is still in a rudimentary stage of development. Thus, understanding the biochemical compositions and digestive enzyme activity of fertilized eggs and yolk sac larvae help to understand the nutritional requirements of fish at the start of exogenous feeding.

The yolk sac stage is an important developmental period for all fish larvae. At this stage, significant body changes take place in anticipation of first feeding. Energy in the yolk is used for growth, development, and activity. Protein and lipid are major energy fuels during the embryonic and yolk sac stages of fish (Fyhn, 1993; Sargent, 1995). However, most information on protein and lipid metabolism in fish is derived from studies on juvenile and adult specimens.

Studies show that marine fish larvae contain eicosapentaenoic acid (EPA) and docosahexaenoic (DHA), two essential highly unsatu-

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rated fatty acids (HUFA), whereas as freshwater fish can synthesize them from their C18 precursors, linoleic (18:2n-6) and linolenic (18:3n-3) acids (Henderson and Tocher, 1987; Sargent et al., 1989). Stoichiometric studies show that amino acids are the major substrates of aerobic metabolism during the development of embryos and yolk sac larvae in marine species that have pelagic eggs (Fyhn, 1989; Ronnestad et al., 1992, 1994, 1999; Finn et al., 1995; Seoka et al., 1997; Sivaloganathan et al., 1998).

Sea bass (*Dicentrarchus labrax* L., 1758) is an important species in the Mediterranean Sea because of its economic value and ability to adapt to variable environmental conditions such as salinity and temperature. Since the essential amino acid profile of a fish body is generally a good indicator of the amino acid requirements of that fish, the aim of this study was to determine the nutrient composition and digestive enzyme activity at different stages during egg and yolk sac development in sea bass.

Materials and Methods

Broodstock, egg incubation, larval rearing. The study was carried out at the Mediterranean Aquaculture Production and Education Institute. Twenty females (3.5 kg mean wt) and twenty males (1.5 kg mean wt) of sea bass (*Dicentrarchus labrax*) broodstock were selected from wild breeders and stocked in a 10-m^3 tank with a sea water supply of 30 l/min. Water temperature was kept at 15±0.5ºC.

Spawned eggs were immediately collected into a recuperator and buoyant viable eggs were separated from sinking dead eggs. The fertilized eggs were disinfected with an iodine solution and incubated in 150-l incubators supplied with a gentle flow of sea water of 15±0.5ºC.

Newly-hatched larvae were transferred from the incubators and stocked at approximately 100 larvae/l in three fiberglass tanks (300 l) with black walls. The water temperature was controlled by pipe heating systems and automatic transformer equipment calibrated at 15±0.5ºC. The rearing tanks were kept in complete darkness and water was exchanged at 20% of the total volume of the tanks every hour using running sea water filtered through a UV filter.

Salinity was 35-38 ppt throughout the experiment. The oxygen level was maintained above 6.5 ppm with liquid oxygen systems. Air and fresh sea water were introduced at the bottoms of the tanks to prevent water stratification.

Sampling and analytical methods. Samples for biochemical analyses were taken before the eggs were placed in the incubators, during hatching, and at the start of exogenous feeding. The samples were immediately stored at **-**196ºC until assayed.

Fertilized eggs and whole body larvae were homogenized in 5 volumes (v/w) of ice-cold distilled water. Amylase activity was assayed according to Metais and Bieth (1968). Trypsin activity was assayed according to Tseng et al. (1982). Alkaline phosphatase (AP), aminopeptidase N (LAP), and leucine alanine peptidase (LEU-ALA) were assayed according to Bessey et al. (1946), Maroux et al. (1973), and Nicholson and Kim (1975), respectively. Amylase activity was expressed as the equivalent enzyme activity required to hydrolyze one mg of starch in 30 min at 37ºC. Activity was expressed as µmoles of substrate hydrolyzed per minute per mg protein (i.e., U**/**mg protein) at 25ºC for trypsin and at 37ºC for AP and LAP. LEU-ALA activity was expressed as nmoles of substrate hydrolyzed per minute per mg protein (i.e., U/min**/**mg protein) at 37ºC.

Fertilized eggs and yolk sac larvae were esterified for fatty acid analysis according to Garces and Mancha (1993). Fatty acid methylesters were analyzed on GC-MS equipped with a SP-2330 fused capillary column (30 x 0.25 mm) using hydrogen as the carrier gas at a temperature gradient of 120 to 220°C (5°C/min). The temperatures of the injector and detector were 240°C and 250°C, respectively. Methylesters were identified by comparison with a known standard mixture of fatty acids.

Amino acid contents of fertilized eggs and yolk sac larvae were analyzed according to

AOAC (1995). Amino acid contents of hydrolyzed samples were determined using GC Varian 3800 equipped with a ZB-AAA(10 m x 0.25 mm) column (Scientific and Technical Research Council of Turkey) using helium as the carrier gas and a temperature gradient of 110 to 310°C (30°C/0.3 min). The temperatures of the injector and the detector were 250°C and 320°C, respectively. The concentration of soluble protein in the fertilized eggs and yolk sac larvae was determined by the Bradford (1976) method using bovine serum albumin as a standard.

The experiment was terminated when the first mouth openings were observed. Results are given as means±SD. Comparisons were made using one-way ANOVA and differences were considered significant at $p<0.05$. SPSS statistical software was used for statistical analyses (SPSS, 1993).

Results

Enzyme activity is given in Table 1. Amino acid contents are given in Table 2. Fatty acid contents are given in Table 3.

Discussion

Amylase activity can be stimulated by dietary changes. Dietary starch can modulate changes in amylase activity (Sheele, 1993; Peres et al., 1998). In the present study, however, dietary components were not used. Therefore, the variations in amylase activity were not due to the dietary components but to genetic programming during larvae development.

Tryptic enzyme activity rose at the start of the exogenous feeding. An increase in tryptic enzyme activity of yolk sac larvae just prior to first feeding was also reported by Alliot et al. (1977), Pedersen and Hjelmeland (1988), Ueberschar et al. (1992), and Chen et al., 2006). Presumably, such an increase is a general feature of fish larvae that hatch in the embryonic stage and with a large yolk sac.

Alkaline phosphatase (AP) activity increased until the end of endogenous feeding. AP is stimulated by phosphorylated substrates such as phosphoproteins and phospholipids (Shirazi et al., 1978; McCarty et al., 1980). Eggs and yolk sac larvae are excellent sources of phosphorylated substrates (Hertrampf, 1992). Acid and AP activity was also detected in the yolk of *Sparus aurata* (Sarasquete et al., 1993).

Since physiological parameters vary during ontogenesis, the activity of the cytosolic enzyme LEU-ALA is not enough to account for the digestive maturation of the enterocyte. The importance of peptide digestion in brush border (BB) membranes compared to that in the intracellular compartment can be

Table 1. Enzyme activity in fertilized eggs and yolk sac larvae of sea bass, *Dicentrarchus labrax* (means±SD; n = 3).

Digestive enzyme	*Fertilized eggs*	*At hatching*	*At end of endogenous feeding*
Amylase (U/mg protein)	16.408±3.57^{a}	30.807±2.109^{b}	13.658±1.509^{a}
Trypsin (mU/mg protein)	45.792±19.583^{a}	45.724±2.755^{a}	70.994±5.358^{b}
AP (mU/mg protein)	89.453±1.835^{a}	178.949±3.627^{b}	575.057±60.206^{c}
LAP (mU/mg protein)	1174.15±135.715^{a}	1457.91±58.168^{b}	1489.079±186.142^{b}
LEU-ALA (mU/mg protein)	2809.157±783.115^{a}	2721.267±88.396^{a}	2773.942±501.601^{a}
LAP (x 1000)/LEU-ALA	431.53±73.891^{a}	535.889±19.703^{b}	540.227±28.418^{b}

Different superscripts within a row indicate significant differences at $p<0.05$.
AP = alkaline phosphatase, LAP = aminopeptidase N, LEU-ALA = leucine alanine peptidase

Table 2. Amino acid (AA) contents (mg/100 g) in fertilized eggs and yolk sac larvae of sea bass, *Dicentrarchus labrax* (means±SD; n = 3).

Amino acid	*Fertilized eggs*	*At hatching*	*At end of endogenous feeding*
Alanine	459.633±2.177^{b}	382.9±6.173^{a}	1700.833±11.46^{c}
Aspartic acid	2798.6±28.653^{c}	338.1±1.873^{a}	1448.666±79.481^{b}
Glutamic acid	661.00±8.389^{b}	413.366±2.309^{a}	641.7±17.066^{b}
Glycine	182.033±2.196^{a}	193.5±6.00^{a}	1387.866±13.031^{b}
Histidine	125.166±1.167^{b}	107.6±0.3^{a}	nd
Isoleucine	128.4±13.398^{a}	337.933±8.146^{b}	1199.166±2.742^{c}
Leucine	635.866±3.496^{b}	477.433±3.419^{a}	1627.4±3.064^{c}
Lysine	332.6±8.229^{b}	200.566±1.747^{a}	nd
Methionine	92.766±0.929^{a}	109.333±1.625^{a}	498.4±14.756^{b}
Phenylalanine	351.6±16.119^{b}	239.1±2.338^{a}	1186.2±39.634^{c}
Proline	446.266±14.299^{b}	176.033±5.024^{a}	644.266±50.657^{c}
Serine	170.133±3.847^{b}	94.4±2.651^{a}	884.633±32.957^{c}
Threonine	254.333±2.929^{b}	227.633±3.611^{a}	nd
Tyrosine	286.3±1.51^{b}	213.333±0.288^{a}	nd
Valine	550.733±6.882^{b}	321.8±2.615^{a}	1220.266±29.954^{c}
Essential AA	2471.467	2021.4	5731.433
Non-essential AA	5003.967	1811.633	6707.966
Total free AA	7475.433	3833.033	12439.4

Different superscripts within a row indicate significant differences at $p<0.05$.

expressed by the ratio of BB peptidase (LAP) activity and cytosolic peptidase (LEU-ALA) activity. The enzymatic activity ratio LAP/LEU-ALA is a good indicator of digestive capacity (Cahu and Zambonino Infante, 1995). Our results show that the digestive capacity of sea bass larvae at hatching and at the beginning of the exogenous feeding is higher than in fertilized eggs.

Free amino acid concentrations increased with time. In eggs, the dominant amino acids were alanine, aspartic acid, glutamic acid, leucine, lysine, phenylalanine, proline, and valine. This profile is consistent with a range of pelagic fish eggs in which alanine, isoleucine, leucine, lysine, serine, and valine were dominant. The similarity is attributed to hydrolysis of a common protein at the time of oocyte hydration (Ronnestad and Fyhn, 1993). Essential amino acids were significantly higher (131.9%) at the start of exogenous feeding than in fertilized eggs, greater than the increase of non-essential amino acids (34.05%). In most fish species, the yolk provides substrates for energy and growth during the egg and yolk sac stages. The general model assumes that yolk lipid is the main energy substrate whereas yolk protein provides amino acids for tissue synthesis (Heming and Buddington, 1988). In marine fish eggs, energy is partly derived from free amino acid pools (Ronnestad et al., 1992;

Table 3. Fatty acid (FA) contents (%) in fertilized eggs and yolk sac larvae of sea bass, *Dicentrarchus labrax* (means±SD; n = 3).

Fatty acid	*Fertilized eggs*	*At hatching*	*At end of endogenous feeding*
C6:0	0.0205±0.0007	nd	nd
C8:0	0.052±0.0014	nd	nd
C10:0	0.042±0.0014	nd	nd
C11:0	nd	nd	0.016±0.0012
C12:0	0.4205±0.0091	nd	nd
C13:0	0.016±0.0009	nd	nd
C14:0	2.4655±0.0516	2.4305±0.0289	2.608±0.0353
C14:1	0.078±0.0042	0.0765±0.0077	0.083±0.0042
C15:0	0.339±0.0042	0.3575±0.0049	0.3775±0.0049
C15:1	0.0175±0.0007	0.03±0.0014	0.023±0.0014
C16:0	11.062±0.1357	8.9735±0.0657	9.334±0.0636
C16:1	6.919±0.1032	7.3835±0.0685	7.6305±0.0869
C17:0	0.5855±0.0148	0.7055±0.0007	0.7265±0.0134
C17:1	0.032±0.0042	0.0325±0.0106	0.025±0.0014
C18:0	1.8685±0.0035	1.301±0.0042	1.243±0.0042
C18:1n9c	23.369±0.1442	21.581±0.065	21.5385±0.1039
C18:1n9t	nd	nd	nd
C18:2n6c	0.2195±0.0162	8.2285±0.0289	8.1895±0.0148
C18:2n6t	0.0215±0.0035	0.0175±0.0007	0.016±0.0014
C18:3n3	1.374±0.0254	1.318±0.0183	1.318±0.0042
C18:3n6	0.0485±0.0049	0.0465±0.0007	0.042±0.0014
C20:0	0.0825±0.0077	0.1795±0.0035	0.1625±0.0007
C20:1n9	0.6965±0.0077	0.8945±0.012	0.813±0.0141
C20:2	0.107±0.007	0.176±0.024	0.229±0.0212
C20:3n3	0.558±0.0311	0.62±0.0042	0.5435±0.0233
C20:4n6	0.833±0.0014	1.2475±0.0205	1.193±0.0183
C20:5n3 (EPA)	5.2855±0.3783	7.098±0.0947	7.1755±0.0968
C21:0	0.454±0.0127	0.4375±0.0261	0.4905±0.0318
C22:0	0.0515±0.0035	0.0255±0.012	0.1245±0.0035
C22:1n9	0.0735±0.0049	0.0675±0.0021	0.0665±0.0035
C22:2	0.0355±0.0007	0.029±0.0013	nd
C22:6n3 (DHA)	18.099±0.1937	23.8915±0.2326	23.842±0.1187
C23:0	0.0955±0.0289	0.07±0.0185	0.071±0.0197
C24:0	0.971±0.0494	0.257±0.1711	0.375±0.0038
C24:1n9	1.1545±0.1605	0.2135±0.1562	0.124±0.0268
Total FA	77.4395±0.1294	87.6745±0.0304	88.193±0.0763
Monounsaturated FA	32.34±0.0989	30.279±0.3238	30.3035±0.1859
Polyunsaturated FA	26.5815±0.4829	42.658±0.2687	42.5485±0.0516
Saturated FA	18.518±0.2545	14.7375±0.0247	15.341±0.1612
DHA/EPA	3.4317±0.2089	3.366±0.0121	3.323±0.0614
n-3	25.3165±0.5154	32.9275±0.3132	32.879±0.0056
n-6	1.1225±0.0261	9.54±0.048	9.4405±0.036
n-3/n-6	22.5651±0.9851	3.4516±0.0502	3.4827±0.0139
Undefined	22.5605±0.1294	12.3255±0.0304	11.807±0.0763

DHA = docosahexaenoic acid, EPA = eicosapentaenoic acid

Fyhn, 1993) while phospholipids fulfill other essential functions for growth and survival (Kanazawa et al., 1985; Sargent et al., 1993). The general consensus in the present study is that lipid has a major role as an energy source.

In fertilized eggs, the monounsaturated and polyunsaturated fatty acid ratios were higher than the saturated fatty acid ratio, as found by Bulut et al. (2004). In addition, the fertilized eggs and yolk sac larvae were rich in the n-3 fatty acids, EPA (20:5n-3) and DHA (22:6n-3). Marine fish contain lower n-6 PUFA levels than freshwater fish. The principal fatty acids in marine fish larvae are 16:0, 18:1n9, 20:5n-3, and 22:6n-3 (Mourente and Odriozola, 1990). The (n-3)/(n-6) ratio in the fertilized eggs and at the beginning of exogenous feeding were 22.5651 and 3.4827, respectively, higher than in many other marine and freshwater species (Henderson and Tocher, 1987; Sargent, 1995; Bulut et al., 2004). While the 22:6n-3 level was close to other research results, the 18:2n-6 level was lower (Bulut et al., 2004). Therefore, the high (n-3)/(n-6) ratio in the fertilized eggs may be related to the low level of 18:2n-6.

Our results reveal that sea bass eggs and yolk sac larvae contain significant pools of free amino acids and fatty acids, contributing information about the nutritional requirements of sea bass larvae at the onset of exogenous feeding. In addition, the changes in enzymatic activity observed from fertilized egg to first feeding provide important information about the digestive enzyme potential of post larvae at the beginning of exogenous feeding. Such information can aid in developing artificial feeds for marine fish larvae. The nutrient dynamics in egg and yolk sac stages can provide a model for overcoming problems in the larvae stage of marine fish. Fertilized eggs and yolk sac larvae contain different molecular forms of proteins, other than free amino acids. Thus, future efforts to determine changes in protein forms will contribute to formulizing appropriate micro diets for larvae. Further studies are needed to explain the absorption dynamics and role of peptides in marine fish larvae nutrition.

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