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Expression and activities of pancreatic enzymes in developing sea bass larvae (Dicentrarchus labrax) in relation to intact and hydrolyzed dietary protein; involvement of cholecystokinin

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Abstract:

In order to assess the influence of dietary protein on digestive enzyme expression and cholecystokinin (CCK) content in sea bass larvae, four groups of larvae were fed experimental diets from mouth opening until day 42: three isonitrogenous diets with increasing protein hydrolysate levels (0%, 14% and 46% of crude matter) and one diet incorporating starch. The groups fed high starch or high protein hydrolysate level exhibited the lowest growth. The final weight in these groups was 9.5 and 5.6 mg, respectively, whereas it reached approximately 20.0 mg in the groups fed 0% or 14% protein hydrolysate level. The highest levels of trypsin secretion were observed in larvae fed the lowest protein hydrolysate level. Paradoxically, the groups fed diet containing starch also exhibited a high trypsin secretion level. There was a clear allometric relationship between larval CCK content and body mass, but there were also differences between dietary groups. On day 42, the CCK level in the group fed diet incorporating starch (40 fmol/mg dry weight of larvae) was more than twice as high as that found in the other groups. The lowest CCK level (13 fmol/mg) was found in the group fed the highest protein hydrolysate level. Our data suggested that dietary protein level and chain length combined with protein intraluminal proteolytic activity regulate the CCK level in fish larvae as in other vertebrates. The CCK concentration assayed in larvae fed diets with a low protein content or different protein hydrolysate levels is compatible with the existence of an indirect mechanism controlling CCK release and mediating pancreatic enzyme secretion.

Keywords: Cholecystokinin; Pancreatic enzyme secretion; Protein hydrolysate; Sea bass larvae; Starch

1. Introduction

Marine fish larvae are not completely developed at hatching and they undergo major morphological and functional changes during the first weeks of life. A proper development of the digestive tract is essential for the transition from an endogenous to exogenous feeding (Boulhic and Gabaudan, 1992; Walford and Lam, 1993). The pancreas acquires progressively its enzymatic capacity (Krogdahl and Sundby, 1999) and its secretory function (Beccaria *et al.,* 1991). Recent studies demonstrated that the developmental process of pancreas could be affected by the composition of the exogenous diet (Zambonino Infante and Cahu, 1994; Perés *et al.,* 1996).

In adult fish and in mammals, cholecystokinin (CCK) plays a major role in the pancreatic enzyme secretion together with neural stimulation (Singer 1993). It is produced in specific cells scattered in the mucosa of the proximal intestine and is secreted into the plasma in response to the presence of nutrients in the lumen (Liddle, 1997). The existence of an endogenous trypsin-sensitive luminal CCK releasing factor was demonstrated in 1989 in rats (Liddle, 1995). This factor is modulated by the nature and chain length of dietary protein (Owyang, 1994), which interacts with and regulates the CCK-secreting cells of the intestine (Liddle, 1995). In rats, CCK secretion is more stimulated by intact proteins than hydrolyzed proteins or amino acids (Green and Miyasaka, 1983), while it is less stimulated by carbohydrates than fat or proteins (Liddle *et al.,* 1986).

It is not known when these mechanisms controlling pancreatic secretion become effective in developing animals. Marine fish larvae constitute an interesting animal model since they acquire progressively their adult mode of digestion. There are very few studies on CCK in fish larvae, and research on this topic is still in its infancy. CCK-producing cells have been

detected from the first day post-hatch in Japanese flounder, *Paralichthys olivaceus* (Kurokawa *et al.,* 2000), tuna, *Thunnus thynnus*, (Kamisaka *et al.,* 2002) and ayu, *Plecoglossus altivelis* (Kamisaka *et al.,* 2003), while CCK has not been detected in the gut at the onset of exogenous feeding in Atlantic halibut, *Hippoglossus hippoglossus*, (Kamisaka *et al.,* 2001, Rojas-García and Rønnestad, 2002). Nevertheless, in older animals, short term experiments performed with tube-fed larvae have strongly suggested the involvement of this hormone in protein digestion in post-larval Atlantic halibut (Rojas-García and Rønnestad, 2002) and also in herring larvae from onset of exogenous feeding (Koven *et al.,* 2002).

Our experiment aimed to study the expression of two major pancreatic enzymes and to assess the involvement of CCK in the regulation of their secretion, during the development of sea bass larvae fed different dietary protein concentration and chain length.

2. Materials and methods

2.1. Animals and diets

Eggs of European sea bass (*Dicentrarchus labrax*) were obtained from *Aquanord.* The larvae were reared at the *Ifremer-Station de Brest* during 42 days, a period which corresponds to the end of larval development. Newly hatched larvae were transferred from incubators to 16 conical fiber-glass tanks (35 L) with black walls (initial stocking density: 60 larvae $\cdot$ L^{-1}, i.e. 2100 larvae per tank). They were supplied with running sea water (20°C; salinity: 35 g $\cdot$ L^{-1}), which had been filtered through a sand filter, then passed successively through a tungsten heater and a degassing column packed with plastic rings. Water exchange rate was up to 30% per hour (flow rate: 0.18 L $\cdot$ min^{-1}) to maintain oxygen level above 6 mg $\cdot$ L^{-1}. The light intensity was a maximum of 9 W $\cdot$ m^{-2} at the surface. All animal procedures and handling were conducted in compliance with the Guide for the Care and Use of Laboratory Animal (NRC 1985).

Larvae were divided into four groups (four tanks per group) before mouth opening (d 6 post-hatching). They were fed from d 6 to d 42 post-hatching on one of four diets (Table 1): three isonitrogenous diets with increasing protein hydrolysate levels (NAP, LHY and HHY) and one diet incorporating starch (SAP). The size of the dietary microparticles was 125-200 µm during the first 5 d, then 200-400 µm. Fish were continuously fed to large excess for 18 hours per day by means of a belt feeder. Larval digestive tracts were observed under a binocular microscope in order to monitor food ingestion, since microparticles are visible through the transparent larvae.

2.2. Samplings and dissection

Water volume in tanks was reduced and ten larvae (n=4 tanks for each dietary group) were taken from each tank in one single stroke using a net, for determination of wet mass at the beginning and the end of the experiment. These larvae were representative of the tank population. At the end of the experiment, the remaining larvae were counted to determine survival rates. On d 25 and d 42, larvae were collected from each tank for enzymatic studies (50 larvae) or CCK determinations (15 larvae) before the morning food distribution; they were immediately stored at -80°C pending dissection and assays or lyophilization and shipment to Norway. Fifty more larvae were collected for mRNA studies from only three tanks per dietary treatment were dissected and RNA were immediately extracted.

Larvae were dissected into four parts under microscope on a glass slide maintained at 0°C; 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. The dissection

enables distinguishing between pancreatic (synthesized enzymes) and intestinal (secreted enzymes) activities (Zambonino Infante *et al*.1997).

2.3. Analytical methods

The pancreatic and intestinal segments were homogenized into five volumes (v/w) of ice-cold distilled water. Amylase (EC 3.2.1.1) activity and trypsin (EC 3.4.21.4) were assayed according to Métais and Bieth (1968) and Holm *et al.* (1988) respectively. Enzyme activities were expressed as specific activities, i.e. mU · mg protein^{-1} Secretion of pancreatic enzymes was calculated as follows: enzyme assayed in the intestinal segment (mU/segment) divided by enzyme assayed in pancreatic plus intestinal segment (Zambonino Infante and Cahu, 1999). Protein was determined according to Bradford (1976).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Total RNA was extracted using the TRIzol® reagent kit procedure (Life Technologies, Grand Island, NY). For synthesis of cDNA, 5 µg of total RNA was treated with FPLC*pure* Moloney-Murine Leukemia virus reverse transcriptase using the Ready-To-Go-T-Primed First Strand Kit (Pharmacia Biotech, Uppsala, Sweden). PCR was carried out by an initial denaturation at 94°C for 1 min, followed by 30 cycles at temperatures of 94°C for 30 sec, specific annealing temperatures for 1.5 min, and 72°C for 1 min; the final extension was conducted at 72°C for 7 min. The PCR mixture contained 0.8 µL of the cDNA, 0.2 Units Taq Polymerase (Appligene, Gaithersburg, MD), 100µmol/L dNTP, 50pmol of each primer and 1X Reaction Buffer (Appligene), in a final volume of 50µL. Sequences and annealing temperatures for the sense and antisense oligonucleotides were as follows: 5'-GCCATCAATGACCCCTT-3' and 5'-GGTGCAGGATGCATTGC-3' (50°C) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-AAGCACATGTGGCCY-3' and

5’-CCAGCGCCACTCRAA-3’ (54°C) for amylase and 5’-CAGGTGTCTCTGAAC-3’ and 5’-CCCARGACACAACACCCTG-3’ (60°C) for trypsin, respectively.

These primers were selected after alignment of sequences from different species of selected mRNA, obtained using the Sequence Retrieval System WWW server at EMBL-EBI (Cambridge, UK). Alignments of cDNA sequences of species from different phyla were necessary as fish RNA sequences were scarce. The resulting PCR products were cloned using the TOPO-TA Cloning Kit (Invitrogen, Leek, The Netherlands) and sequenced by Cybergene (St Malo, France); the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), amylase and trypsin sequences obtained have been registered by EMBL under accession numbers AJ006883, AJ310653 and AJ006882 respectively.

GAPDH, amylase and trypsin were separately amplified using the same cDNA sample; 1 to 10 µl of each PCR product were applied on a 1.2% agarose-1mg/L ethidium bromide gel. cDNA bands were quantified using a Fluor-S^{TM} Multi-imager and its MultiAnalyst Software (BIORAD, Hercules, CA), using an appropriate calibration (Freeman *et al.,* 199). We generated for each gene a standard curve by plotting the UV absorbance of the bands, resulting from a 30 cycle PCR, against a known start concentration of the studied cDNA (using at least 7 different dilutions of the studied cDNA). We determined the limits of the exponential phase and the beginning of the saturation phase of the amplification reaction; this enabled us to ensure that there was a linear relationship between input RNA and final RT-PCR product, or to maintain it by an appropriate dilution of the input cDNA. The number of cycles has been optimized in order to produce easily visualized products and to remain out of the plateau phase of the reaction. We also sequenced the amplified cDNA products obtained in gels in order to control they were derived from amylase and trypsin.

The values of cDNA obtained were normalized relative to the GAPDH cDNA, by calculating the amylase/GAPDH and trypsin/GAPDH ratios. Indeed, Sölch and Arnold (1996) have shown that this normalization relative to GAPDH provides a widely applicable value for comparative studies of gene expression at the mRNA level.

Radio-immunoassay (RIA).

Lyophilized individual larvae were transferred to tared Eppendorf tubes and weighed; CCK was subsequently extracted in methanol as described by Rojas-García *et al.,* (2001). The methanol supernatants were evaporated and the remaining dried extracts analysed for CCK by a competitive radio-immunoassay (RIA) kit (EURIA-CCK, Euro-Diagnostica, Malmö Sweden) using a non-piscine antiserum anti-CCK-8 with high specificity for sulphated forms of CCK-8, CCK-22, CCK-33 and CCK-58 (Rehfeld 1998). CCK levels in the samples were calculated in pmol· L^{-1} by interpolation from the standard curve with corrections for non-specific binding and recovery determined for every run. CCK is presented as fmol · ind^{-1} and fmol · mg^{-1} Dry Matter ($\pm$ SD)

2.5. Statistical analyses.

Results are given as mean $\pm$ SD (n = 4; n = 3 for mRNA studies; n = 4 to 6 for CCK). Survival rates, malformation rates and ratios of mRNA were arcsin($x^{1/2}$) transformed. The variance homogeneity of the data was checked using Bartlett's test (Dagnelie, 1975). Weight, CCK, survival rate and ratios of enzymatic segmental activity data were compared by one-way ANOVA followed by the Newman Keuls multiple range test (Dagnelie, 1975) when significant differences were found at the 0.05 level.

3. Results

3.1. Rearing performances

The microdiets were efficiently ingested by the larvae of the four dietary treatments from starting exogenous feeding. Larval growth was more than two times as high in groups fed diets that incorporated native protein (NAP) and low hydrolysate levels (LHY) as in the two other groups (Table 2). Survival on day 42 was significantly lower in the group fed a high hydrolysate level diet (HHY).

3.2. Enzyme expression

Lower trypsin activity levels (Table 3) were observed in the homogenates of pancreatic segment on day 25 as well as on day 42 in the group fed diet incorporating starch and low protein level (SAP). At day 42, only the two diets incorporating a high percentage of native protein (NAP and LHY) induced high trypsin activity. In the same way, diets NAP and LHY also induced high level of trypsin-coding RNA on day 42. The lowest level of trypsin activity assayed in intestinal segments was observed in the group fed the SAP diet on days 25 and 42 (Fig. 1). The highest amylase activities were recorded on day 25 and day 42 in the group which was fed the diet incorporating starch (SAP). Differences in amylase/GAPDH ratios only appeared on day 42: groups fed SAP and HHY exhibited the highest ratios, while the NAP group showed the lowest (Table 3).

3.3. Variation in CCK related with variations in pancreatic secretion

There was an allometric increase in larval CCK content with development. Expressed on a weight-specific basis the CCK content displayed a significant (second-order polynomial ANCOVA using dry weight as covariate, $f=64.05$; $p<0.0001$) decline with development (Fig. 2). On day 25, the highest level of CCK was assayed in the NAP and LHY groups (Fig 3A);

when expressed as fmol·mg^{-1} dry weight, a high level of CCK was found in the SAP group (Fig. 3B). On day 42, the CCK level/mg dry weight in the group fed the SAP diet was more than twice as high as that found in fish of the other dietary groups collected on the same date (Fig. 3B). The lowest CCK level was found in the group fed HHY diet. The same trend appeared when CCK was expressed in terms of fmol per larvae. The highest levels of trypsin secretion were observed in larvae fed the highest levels of native protein (NAP and LHY) and the lowest level was observed in the group fed a high level of hydrolysate (HHY). Paradoxically, the group which was fed SAP exhibited a level of trypsin secretion very much higher than that of the HHY group (Fig. 3C). On day 25, amylase secretion was the highest in the SAP group, while on day 42, the HHY group exhibited a rate of amylase secretion significantly lower ($P<0.05$) than that of the three other groups (Fig. 3D).

4. Discussion

The purpose of this experiment was to investigate the mechanisms that regulate exocrine pancreatic secretion, in particular, the involvement of CCK, during sea bass development. Nutrient delivery into the intestinal lumen is the most important stimuli of exocrine pancreatic secretion (Liddle *et al.*, 1986). Digestive products of dietary protein, i.e. protein hydrolysates and amino acids, modulate the action of pancreatic proteases, and particularly trypsin, which in turn control CCK release in mammals (Owyang, 1994). We decided to formulate 3 isoproteic diets containing different amount protein hydrolysates and evaluate the consequent effect on CCK release and pancreatic secretion. An additional diet containing a low level of protein was also considered in order to assess the influence of the dietary protein content. In this diet, SAP, the lowering in protein level was compensated in energy by a moderate supply of starch. Indeed, a previous study demonstrated that 17% starch incorporation in diet allowed a good growth and survival in sea bass larvae, when higher starch levels negatively affected

these parameters (Péres et al. 1996). It must be pointed out that the amount of fish meal and hydrolysed fish meal (CPSP G) used in each diet provided enough n-3 polyunsaturated fatty acid (around 1.5% of diet dry matter) to sustain larval growth and survival (Cahu *et al.*, 2003).

As expected, the growth rate of sea bass larvae was depressed in the group that was fed a diet that contained only 47% protein (SAP), and in the group fed a diet containing a high concentration of hydrolysate (HHY). These effects were not due to a low ingestion of the diet. In fact, it had previously been shown that optimal growth and survival in sea bass are obtained on a diet that contains 50-60% protein (Perés *et al.,* 1996). Moreover, high levels of hydrolysate in the diet have negative effects on both growth and survival, as demonstrated by Zambonino Infante *et al.,* (1997) and Cahu *et al.,* (1999). Several studies had shown that the inclusion of a moderate proportion of protein hydrolysate improves larval development in several species of fish such as carp (Carvalho *et al.,* 1997), goldfish (Slaminska *et al.,* 1991), sea bream (Kolkovski and Tandler, 2000), and sea bass (Cahu *et al.,* 1999). Nevertheless, growth was efficient in the four groups, allowing valuable physiological studies to be obtained.

This study suggested that, as early as day 25, trypsin activity in larvae is modulated at the translational level by the protein content of the diet. This result is original for marine fish larvae. Indeed, the fact that Perés *et al.* (1996; 1998) failed to demonstrate such regulation in early stages of sea bass may have been a consequence of the poor growth of larvae in that study. On day 42, a transcriptional control was evidenced in trypsin regulation by the dietary protein content. Several studies have shown that trypsin regulation is mainly transcriptional in vertebrates (Wicker *et al.,* 1983; Brannon 1990). Lhoste *et al.* (1994) demonstrated that the nature of protein, casein, soybean or fish meal protein modulates trypsin mRNA transcription. Our study suggests that the chain length of the protein may also modulate trypsin transcription

in sea bass larvae. Additional assays earlier than day 25 and after day 42 would allowed to confirm the switch from translational to transcriptional control of trypsin secretion, depending on the larval developmental stage.

Amylase expression decreases during larval development in several carnivorous marine fish: sea bass (Zambonino Infante and Cahu, 1994), sea bream (Moyano *et al.,* 1996), sole (Ribeiro *et al.,* 1999), red drum (Buchet *et al.,* 2000), winter flounder (Douglas *et al.,* 2000). The changes in amylase expression were not necessarily dietary induced, and could to correspond with the different feeding habits in the wild (Krogdahl and Sundby, 1999). Such a decrease in amylase can be likened to the decrease in lactase which has been described in mammals (Henning, 1987). High lactase activity characterizes the post-natal stages in mammals and its decrease is associated with the maturation of pancreatic functions. Similarly, amylase activity can be regarded as an indicator of pancreas maturation in marine fish larvae (Cahu and Zambonino Infante, 1994). In our study, in the three groups fed isoprotein diets, amylase expression (activity and mRNA levels) was highest on days 25 and 42 in the HHY-group that exhibited the lowest rate of growth. This reveals a delay in the process of pancreas maturation. The high level of amylase activity in the SAP group is the result of both a delay in maturation and a modulation of amylase by its substrate, starch. Our results suggest that the regulation of amylase expression is posttranscriptional in the early stages (day 25) and becomes transcriptional towards the end of the larval period. We can hypothesize that amylase regulation is transcriptional in juvenile fish, as has been reported in developing and adult mammals (Wicker *et al.,* 1984).

Previous studies conducted in fish suggested that CCK expression is both tissue-specific and developmentally regulated, as in other vertebrates. In a recent study only trace amounts of

CCK (0.2 fmol·ind^{-1}) could be demonstrated in the gut of Atlantic halibut one week after the onset of exogenous feeding, and this represented only about 2% of the whole-body content of CCK (Rojas-García and Rønnestad, 2002). However, after a further three weeks of development it increased 100-fold to ca 20 fmol·ind^{-1}; representing 60% of the whole body CCK content (Rojas-García and Rønnestad, 2002). In that study the CCK content of the eviscerated body, which probably derived primarily from sources in the central nervous system (CNS), was kept almost at a constant level of 10-12 fmol·ind^{-1}, regardless of body size during the period studied. Such a finding can be explained by a more rapid increase in non-CCK-producing tissues (i.e. muscle) than in the gut and CNS. The clear allometric relationship between CCK content and body mass in our study (Fig. 2) indicate that a comparable relationship also occurs in developing sea bass.

Despite the large differences between treatments in larval weight at the end of the experiment (Table 2) and the allometric relationship of CCK per dry weight (Fig. 2), statistical evaluation demonstrated significant differences between dietary groups, as reported here. We assume that the differences in CCK levels found in this study were mainly those of the gastrointestinal tract, as the different dietary treatments were the only variable to which the larvae were exposed. Similar assumptions were applied in an experiment on herring larvae by Koven *et al.* (2002) using the same CCK assay method as in the present study. However, it is evident that in order to properly define the role of CCK in digestive function of larval fish, future studies should focus on separating neural and gastrointestinal sources as well as differentiating between the CCK stored in the producing cells (transcription and translation) and CCK release.

Earlier studies on mammals have shown that a rise in dietary protein levels stimulates pancreatic hypertrophy and leads to increased expression of pancreatic enzymes (Green *et al.,* 1986). These changes in pancreatic function are believed to result from elevated plasma CCK concentrations (Green *et al.,* 1985). However, a study of gene knockout mice has shown that CCK is not required as a mediator for the dietary protein-induced increase in pancreatic proteolytic enzymes (Lacourse *et al.,* 1999). This supports earlier notions that not all effects on pancreatic secretion can be attributed to circulating CCK, since other hormones and neural stimulation might also be involved (Liddle, 2000).

In our study, the highest levels of trypsin activity in intestine were found in the three groups that had been fed high isoprotein diets. The CCK level in larvae did not follow the same pattern, in that it was largely depressed in the group that had been fed a diet incorporating protein hydrolysate (low level of native protein), compared to the other groups and including the group fed diet a starch-rich diet. This observation must be considered in conjunction with the depressed pancreatic secretion of both trypsin and amylase in the group fed diet HHY (high protein hydrolysate and low intact protein levels). Intact proteins are known to be the only stimulants of CCK release in rats (Liddle *et al.,* 1986). Owyang (1994) showed that casein hydrolysate, which induced trypsin activity, failed to stimulate CCK release and pancreatic secretion in rat. We obtained similar results in our study on fish larvae by using fish meal hydrolysate and intact fish meal. These observations suggest that dietary protein level and chain length combined with intraluminal proteolytic activity may regulate the CCK level in fish larvae just as in rats.

The high level of CCK associated with high pancreatic enzyme secretion observed in larvae fed the diet containing starch, may be due to the combined effects of a low level of trypsin

(because of a low dietary protein concentration) in the intestinal lumen and a delay in larval development. The fact that high level of CCK can be found with low or high dietary protein level is compatible with existence of an indirect mechanism controlling CCK secretion in seabass larvae. The presence of a peptide that regulates CCK release and mediates pancreatic enzyme secretion has been demonstrated in some mammals, including rats and humans (Liddle, 1994). Under basal conditions, trypsin degrades the CCK-releasing factor. Food, particularly intact protein, which is a substrate for trypsin, may temporarily bind trypsin and prevent degradation of the CCK-releasing factor. Intact CCK-releasing factor in turn stimulates the release of CCK (Chey, 1993). The existence of such mechanism controlling CCK secretion in sea bass larvae could explain some of our experimental results.

5. Conclusion

This study demonstrates that different levels of proteins and inclusions of hydrolysate in the diets modulate trypsin expression and in turn affect CCK content of larvae. The study supports the hypothesis that CCK is involved in protein digestion via an indirect mechanism. We also demonstrate that high larval CCK content is not correlated with a high rate of growth.

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The experiments conducted in this study comply with the current laws of the country in which the experiments were performed.

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Table 1. Composition of the experimental diets

Ingredients[1] *(in %)*	DIETS			
	NAP	**LHY**	**HHY**	**SAP**
Fish meal	74.0	62.0	30.0	45.7
Hydrolyzed fish meal	-	14	46	10.3
Precooked Potato starch	-	-	-	17.7
Cod liver oil	2	-	-	2.3
Soy lecithin	11	11	11	11
Vitamin Mixture[2]	8	8	8	8
Mineral Mixture[3]	4	4	4	4
Betaine	1	1	1	1
Proximal composition				
Proteins (Nx6.25)	61.8	62.3	61.1	47
Lipids	17.4	17.9	19.1	17.4
Ash	17.1	15.8	13.5	13.7
% dry matter	94.1	93.4	92.6	94.4

[1]Dietary ingredients were commercially obtained. Fish meal, Hydrolyzed fish meal (CPSP G, Concentré de Protéines Solubles de Poisson) and cod liver oil were from *La Lorientaise* (Lorient, France). The soy lecithin was from *Ets Louis François* (St Maur des Fossés, France). The potatoe precooked starch (Nutralys) was from *Roquette* (Lille, France).

[2]Per kg of vitamin mix: retinyl acetate 1 g; cholecalciferol 2.5 mg; all-*rac*- α-tocopherol acetate 10 g; menadione 1 g; thiamin 1 g; riboflavine 0.4 g; D- calcium pantothenate 2 g; pyridoxine HCl 0.3 g; cyanocobalamin 1 g; niacin 1 g; choline chloride 200 g; ascorbic acid 20 g; folic acid 0.1 g; biotine 1 g; meso-inositol 30 g.

[3]Per kg of mineral mix: KCl 90 g; KI 40 mg; $CaHPO_4 \cdot 2H_2O$ 500 g; NaCl 40 g; $CuSO_4 \cdot 5H_2O$ 3 g; $ZnSO_4 \cdot 7H_2O$ 4 g; $CoSO_4 \cdot 7H_2O$ 20 mg; $FeSO_4 \cdot 7H_2O$ 20 g; $MnSO_4 \cdot H_2O$ 3 g; $CaCO_3$ 215 g; $MgSO_4 \cdot 7H_2O$ 124 g; NaF 1 g.

Table 2. Growth and survival rate at day 42 of sea bass larvae fed the four experimental diets. Means ± S.D. (n=4) with different letters in a same row are significantly different (P<0.05).

	DIETS			
	NAP	**LHY**	**HHY**	**SAP**
Larval growth				
Initial weight (mg)	1.0 ± 0.92			
Final weight (mg)	21.6 ± 4.60a	19.7 ± 4.30a	5.6 ± 1.50b	9.5 ± 2.40b
Survival rate (%)	44.9 ± 2.68a	40.7 ± 1.31a	29.2 ± 6.48b	38.8 ± 5.31a

Table 3. Specific activity and RNA levels of trypsin and amylase in homogenates of pancreas segment of sea bass larvae fed the different experimental diets. Means ± S.D. (n=4 for enzyme activity; n=3 for RNA ratio) with different letters in a same row are significantly different (P<0.05).

Enzyme	DIETS			
	NAP	**LHY**	**HHY**	**SAP**
Trypsin				
Activity in mU/mg protein				
Day 25	64 ± 14.5a	60 ± 9.2 a	74 ± 8.6a	40 ± 4.8b
Day 42	90 ± 4.2a	95 ± 13.6a	57 ± 3.5b	62 ± 8.3b
RNA ratio				
Day 25	5.1 ± 0.30	4.6 ± 0.59	4.7 ± 0.46	5.1 ± 0.09
Day 42	5.9 ± 0.68a	5.2 ± 0.12a	3.5 ± 0.64b	4.0 ± 0.06b
Amylase				
Activity in U/mg protein				
Day 25	1.7 ± 0.25b	2.0 ± 0.22ab	2.3 ± 0.61ab	2.8 ± 0.60a
Day 42	1.1 ± 0.13b	1.0 ± 0.21b	1.7 ± 0.82ab	2.4 ± 0.50a
RNA ratio				
Day 25	0.4 ± 0.03	0.3 ± 0.07	0.4 ± 0.05	0.4 ± 0.07
Day 42	0.9 ± 0.18c	1.2 ± 0.07bc	1.7 ± 0.82a	1.4 ± 0.08ab

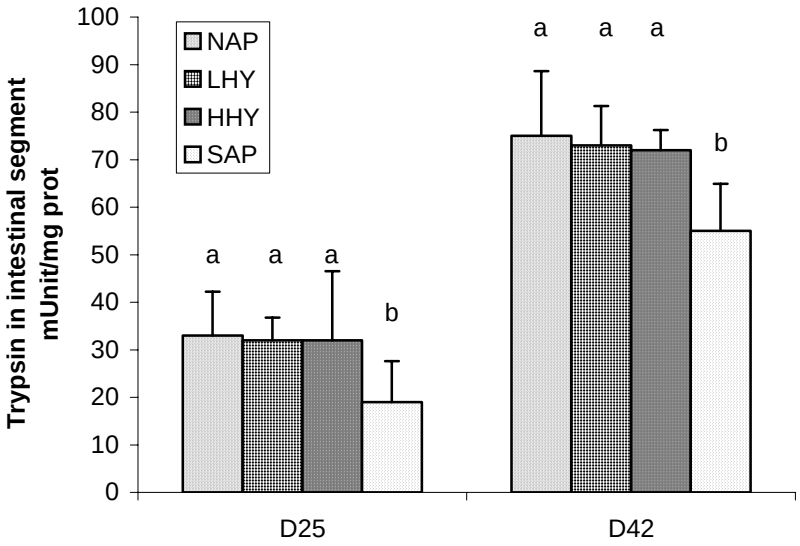

Figure 1. Specific activity of trypsin assayed in intestinal segment of sea bass larvae fed the four experimental diets. Means ± S.D. (n=4) with different superscript letters for a same day are significantly different (P<0.05).

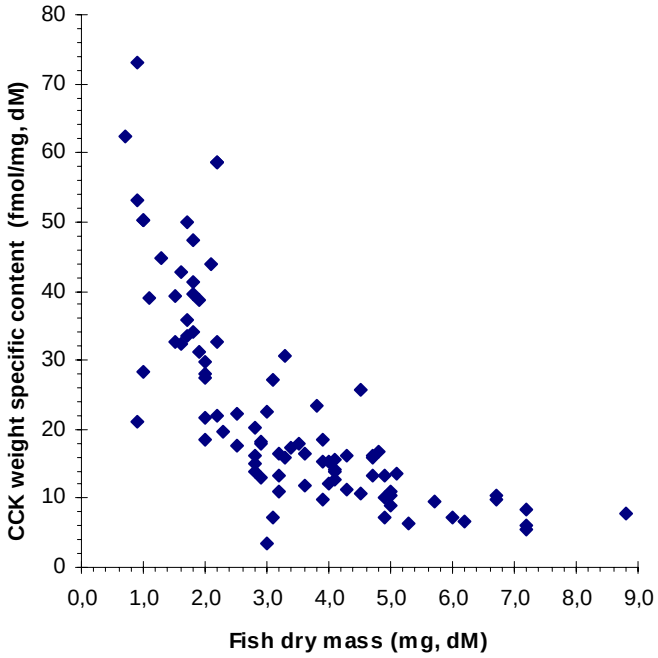

Figure 2. Relationship between amount of CCK and dry mass of whole sea bass larvae.

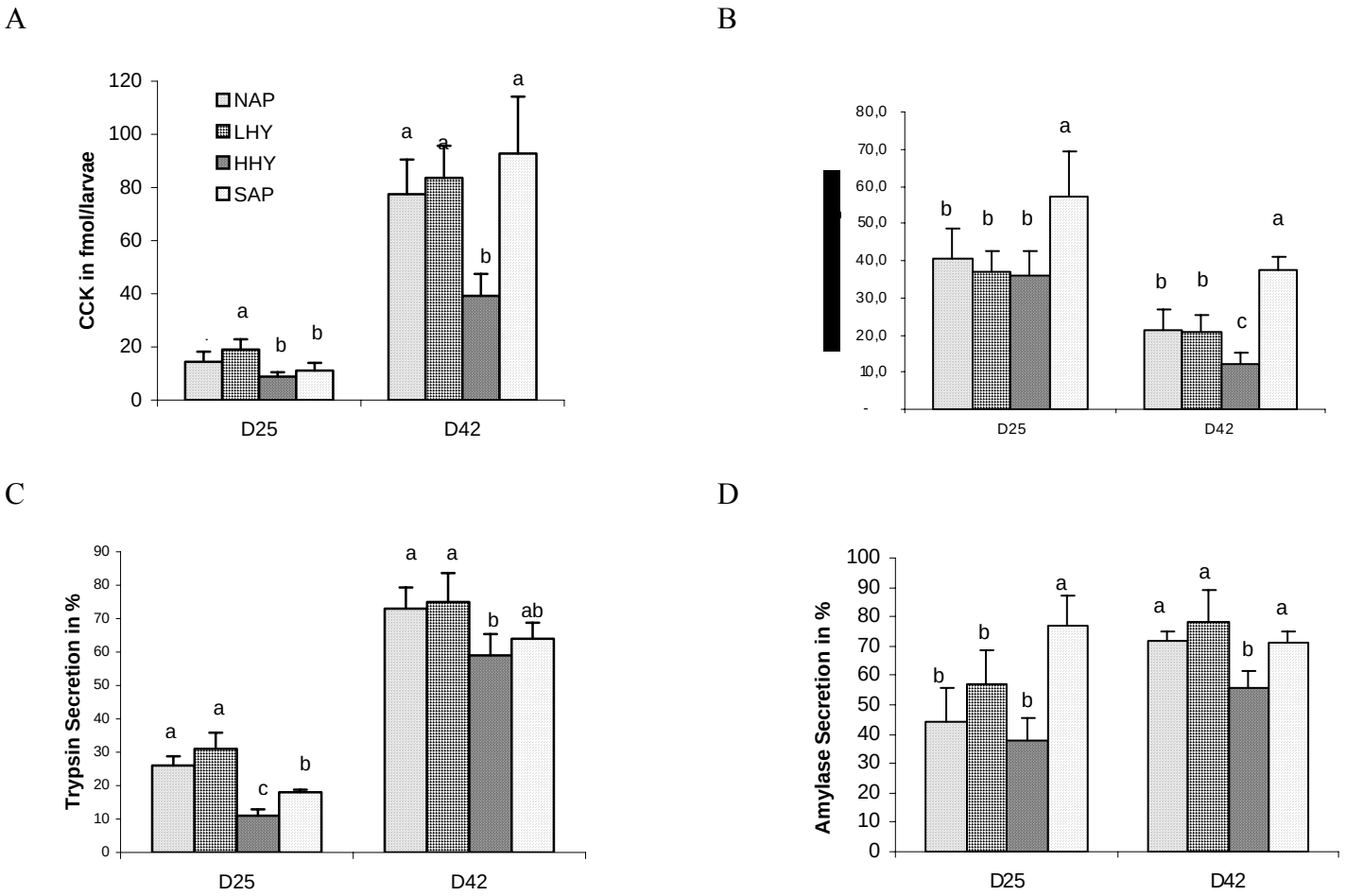

Figure 3. CCK in whole body larvae (3A) or related to dry weight larvae (3B). Secretion level [% of enzyme assayed in the intestinal segment (mU/segment) divided by enzyme assayed in pancreatic plus intestinal segment] of trypsin (3C) and amylase (3D) in sea bass larvae. Means ± S.D. (n=4) with different letters for a same day are significantly different ($P<0.05$).