The changes in digestive enzymes and hormones of gilthead seabream larvae (*Sparus aurata*, L 1758) fed on *Artemia* nauplii enriched with free methionine

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Abstract   Variations in digestive enzymes and hormones during the larval development of gilthead seabream (*Sparus aurata*) fed on live prey (*Artemia* nauplii) enriched with free methionine were investigated for 16 days (from day 24 to day 40). Prior to initiation of the experiment, newly hatched larvae were transferred from incubators to fiber glass tanks (300 l) with black walls and fed with same diets until day 24. Each experiment was performed in triplicate. In the experimental group, the content of the free methionine in the *Artemia* nauplii was increased by adding a 5.3 mM free methionine solution to the culture water during a 16-h enrichment period. The larvae of both the control and enriched-methionine groups were sampled four times, with 4-day intervals between samplings, during a 16-day period. The larvae in the control group had a significantly lower growth than those of the methionine group at the end of the study ($P < 0.05$). The highest trypsin activity and leucine aminopeptidase N/leucine–alanine peptidase ratios were observed in the control group. A significant difference between bombesin activities in the treatment groups was not found at 5th minute after the initiation of feeding ($P > 0.05$), but they were significant at 15th minute post-initiation of feeding ($P < 0.05$). A significant difference between the cholecystokinin levels of the treatment groups was found ($P < 0.05$).

Keywords   Alkaline phosphatase · Aminopeptidase N · Amylase · Bombesin · Cholecystokinin · Digestive enzymes · Leucine alanine peptidase · *Sparus aurata* · Trypsin

Introduction

The larvae of marine fish species generally initiate exogenous feeding when their digestive system is still in a rudimentary stage of development. Despite many studies on the larval digestive system, the understanding of the ontogeny of the digestive function in larval fish is still incomplete.

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The regulation of proteolytic enzyme secretion during feeding is controlled by multiple components, including digestive hormones which are the key factors for proper gastrointestinal function in vertebrates (Bentley 1998). It is well documented that nutrients, such as free amino acids (FAA) and free fatty acids (FFA), entering the mammalian digestive tract stimulate an endocrine response that controls digestion and nutrient assimilation and influences feeding behavior and food intake.

Bombesin and cholecystokinin, both pituitary neuropeptides, are an integral part of this gastro–entero–pancreatic endocrine system (Moons et al. 1992; Himick and Peter 1994a, b). The effect of nutrient factors present in live feed on the activation of these digestive hormones in developing fish larvae has been a major focus of research (Kolkovski et al. 1997; Ronnestad et al. 1999). The hormone cholecystokinin is particularly interesting due to its key role in the regulation of the release of bile and pancreatic enzymes, such as trypsin, during digestion in vertebrates (Bentley 1998). Trypsin serves as a key enzyme since it also activates the other proteolytic enzymes upon entrance into the gut lumen (Holst and Schmidt 1994; Liddle 1995). On the other hand, bombesin influences digestion by activating the peristaltic movement of the gut and the release of HCl as well as increasing blood circulation to the gut wall (McDonald et al. 1979).

The need for FAA in diets of marine fish larvae is suggested by the large pool of FAA found in marine invertebrates, such as copepods, which are the natural food of marine fish larvae (Fyhn et al. 1993). In contrast, the nauplii of *Artemia fransiscana*, used in aquaculture, contain markedly lower levels of FAA than wild copepods (Fyhn et al. 1993; Helland 1995). This is especially true for the essential amino acid methionine, which in *Artemia* nauplii is only present at low levels (Fyhn et al. 1993; Helland 1995), suggesting that methionine is a limiting amino acid for fish larvae when fed *Artemia* nauplii (Conceicao et al. 1997). Based on the above findings, it would seem to be appropriate to enrich *Artemia* with free methionine in order to meet their nutritional requirements for marine fish larvae.

The aim of this study was to determine the changes in the digestive enzymes and hormones of gilthead sea bream larvae fed on *Artemia* enriched with free methionine. In other words, FAA pools in the live prey of the larvae were investigated in terms of their effects on the native endocrine factors to complete the digestive process in the developing and not fully functional larval digestive tract.

Materials and methods

The study was carried out at the Mediterranean Aquaculture Production and Education Institute. Water temperature was controlled by pipe heating systems, and automatic transformer equipment was calibrated at $\pm0.5°C$. Fertilized eggs of gilthead seabream (*Sparus aurata*) were collected from the broodstock tanks and incubated in conical fiber glass tanks at a temperature of 16°C. Newly hatched larvae were transferred from the incubators to fiber glass rearing tanks (300 l) with black walls and fed identical diets until day 24. All experiments were carried out in triplicate. Each rearing tank was stocked with approximately newly hatched larvae (approximately 100 larvae l^{-1}). The tanks were supplied with running sea water that had been filtered through a UV filter. Larvae were reared for the first 4 days following hatching at $16 \pm 0.5°C$, followed by 16 days at $18 \pm 0.5°C$, and then 20 days at $20 \pm 0.5°C$. Salinity was 35–38 g l^{-1} during the experiment. The oxygen levels were maintained above 6.5 mg l^{-1} with liquid oxygen systems. Air and fresh sea water were introduced into the bottoms of the tanks to prevent

water stratification. Each day 10% of the water was exchanged with green water (*Nannochloropsis* sp.). Light intensity was a maximum of 100–150 lux at the water surface. All tanks were exposed to a 24-h photoperiod.

Artemia cysts were incubated in filtered seawater at 30°C under continuous aeration and illumination. After 24 h, the nauplii were collected and then washed with tap water. The enrichment was performed in a 15-l, round-bottom beaker containing 12 l of filtered seawater. *Artemia* nauplii were added to the beaker to obtain a density of 600 nauplii ml^{-1}, and a gentle aeration ensured a homogeneous distribution of the nauplii during the enrichment. The content of the free methionine in the *Artemia* nauplii was increased by adding a 5.3 mM free methionine solution to the culture water during a 16-h enrichment period (Tonheim et al. 2000). The feeding regime consisted of *Brachionus plicatilis* from day 4 to day 20, reaching a maximum concentration of 10 prey ml^{-1}, *Artemia* nauplii from day 15 onwards, with a maximum density of 2 prey ml^{-1}, and *Artemia* metanauplii enriched with free methionine from day 24 onwards, with a maximum density of 2 prey ml^{-1}. Rotifers were reared on baker's yeast and enriched with a Selco (INVE products) prior the their transfer to the larval feeding tanks.

Prior to the initiation of the experiment, all larvae were fed the same diets until day 24. In the control group, larvae were fed with unenriched *Artemia*, while in the experimental group, larvae were fed on *Artemia* enriched with free methionine until the end of experiment (day 40).

Sampling

Larvae were sampled four times, with 4-day intervals between samplings, during the 16-day sampling period. In order to monitor growth, three samples (40 larvae/sample) were taken from each group on each sampling occasion. Larvae weights were determined using a microbalance (Precisa XB 220A; ±0.1 mg). Three samples (50 larvae/sample) for the measurement of enzymatic and cholecystokinin activities were also taken from each group before the morning feeding and immediately stored in liquid nitrogen (-196°C) to prevent protein autolysis. The larval samples for the testing of bombesin activities were taken in the 5th and 15th minute after the initiation of feeding and immediately stored in liquid nitrogen (-196°C) to prevent protein autolysis.

Analytical methods

Enzyme analyses

Whole body larvae were homogenized in five volumes (v/w) of ice-cold distilled water. Trypsin and amylase activities were assayed according to Tseng et al. (1982) and Metais and Bieth (1968), respectively. Alkaline phosphatase (AP), leucine 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. Soluble protein was determined according to Bradford (1976). Enzyme activities were expressed as micromoles of substrate hydrolyzed per minute per mg protein (i.e., U mg^{-1} protein) at 37°C for AP and Lap and at 25°C for trypsin. Amylase activity was expressed as the equivalent enzyme activity which was required to hydrolyze 1 mg of starch in 30 min at 37°C, while the leu–ala peptidase activity was expressed as nanomoles of substrate hydrolyzed per minute per mg protein (i.e., U mg^{-1} protein) at 37°C. The amino acid composition of *Artemia* enriched with free methionine was analyzed according to the recommendations of the AOAC (1995).

Hormone analyses

Cholecystokinin extraction   Lyophilized larvae were transferred to tared Eppendorf tubes and their weights determined using a microbalance (Precisa XB 220A; ±0.1 mg). The tubes were then submerged into liquid nitrogen and the frozen tissues finely ground with a fitted motorized pestle. One milliliter of methanol was added to the pulverized samples, and the mixture was then vortex-mixed for 30 s and left standing for at least 30 min on the bench at 4°C before extraction. The tubes were centrifuged at a minimum of 6000g (15 min, 4°C), following which the methanol supernatants [for radioimmunoassay (RIA) of CCK] were carefully transferred to clean Eppendorf tubes. The methanol supernatants were evaporated using a speed Vac, and the remaining dried extracts were used for the RIA CCK. The kit reagent was brought to room temperature before being used and reconstituted as specified by the supplier. Standards containing 0.78–25 pmol CCK-8 l^{-1} were prepared in a dilute buffer (0.05 mol l^{-1}, pH 7.4). The dried CCK extracts from the samples of the pooled of gilthead sea bream larvae were solubilized in 0.5–1.0 ml of this buffer before the assay. Aliquots of 200 μl of samples and standards were pipetted in triplicate into 4.5-ml polystyrene cryotubes. The CCK in the extracts was assayed by competitive RIA using anti-CCK-8 sulfate and the trace ^{125}I-CCK-8 (Rojas-Garcia et al. 2001). The antibody-bound tracer was separated from the unbound fraction using double antibody solid phase RIA procedure, and its radioactivity was measured in a gamma counter for 3 min. The CCK content was calculated as pmol per liter by interpolation.

Bombesin   Bombesin contents were determined according to Kolkovski (1995). Frozen larvae (previously washed with double-distilled water and frozen in liquid nitrogen) were defrosted and kept at 4°C during the study. The larvae were mixed with buffer phosphate that includes EDTA (7.2 mg ml^{-1}) and Trasylol (500 KIU ml^{-1}; Sigma, St. Louis, MO) at the ratio of 1:50 and homogenate using Ultra Turax homogenizer (Ultra Turax T-25, IKA) with 15-s intervals to prevent temperature rise. The homogenate was filtered through sintered glass filter (Kimax #2) and centrifuged (Sorvall Combi Plus, cooler centrifuge; Thermo Fisher Scientific, Waltham, MA) at 25,000 g for 30 min. The supernatant was then frozen at −80°C. Bombesin activity was detected using a RIA test kit (Bombesin RIA-7113; Penninsula Laboratories, Belmont, CA). The antigen in the kit is specific to bombesin originating in frog skin. The kit is specific for mammalian gastrin-releasing peptide (GRP) with an efficiency of 50%.

Statistical analysis

Data obtained from the study were analyzed according to two factors repeated measured treatment plan. Differences were considered to be significant at $P < 0.05$. The Bonferroni test for differences between averages for each treatment period was performed. The SPSS ver. 9.0 statistical program were used to evaluate data (SPSS 1993).

Results

The content of free methionine in *Artemia* nauplii after 16 h of direct enrichment in 5.3 mM of free methionine was higher than that in the unenriched nauplii (control) (Fig. 1).
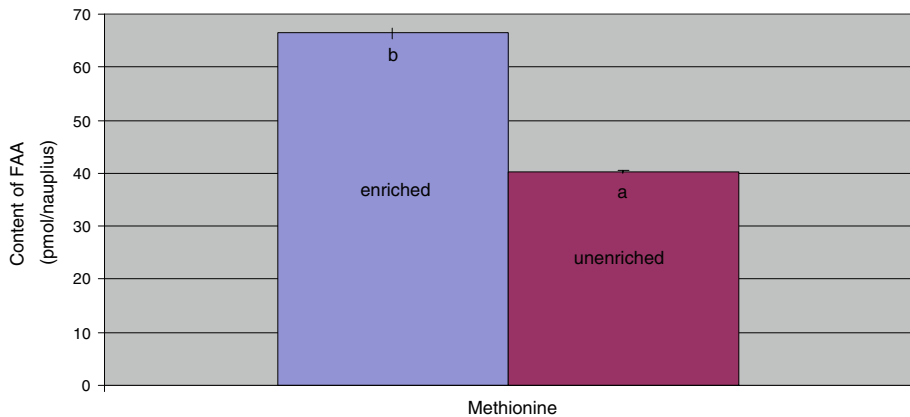
Fig. 1 Content of free methionine in *Artemia* nauplii after 16 h of direct enrichment in 5.3 m*M* of free methionine compared with the unenriched nauplii (control). The *letters a* and *b* indicate to significant differences ($P < 0.05$). FAA—Free Amino Acids

The growth values are shown in Table 1. Larvae of the control group had a significantly lower growth than those of the methionine group at the end of the study ($P < 0.05$). Also, the differences between periods and period × treatments interactions were statistically significant ($P < 0.05$).

The length values of the experimental groups are given in Table 2. Larvae that were fed on the methionine-enriched *Artemia* nauplii obtained a higher mean total length than those of the control group, but the difference was not statistically significant ($P > 0.05$). However, the differences between periods and period × treatment interactions were statistically significant ($P < 0.05$).

Protein contents of the treatment groups are given in Table 3. The results showed that the differences between the control and treatment groups, periods, and period × treatments interactions were statistically significant ($P < 0.05$). The larvae fed methionine-enriched *Artemia* nauplii had the highest protein value at the end of the study.

Amylase, leu–ala, Lap, AP, trypsin enzyme activities, and Lap/leu–ala ratios are given in Tables 4, 5, 6, 7, 8, and 9, respectively.

The differences between treatments, periods, and period × treatment interactions were statistically significant for the amylase, leu–ala peptidase, Lap, and trypsin enzymes and for the Lap/leu–ala ratios ($P < 0.05$). The results revealed that *S. aurata* larvae fed on the methionine-enriched *Artemia* nauplii had the highest leu–ala peptidase and Lap values but not the highest amylase activity. The highest trypsin activity was observed in the control group ($P < 0.05$). The results on AP activity demonstrated that the differences between the treatment groups and period × treatment interactions were not statistically significant ($P > 0.05$). However, the differences between the periods were statistically significant ($P < 0.05$). Table 9 presents the ratios in the activity of the brush border (BB) peptidase (Lap) and cytosolic peptidase (leu–ala); this ratio is known to be a good indicator of digestive capacity. The highest Lap/leu–ala peptidase ratio was observed in the control group ($P < 0.05$).

Bombesin activities in 5th and 15th minutes after the initiation of feeding are given in Tables 10 and 11, respectively. A significant difference between the treatments groups was not found at 5th minute after the initiation of feeding ($P > 0.05$). However, the differences

Table 1 The average weights according to period and treatment group of *Sparus aurata* larvae fed on *Artemia* nauplii enriched or not enriched with free methionine

Treatment groups	Average weight (mg) in period:[A]				T_{ave}
	I	II	III	IV	
Methionine	2.97 ± 0.01	4.31 ± 0.02	12.86 ± 0.14	28.58 ± 0.34	12.18 ± 3.07[b]
Control	2.98 ± 0.00	4.30 ± 0.03	11.53 ± 0.18	26.60 ± 0.01	11.35 ± 2.82[a]
P_{ave}	2.98 ± 0.00[a]	4.30 ± 0.01[b]	12.19 ± 0.31[c]	27.59 ± 0.47[d]	

Values (mean ± standard error) followed by different letters in the same column and rows are statistically different

T_{ave}, Averages of treatment groups; P_{ave}, averages of periods

[A] I, II, III, and IV, Days 28, 32, 36, and 40, respectively, of the experimental period

Table 2 The average lengths according to period and treatment group of *S. aurata* larvae fed on *Artemia* nauplii enriched or not enriched with free methionine (mean ± standard error) (mm)

Treatment groups	Average length (mm) in period:				T_{ave}
	I	II	III	IV	
Methionine	7.02 ± 0.00	7.68 ± 0.09	10.39 ± 0.00	13.27 ± 0.02	9.59 ± 0.74[a]
Control	6.92 ± 0.01	7.76 ± 0.08	10.70 ± 0.00	12.91 ± 0.01	9.57 ± 0.71[a]
P_{ave}	6.97 ± 0.02[a]	7.72 ± 0.05[b]	10.55 ± 0.07[c]	13.09 ± 0.08[d]	

Values (mean ± standard error) followed by different letters in the same row are statistically different

Table 3 Protein averages according to period and treatment group of *S. aurata* larvae fed on *Artemia* nauplii enriched or not enriched with free methionine (mean ± standard error)

Treatment groups	Protein content (µg protein/larva) in period:				T_{ave}
	I	II	III	IV	
Methionine	7.77 ± 0.03	8 ± 0.00	23.60 ± 0.01	55.96 ± 0.02	23.83 ± 5.91[b]
Control	7.85 ± 0.05	8 ± 0.00	20.94 ± 0.01	50.50 ± 0.00	21.82 ± 5.24[a]
P_{ave}	7.81 ± 0.03[a]	8 ± 0.00[b]	22.27 ± 0.59[c]	53.23 ± 1.22[d]	

Values (mean ± standard error) followed by different letters in the same column and row are statistically different

Table 4 Average amylase enzyme values according to period and treatment group of *S. aurata* larvae fed on *Artemia* nauplii enriched or not enriched with free methionine

Treatment groups	Average amylase enzyme values (U mg^{-1} protein) in period:				T_{ave}
	I	II	III	IV	
Methionine	2.54 ± 0.08	1.84 ± 0.08	1.4 ± 0.01	6.53 ± 0.12	3.08 ± 0.61[a]
Control	2.68 ± 0.14	22.2 ± 0.28	25.84 ± 0.6	11 ± 0.41	15.43 ± 2.77[b]
P_{ave}	2.61 ± 0.07[a]	12.03 ± 4.55[c]	13.62 ± 5.47[c]	8.77 ± 1.01[b]	

Values (mean ± standard error) followed by different letters in the same column and row are statistically different

Table 5 Average leucine alanine peptidase enzyme values according to period and treatment group of *S. aurata* larvae fed on *Artemia* nauplii enriched or not enriched with free methionine

Treatment groups	Average leucine alanine peptidase enzyme values (U mg^{-1} protein) in period:				T_{ave}
	I	II	III	IV	
Methionine	351.58 ± 0.75	1486.94 ± 2.62	2739.61 ± 5.19	2557 ± 18.7	1783.78 ± 288.07[b]
Control	351.2 ± 0.65	757.78 ± 2.84	1044.94 ± 3.18	2945.50 ± 3.57	1274.86 ± 300.16[a]
P_{ave}	351.39 ± 0.45[a]	1122.37 ± 163.05[b]	1892.28 ± 378.94[c]	2751.25 ± 87.28[d]	

Values (mean ± standard error) followed by different letters in the same column and row are statistically different

Table 6 Average aminopeptidase N enzyme values according to period and treatment group of *S. aurata* larvae fed on *Artemia* nauplii enriched or not enriched with free methionine

Treatment groups	Average aminopeptidase N enzyme values (mU mg^{-1} protein) in period:				T_{ave}
	I	II	III	IV	
Methionine	448.05 ± 0.92	1051.46 ± 7.18	1398.85 ± 4.59	1188.33 ± 1.74	1021.67 ± 106.61[b]
Control	449.17 ± 1.6	782.98 ± 7.5	1046.06 ± 3.15	1378.49 ± 4.37	914.18 ± 102.97[a]
P_{ave}	448.61 ± 0.86[a]	917.23 ± 60.21[b]	1222.45 ± 78.92[c]	1283.41 ± 42.57[d]	

Values (mean ± standard error) followed by different letters in the same column and row are statistically different

Table 7 Average alkaline phosphatase enzyme values according to period and treatment group of *S. aurata* larvae fed on *Artemia* nauplii enriched or not enriched with free methionine

Treatment groups	Average alkaline phosphatase enzyme values (mU mg^{-1} protein) in period:				T_{ave}
	I	II	III	IV	
Methionine	430.62 ± 1.21	433.1 ± 2.03	375.35 ± 42.17	776.85 ± 88.8	503.98 ± 52.38[a]
Control	431.6 ± 1.46	513.05 ± 2.09	594.2 ± 64.09	879.1 ± 104.76	604.49 ± 57.19[a]
P_{ave}	431.11 ± 0.88[a]	473.07 ± 17.92[b]	484.78 ± 59.76[a, b]	827.98 ± 65.53[c]	

Values (mean ± standard error) followed by different letters in the same row are statistically different

Table 8 Average trypsin enzyme values according to period and treatment group of *S. aurata* larvae fed on *Artemia* nauplii enriched or not enriched with free methionine

Treatment groups	Average trypsin enzyme values (mU/mg protein) in period:				T_{ave}
	I	II	III	IV	
Methionine	108.27 ± 0.28	93.16 ± 0.97	90.39 ± 0.58	181.69 ± 3.36	118.38 ± 11.23[a]
Control	108.27 ± 0.55	143.99 ± 0.84	159.6 ± 1.01	138.61 ± 1.23	137.62 ± 5.62[b]
P_{ave}	108.27 ± 0.27[a]	118.57 ± 11.38[b]	124.99 ± 15.48[c]	160.15 ± 9.76[d]	

Values (mean ± standard error) followed by different letters in the same column and row are statistically different

Table 9 Average aminopeptidase N/leucine alanine peptidase values according to period and treatment group of *S. aurata* larvae fed on *Artemia* nauplii enriched or not enriched with free methionine

Treatment groups	Average aminopeptidase N/leucine alanine peptidase values (Lap × 1000/leu–ala) in period:				T_{ave}
	I	II	III	IV	
Methionine	1274.38 ± 1.65	707.15 ± 6.07	510.61 ± 2.42	464.77 ± 2.88	739.23 ± 97.13[a]
Control	1278.94 ± 4.45	1033.25 ± 9.38	1001.06 ± 0.66	467.99 ± 1.23	945.31 ± 89.21[b]
P_{ave}	1276.66 ± 2.36[d]	870.2 ± 73.08[c]	755.83 ± 109.67[b]	466.38 ± 1.57[a]	

Values (mean ± standard error) followed by different letters in the same column and row are statistically different

Table 10 Bombesin activity values at 5th minute after the initiation of feeding of seabream larvae fed on either *Artemia* nauplii enriched with free methionine or unenriched *Artemia* (mean ± standard error)

Treatments groups	Bombesin activity values (pg mg^{-1} protein) in period:				T_{ave}
	I	II	III	IV	
Methionine	0.37 ± 0.00	0.42 ± 0.00	0.45 ± 0.00	0.32 ± 0.00	0.39 ± 0.01[a]
Control	0.63 ± 0.01	0.37 ± 0.00	0.3 ± 0.01	0.33 ± 0.00	0.41 ± 0.03[a]
P_{ave}	0.5 ± 0.05[c]	0.4 ± 0.01[b]	0.37 ± 0.03[b]	0.33 ± 0.00[a]	

Values (mean ± standard error) followed by different letters in the same row are statistically different

Table 11 Bombesin activity values at 15th minute after the initiation of feeding of seabream larvae fed on either *Artemia* nauplii enriched with free methionine or unenriched *Artemia*

Treatment groups	Bombesin activity values (pg mg^{-1} protein) in period:				T_{ave}
	I	II	III	IV	
Methionine	0.31 ± 0.00	0.48 ± 0.01	0.51 ± 0.01	0.31 ± 0.00	0.4 ± 0.02^b
Control	0.42 ± 0.00	0.34 ± 0.00	0.21 ± 0.00	0.21 ± 0.00	0.3 ± 0.02^a
P_{ave}	0.37 ± 0.02^b	0.41 ± 0.03^c	0.36 ± 0.06^b	0.26 ± 0.02^a	

Values (mean ± standard error) followed by different letters in the same column and row are statistically different

Table 12 Average cholecystokinin hormone values according to periods and treatment groups of *S. aurata* larvae fed on *Artemia* nauplii enriched or not with free methionine

Treatment groups	Average cholecystokinin hormone values (fmol/mg dry weight) in period:				T_{ave}
	I	II	III	IV	
Methionine	1.54 ± 0.00	0.83 ± 0.02	0.65 ± 0.15	1.92 ± 0.14	1.24 ± 0.16^a
Control	1.55 ± 0.02	1.56 ± 0.01	1.7 ± 0.15	1.51 ± 0.13	1.58 ± 0.05^b
P_{ave}	1.55 ± 0.01^a	1.2 ± 0.16^a	1.18 ± 0.25^a	1.71 ± 0.12^a	

Values (mean ± standard error) followed by different letters in the same column are statistically different

at 15th minute after the initiation of feeding were statistically significant ($P < 0.05$). Bombesin hormone values also demonstrated that the differences between the periods and period × treatment interactions were statistically significant ($P < 0.05$). These results revealed that bombesin activities tended to be higher in the larvae fed on the live prey enriched with free methionine than in the control group except at the 5th minute point on day 28.

CCK results are given in Table 12. The results indicate that CCK levels tended to be lower in the larvae fed on the live prey enriched with free methionine groups than in those of the control group except on day 40. A significant difference between the treatments groups was found ($P < 0.05$). However, the differences between the periods and period × treatment interactions were statistically insignificant ($P > 0.05$).

Discussion

In order to follow the development of the intestinal digestive functions of *S. aurata* larvae, we analyzed two peptidases that are mainly found in two different areas of the cell: 90% of leu–ala is located in the cytosol, whereas Lap is primarily found in the BB membranes (Nicholson et al. 1974). The decrease in leu–ala activity observed on day 40 in the sea bream larvae fed on *Artemia* nauplii enriched with free methionine suggests a decrease in the intracellular digestion of this peptide associated with the maturation of enterocystes. This developmental process has been described in mammals during the first post-natal week (Himukai et al. 1980; Henning 1987). In contrast, the high level of leu–ala activity in

the control group shows that the intestinal changes of larvae fed *Artemia* nauplii that had not been enriched in free methionine remained the same on day 40.

Since all physiological parameters vary during ontogenesis, the activity of only the cytosolic enzyme (leu–ala) is not enough to account for the evolution of the maturation of enterocytes involved in digestion. The importance of peptide digestion in the BB membranes compared to that in the intracellular compartment can be expressed by the ratio of the activities of BB peptidases (Lap) and cytosolic peptidase (leu–ala). The enzymatic activity ratio of Lap to leu–ala peptidase (Lap/leu–ala) is known to be a good indicator of digestive capacity (Cahu and Zambonino Infante 1995a). The increase in these ratios in the control groups compared that in the larvae of the free methionine groups during the experimental period is consistent with an increase in the digestive capacity of the BB during larval development. However, the ratios observed in the experimental groups on day 40 revealed that the development of the digestive capacities of seabream larvae fed on the live prey enriched with free methionine was similar to that of the control group. Our results indicate that the larval feeding on live prey enriched with the free methionine tended to stimulate the intestinal maturation—in comparison to the sharp decreases observed in the control group, especially from day 36 to 40. On the other hand, leu–ala is perhaps not a good indicator of intracellular digestion, while Lap may be a good indicator of the development of the BB membrane (Kvale et al. 2007). In other studies, leu–ala levels failed to show the expected decrease in both halibut and cod, while the expected decrease of leu–ala was found in Senegalese sole (*Solea senegalensis*) and sea bass (*Dicentrarchus labrax*) between 20 and 30 days (Ribeiro et al. 1999; Zambonino Infante and Cahu 2001). Our results are supported by those of Kvale et al. (2007).

Previous studies have reported that trypsin levels are related to the protein content in the lumen (Tseng et al. 1982; Cahu et al.1999), whereas this regulatory process may not be functional in younger larvae (Peres et al. 1996). Our results revealed that the amounts of tryptic activity in the control group were higher than that in the free methionine group until day 36. However, at day 40, tryptic activity tended to be higher in the seabream larvae fed on the live prey enriched with the free methionine than in those of the control group. These findings indicate that *Artemia* nauplii enriched with free methionine have no effect on trypsin activities of seabream larvae until day 36. From this date onwards, free methionine had a positive effect on the mechanism controlling the adaptation of the trypsin activity in seabream larvae.

It is know that AP activity is stimulated by phosphorylated substrates, such as phosphoproteins and phospholipids (Shirazi et al. 1978; McCarthy et al. 1980). *Artemia* contains approximately 1% organic phosphorus. Our study showed that the effect of methionine was not clear since the live prey used in the feeding of larvae had a similar organic phosphorus content.

In general, amylase activity is stimulated by dietary change—i.e., the dietary starch content can modulate changes in amylase activity (Peres et al. 1998; Sheele 1993). The starch contents of the live prey used in the experimental groups were similar. Our results revealed that the variations in amylase activities were not due to the dietary components but to genetical programming during the larval development.

Studies on humans have revealed that the most potent stimulants of CCK secretion are the partial digestion products of fat and protein, including di- and tri-peptides (Liddle 2000). In contrast, based on a study of the first-feeding stages of Atlantic herring, Koven et al. (2002) reported that soluble protein caused a more rapid and greater increase in CCK content (whole body homogenate) than FAA. In addition, tryptic activity increased in larvae fed protein, while no changes occurred in larvae fed FAA (Koven et al. 2002).

Another study on seabass (*Dicentrarchus labrax*) showed that trypsin secretion was stimulated in larvae fed a mixture of FAA, while a protein hydrolysate (casein) actually reduced secretion (Cahu and Zambonino Infante 1995a, b). It has been demonstrated that digestive end-products, such as L-lysine, can act directly on the pancreatic acinar cells to stimulate enzyme secretion (Grendell and Rothman 1981). Tryptophan and phenylalanine are also potent amino acids capable of stimulating CCK secretion (Liddle 2000). The two latter studies support the notion that FAA are themselves stimulants for pancreatic exocrine secretions. Our results for day 40 revealed that, in support of these findings, free methionine content in the live prey of the larvae triggered native endocrine factors to complete the digestive process in the developing and not fully functional larval digestive tract.

Kolkovski et al. (1997) showed that the level of bombesin increased by 300% when *Artemia* nauplii were given as the sole food to seabream larvae, compared to the levels that were found in the larvae offered only as a microdiet. However, the nutrient factors in *Artemia* responsible for eliciting this endocrine response were not determined by Kolkovski et al. (1997). Our results indicated that the free methionine content in *Artemia* nauplii could not solely explain this endocrine response.

In conclusion, research is still inadequate in terms of understanding the digestive enzymes and hormones in marine fish larvae. When such data becomes available, they will serve as the basis for developing a microdiet that is easily accepted and digested by the larvae from the onset of exogenous feeding. However, based on our results, we present two main concepts. Firstly, bombesin and cholecystokinin activities can be stimulated by free methionine. Secondly, the mechanisms controlling the adaptation of the activity of the trypsin to the amount of dietary protein were not active until day 36. We hypothesize that if the mechanism controlling the activity of the trypsin could be stimulated earlier, then the replacement of live prey with a microdiet could be successful. For this reason, the effects of the different molecular forms of the dietary nitrogen sources used in the microdiets on the tryptic activities of marine larvae should be investigated in future studies.

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