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Effect of nature of dietary lipids on European sea bass morphogenesis: implication of retinoid receptors

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Abstract: The effect of the nature and form of supply of dietary lipids on larval development was investigated in European sea bass larvae, by considering the expression of several genes involved in morphogenesis. Fish were fed from 7 to 37 d post-hatch with five isoproteic and isolipidic compound diets incorporating different levels of EPA and DHA provided by phospholipid or neutral lipid. Phospholipid fraction containing 1·1 % (PL1 diet) to 2·3 % (PL3 diet) of EPA and DHA sustained good larval growth and survival, with low vertebral and cephalic deformities. Similar levels of EPA and DHA provided by the neutral lipid fraction were teratogenic and lethal. Nevertheless, dietary phospholipids containing high levels of DHA and EPA (PL5 diet) induced cephalic (8·5 %) and vertebral column deformities (35·3 %) adversely affecting fish growth and survival; moreover, a down-regulation of retinoid X receptor α (RXRα), retinoic acid receptor α, retinoic acid receptor γ and bone morphogenetic protein-4 genes was also noted in PL5 dietary group at day 16. High levels of dietary PUFA in neutral lipid (NL3 diet) first up-regulated the expression of RXRα at day 16 and then down-regulated most of the studied genes at day 23, leading to skeletal abnormalities and death of the larvae. A moderate level of PUFA in neutral lipids up-regulated genes only at day 16, inducing a lesser negative effect on growth, survival and malformation rate than the NL3 group. These results showed that retinoid pathways can be influenced by dietary lipids leading to skeletal malformation during sea bass larvae development.

Keywords: Phospholipids; Neutral lipids; Dicentrarchus labrax larvae; Skeletal malformations; Retinoic acid receptors

INTRODUCTION

Around 30% of marine fish larvae reared in commercial hatcheries exhibit morphological and skeletal deformities (Andrades *et al.* 1994 ; Boglione *et al.* 2001). These abnormalities (e.g., lordosis, scoliosis, vertebral fusion and compression, lower jaw deformity, reduced maxillary bones, and a reduced or incomplete operculum) lead to a depreciated market price for the fish farmer, a setback to the quality image of aquaculture and represent one of the most important bottlenecks in actual marine aquaculture.

Different factors, such as genetic or environmental parameters, are involved in the formation of the skeleton and are supposed to be responsible for the apparition of skeletal abnormalities during larval development. Among them, the influence of diet composition on larval malformation has been recently shown experimentally. Several studies have demonstrated that nutrients are responsible for the apparition of skeletal deformities when their level and/or form of supply in the diet were inappropriate (Zambonino-Infante *et al.* 1997 ; Cahu *et al.* 2003*a*). These perturbations in development are linked to an action of these molecules on the target genes involved in morphogenesis, such as the Hox and sonic hedgehog (shh) family genes. For example, a lack or an excess of some vitamins, such as vitamin A, are known to have teratogenic effects on the development of mammals, birds, amphibians and fish, through the disturbance of the normal expression of morphogenetic genes (Ogura & Evans, 1995 ; Suzuki *et al.* 1998 ; Ross *et al.* 2000 ; Villeneuve *et al.* in press). Dietary lipid levels and form affect the growth and the malformation rate of marine fish larvae (Cahu *et al.* 2003*b*).

Lipids are the main energy source in developing larvae and the lipid requirements of marine fish larvae have been extensively studied. Particular attention has been paid to phospholipids (PL) and polyunsaturated fatty acids (PUFA) (Sargent *et al.* 1999). It has been reported recently that a diet containing 19% lipids with almost 9% phospholipids induced good growth in European sea bass first feeding larvae (Cahu *et al.* 2003*b*). In fact, the former authors demonstrated that the apparition of skeletal deformities in European sea bass during early development was linked more to the proportion of dietary phospholipids:neutral lipids than to the total dietary lipid content. Marine fish are unable to synthesize *n*-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are particularly important as they occur in large amounts in fish cell membranes. The optimal level of these essential dietary components for marine fish is known to be around 3% of dry matter for EPA+DHA (Sargent *et al.* 1999).

In addition to their nutritional role, fatty acids are essential biological components able to rapidly modulate the transcription of genes involved in their metabolism (Kliewer *et al.* 1997). This modulation involves nuclear hormone receptors, called peroxisome proliferator-activated receptors (PPAR). These receptors are known to form heterodimers with other cellular receptors called retinoid X receptors (RXR), which are involved in the retinoid pathways (Ross *et al.* 2000). This heterodimerization indicates that the retinoids and the peroxisome proliferator signaling pathways converge through the direct interaction of their respective nuclear receptors (Bonilla *et al.* 2000). These dimers regulate the expression of target genes by binding to DNA sequence elements (Ross *et al.* 2000) present in the regulatory regions of a variety of genes involved in lipid metabolism, energy balance or morphogenesis (Kliewer *et al.* 1997 ; Balmer & Blomhoff, 2002). The retinoid pathways control the expression of morphogenetic genes through the action of two types of nuclear receptors (the Retinoic Acid Receptors, RAR and the Retinoid X Receptors, RXR). These morphogenetic genes involved many gene families, such as Hox genes (Krumlauf 1994), Bone Morphogenetic Protein (BMP; Sasagawa *et al.* 2002) or Insuline-like Growth Factors (IGF; Fu *et al.* 2001). A modification in the equilibrium between the nuclear receptors, such as an increase of PPARs, results in the perturbation of the number of receptors involved in retinoid pathways (Bonilla *et al.* 2000). Thus, the nature (phospholipids vs. neutral lipids) and level of dietary lipids might be able to modify the expression of certain receptors involved in growth, differentiation and cellular homeostasis from the above-mentioned signalization pathways (Bonilla *et al.* 2000). The aim of the present study was to evaluate the effect of the nature (phospholipid vs. neutral lipid) and level of *n*-3 PUFAs from the dietary lipid supply on 1) the apparition of skeletal abnormalities and 2) the regulation of several genes (RXRα, RARα, RARγ, BMP4 and IGF-I) directly or indirectly involved in the retinoid pathways of European sea bass larvae.

MATERIALS AND METHODS

Animals and diets

Three-day old European sea bass (*Dicentrarchus labrax*) larvae were obtained from the *Ecloserie Marine de Gravelines* (Gravelines, France) and shipped to the *Fish Nutrition Laboratory* at the *Ifremer* (Centre de Brest). Fish were acclimated and divided into fifteen 35 L-cylindroconical fibre glass tanks (2100 larvae/tank) at a initial density of 60 larvae/L. Tanks were supplied with running sea water, which had been previously 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 20°C and 35 ‰, respectively, and the oxygen level was maintained above 6 mg/L by setting the water exchange of the tank at 30% per hour (flow rate: 0.18 L/min). Photoperiod was 24L:0D and maximum light intensity was 9 W/m^2 at the water surface. All animal procedures and handling were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1985).

At 4 days post hatch (dph), larvae were divided into 5 experimental groups (3 replicates per group) that were fed from first feeeding (7 dph) with experimental compound microdiets. Five isonitrogenous and isolipidic diets (Table 1) were formulated to incorporate different levels of PUFAs, which differed in their form of supply : phospholipids (PL) or neutral lipids (NL). Fish meal, naturally containing 17% lipid, was defatted in order to control the experimental dietary lipid composition by only the addition of oils. Fish meal was defatted in the presence of dichloromethane for 20 min at 40°C, rinsed and dried until complete solvent evaporation. Among lipid sources, marine lecithin was used as a source of phospholipids including EPA and DHA, soybean lecithin was a phospholipid devoid of EPA and DHA and cod liver oil was the source of neutral lipid including EPA and DHA. Diet names were chosen according to the percentage of dietary eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) contained in the phospholipid and neutral lipid fraction of the diet: PL5, PL3, PL1, NL1 and NL3. The marine phospholipid fraction in PL diets varied in inverse relation to soybean lecithin, whereas NL diets contained soybean lecithin in an inverse proportion to marine triglycerides (cod liver oil). The composition of PL1 was close to a previously used one that had supported good growth and survival in European sea bass larvae (Cahu *et al.* 2003*b*) and it was considered in this study as a control diet. Microdiets were processed as previously described (Cahu *et al.* 2003*b*) and pellet size was 200-400µm. During all the experimental period, larvae were continuously fed to excess for 24 hours per day using a belt feeder. Food ingestion was monitored by observing the larval digestive tract under a binocular microscope and dietary microparticles being visible by transparency. At the end of the trial (37 dph), fish **can no longer be considered as larvae since this date correspond to the**

Table 1 Composition of the experimental compound microdiet.

	Experimental diets				
	PL1	**PL3**	**PL5**	**NL1**	**NL3**
Ingredients[1]	**g/kg dry matter**				
Defatted Fish Meal			510		
Hydrolyzed fish meal (CPSP)			140		
Cod liver oil	0	0	0	7	14
Marine lecithin	7	14	21	0	0
Soy lecithin	14	7	0	14	7
Vitamin mixture[2]			80		
Mineral mixture[3]			40		
Betaine			20		
Proximate composition			**%**		
Proteins (Nx6.25)	59.9	61.6	60.4.	58.7	59.6
Lipids	16.4	16.7	16.7	17.1	18.2
Including:					
Phospholipids	13.2	13.0	12.1	11.1	8.1
EPA + DHA	1.1	2.3	4.8	0.3	0.4
Neutral lipids	4.8	3.8	3.0	6.2	11.0
EPA + DHA	0.3	0.3	0.3	1.3	2.6
Ash	17.4	17.0	16.8	15.6	15.6
Moisture	6.8	6.3	7.4	6.5	6.4
Energy (kJ/kg)[4]	1618	1658	1638	1624	1681

[1] All dietary ingredients were commercially obtained. Fish meal (La Lorientaise, Lorient, France), hydrolyzed fish meal (CPSP, Soluble Fish Protein Concentrate, Sopropêche, Boulogne sur Mer, France), cod liver oil (La Lorientaise, Lorient, France), marine lecithine (LC60, Phosphomins ^{TM}, Phosphotech, Saint Herblain, France) and soy lecithin (Ets Louis François, St Maur des Fossés, France).

[2] Per kg of vitamin mixture: choline concentrate 50% 200 g, vitamin E (500 UI/g) 10 g, vitamin D3 (500,000 UI/g) 500 mg, vitamin B3 1 g, vitamin B5 2 g, vitamin B1 100 mg, vitamin B2 400 mg, vitamin B6 300 mg, vitamin C 20 g, vitamin B9 100 mg, vitamin concentrate B12 (1g/kg) 1g, biotin 1 g, vitamin K3 1g, meso-inositol 30 g, cellulose 732.1 g.

[3] Per kg of mineral mixture: KCl 90 g, KI_4O 40 mg, $CaHPO_4$ $2H_2O$ 500 g, NaCl 40 g, $CuSO_4$ $5H_2O$ 3 g, $ZnSO_4$ $7H_2O$ 4 g, $CoSO_4$ $7H_2O$ 20 mg, $FeSO_4$ $7H_2O$ 20 g, $MnSO_4$ H_2O 3g, $CaCO_3$ 215 g, $MgSO_4$ $7H_2O$ 124 g, NaF 1g.

[4] Calculated as: fat x 37.7 J/kg; protein x 16.7 J/kg.

transition from larvae to juveniles; consequently, experimental microdiets could not be used after this date considering that the particle size and the composition of the larvae feeds are not adapted to juvenile. Therefore, fish from a same experimental **group** were pooled and transferred to a 700-L square fiberglass tank (one tank per diet) and reared until 71 dph under the above-mentioned environmental conditions. During this period, all fish were fed with the same commercial diet (Neo Soupra AL4, Le Gouessant, Lamballe, France) containing 58% protein, 13% lipid, 12% ash and 1.2% cellulose (proximate composition provided by the feed manufacturer). Food ingestion was monitored by observing digestive tracts of larvae under a binocular microscope.

Sampling. To evaluate growth, ten specimens were randomly sampled from each experimental tank (30 larvae per experimental diet) at 12, 16, 23, 30 and 37 dph, killed with an overdose of anesthetic (tricaine methanesulfonate, MS 222) and their wet body weight measured to the nearest 0.1 mg. Fifty larvae were collected for mRNA studies from all experimental tanks at 16, 23, 30 and 37 dph, and total RNA immediately extracted. In all cases, sampling procedures were performed as previously described (Cahu *et al.* 2003*b*). The incidence of skeletal body malformations (splachnocranium, neurocranium and vertebral column deformities) was counted at 71 dph (100 larvae per experimental diet**; indeed, this developmental stage allows to correctly observe and identify all deformities appeared during the larval period (before day 37).** It was not possible to determine real survival (percentage of surviving individuals in relation to the initial number of larvae) due to repetitive sampling, however, relative survival, expressed as the percentage of surviving fish fed on each diet in relation to the surviving specimens fed on the control diet (PL1), was calculated at 71 dph.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Different cDNA fragments of genes coding for retinoic acid receptors and for signaling molecules known for interacting with the retinoic acid were purified in European sea bass larvae by RT-PCR, cloned (Villeneuve *et al.* 2004), then sequenced and registered by the European Molecular Biology Laboratory (EMBL): Retinoid X Receptor α (RXRα; accession number AJ 567907), Retinoic Acid Receptor α (RARα; AJ 496189), Retinoic Acid Receptor γ (RARγ; AJ 496181), Bone Morphogenetic Protein-4 (BMP4; AJ 567451) and Insulin-like Growth Factor-I (IGF-I; AJ 579342). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AJ 567450) was chosen as the house-keeping gene (Table 2).

Table 2 Oligonucleotide primers used in PCR reactions.

Gene	Forward primer	Reverse primer	Annealing temperature (°C)
GAPDH	CACCACGCTCACCATCGC	CATCTTGGGGAACATGTG	54
RARα	ACCACGCTCACCATCGC	ATCTTGGGGAACATGTG	56
RARγ	GAGGTGGGCATGTCCAAG	ATCCATCTCCAGGGGCAG	58
RXRα	TGCCAGTACTGCCGCTAC	CAGCATCTCCATGAGGAA	56
BMP4	CCAGCCCGAGCCAACA	CACAATCCAGTCATTCCA	52
IGF-I	TAGCCACACCCTCTCACT	ATGCCAAGAGCCCAAG	54

Real-time RT-PCR

cDNA samples were treated with DNase (SIGMA AMP-D1) and Real-time PCR was performed using the iCycler iQ^{TM} (Bio-Rad Laboratories Inc.). Quantitative PCR analyses for each gene were performed in a total volume of 15µL containing 5 µL cDNA (dilution: 10^{-3}), 1.5 µL fluorescein (100 nmol/L, Bio-Rad), 0.5 µL primers (10 µmol/L) and 7.5 µL QuantiTect SYBR Green PCR Master Mix 2X (QIAGEN GmbH, Germany). For each target gene, forward and reverse primers (Table 3) were chosen in the sequences previously cloned. Thermal cycling was initiated with incubation at 95°C for 13.5 min for activation of HotStarTaq^{TM} DNA Polymerase. After this initial step, 45 PCR cycles were performed. Each PCR cycle consisted of heating at 95°C for 30 sec for denaturing, as well as at 60°C for 1 min for annealing and extension. CT (cycle threshold) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. Melting curve analysis was performed to confirm production of a single product in these reactions and the products were sequenced by MilleGen (Labège, France). Standard curves were established for each gene by plotting the CT values against the $\log_{10}$ of 5 different dilutions (in triplicate) of the cDNA sample solutions. Real-time PCR efficiency was determined for each gene from the slopes given by the BIORAD software, according to the equation $E = 10^{[-1/slope]}$. The relative expression ratio of each gene was calculated using REST © software (http://www.gene-quantification.info). The relative expression ratio for a considered gene is based on the PCR efficiency (E) and the CT of a sample (PL3, PL5, NL1 or NL3) versus the control (PL1), and expressed in comparison to the reference gene (GAPDH), according to Pfaffl's mathematical model (Pfaffl, 2001):

$$\text{Ratio} = [(E \text{ gene})^{\Delta CT \text{ gene (control-sample)}}] / [(E \text{ GAPDH})^{\Delta CT \text{ GAPDH (control-sample)}}].$$

Normalization relative to GAPDH provided a widely applicable value for comparative studies of gene expression at the mRNA level, since its expression is constant during activation and proliferation of cells (Gause & Adamovicz, 1994).

Statistical analyses

Results are given as mean ± SD. All data were checked for variance homogeneity using the Barlett's test (Dagnelie, 1975). Growth was compared by means of a one-way ANOVA followed by Newman Keuls multiple range test when significant differences were detected ($P < 0.05$). Statistical differences in gene expression between the control and samples were evaluated in group means by randomization tests (Pfaffl *et al.* 2002) using REST© Software; 2000 random allocations were performed and significant differences were considered at $P = 0.05$.

Table 3 Nucleotide sequences of the PCR primers used to assay gene expression by real-time quantitative PCR.

Gene	Forward primer	Reverse primer	PCR product length (nt)
GAPDH	GAGGTCAAGGTTGAGGGTGA	CCAGTGGACTCAACCACGTA	125
RXR α	CTGGTAGAGTGGGCCAAGAG	GTTCTGTGAGCACCCTGTCA	223
RAR α	CGCTAAACCGAACCCAGA	CTTCTCGGCCTGTTCCAA	170
RAR γ	GCAAAGCTCACCAAGAGACC	GCGTAGTGAAGCCTGGTAGC	180
BMP4	CTGCTCTCTTCCGCTGAACT	GGCTCACACTAAAGCTCTCC	205
IGF-I	GTCTTGGCAGGTGCACAGTA	ACACGCTGCAGTTTGTGTGT	157

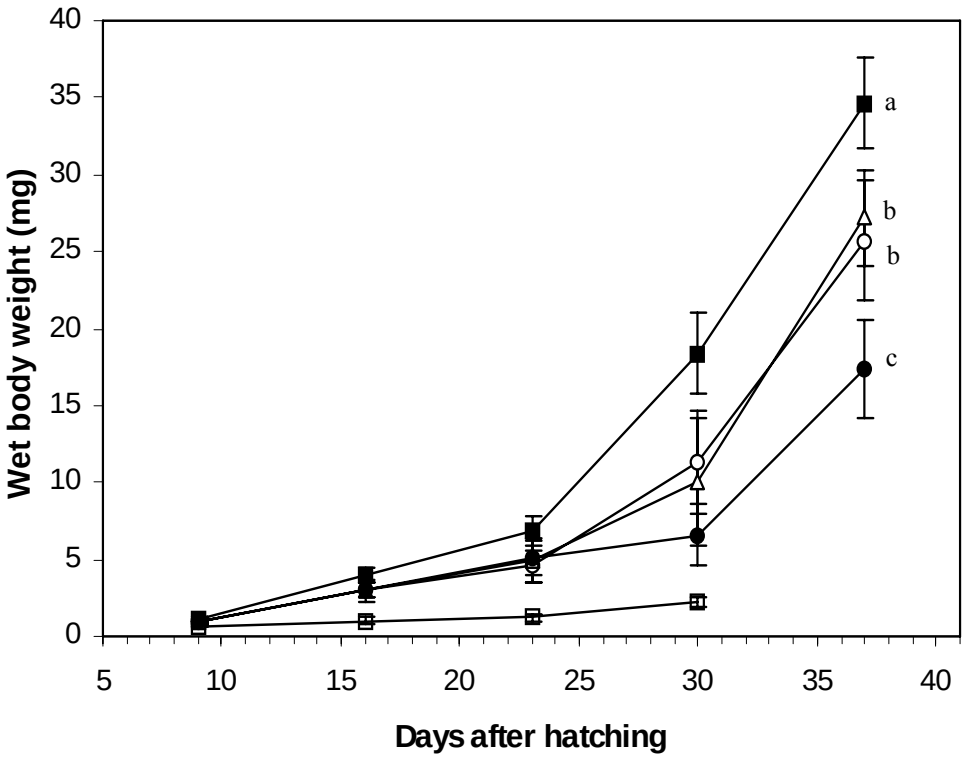
RESULTS

Growth and survival

Observation of the digestive tract under the binocular microscope revealed an effective ingestion of the microparticulated diets in all the groups. At the end of the rearing period (37 dph), statistically significant differences in larval growth were observed between experimental groups (ANOVA, $P < 0.05$, Figure 1). Larvae fed PL3 exhibited the best growth performance, being 21.4% higher than that in the control group, PL1, and 25.7 and 49.7% higher in body weight than those fed PL5 and NL1, respectively. As the fish in the NL3 group died before 37 dph, their larval body weight could not be compared to those of other experimental groups, although it was significantly lower at early stages of development (23 and 30 dph).

Compared to the control group (PL1), the relative survival was 226.1, 31.5 and 128.7% in larvae fed PL3, PL5 and NL1, respectively. The best survival was observed in the group fed with the PL3 diet (n = 710).

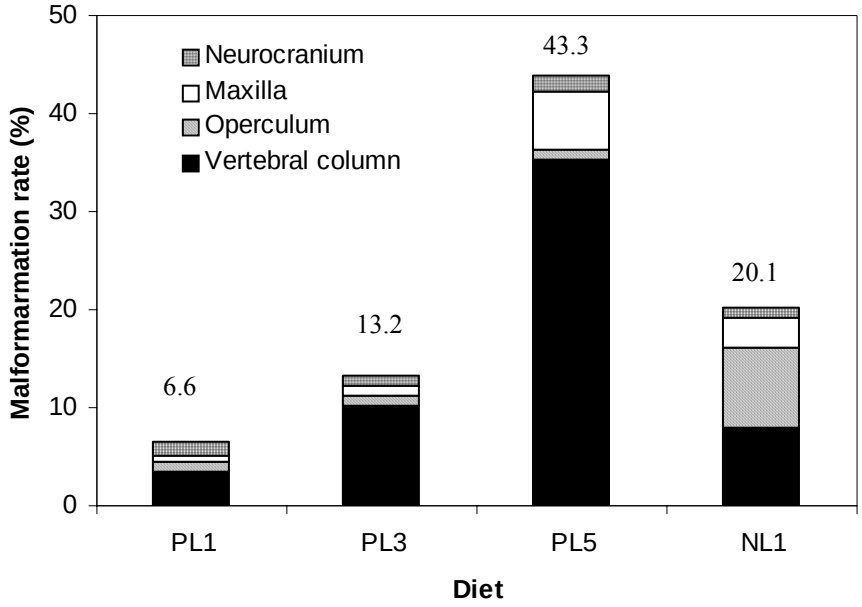
Figure 1. Growth of European sea bass larvae fed isonitrogenous and isolipidic diets containing different levels of eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA). Diets are named according to the percentage of dietary EPA and DHA contained in the phospholipid (PL) and neutral lipid (NL) fraction of the diet: PL5 (○), PL3 (■), PL1 (△), NL1 (●) and NL3 (□). Means ± S.D. (n = 3) with different superscript letters for the same day are significantly different ($P < 0.05$).



Skeletal malformations

The incidence of skeletal malformations in European sea bass juveniles (age: 71 dph, mean weight: 2.4 ± 0.59 g) is presented in Figure 2. Almost half of the fish fed the diet including the highest EPA + DHA concentration (4.8% in PL5) exhibited deformities. This value was very low (6.6%) in the group fed the diet PL1 containing 1.1% EPA + DHA in the PL fraction. Fish fed diets containing the two highest concentrations of EPA and DHA in the phospholipid fraction, PL5 and PL3, presented a high prevalence of scoliotic vertebral columns (35.3 and 10.2%, respectively), while the other categories of skeletal malformations (deformities of the neurocranium, maxilla and operculum) were less frequent. Fish fed with the diet NL1 containing the lowest level of EPA and DHA in the PL fraction (0.3%) showed an equal percentage of skeletal malformations affecting the vertebral column and operculum (7.9 and 8.2%, respectively), this percentage of deformities was higher than that observed in the control group.

Figure 2. Incidence of skeletal malformations in European sea bass juveniles (age: 71 days post hatch) fed isonitrogenous and isolipidic diets containing different levels of eicosapentanoic acid and docosahexaenoic acid. As the fish fed NL3 diet died before 37 dph, the incidence of skeletal deformities could not have been determined.



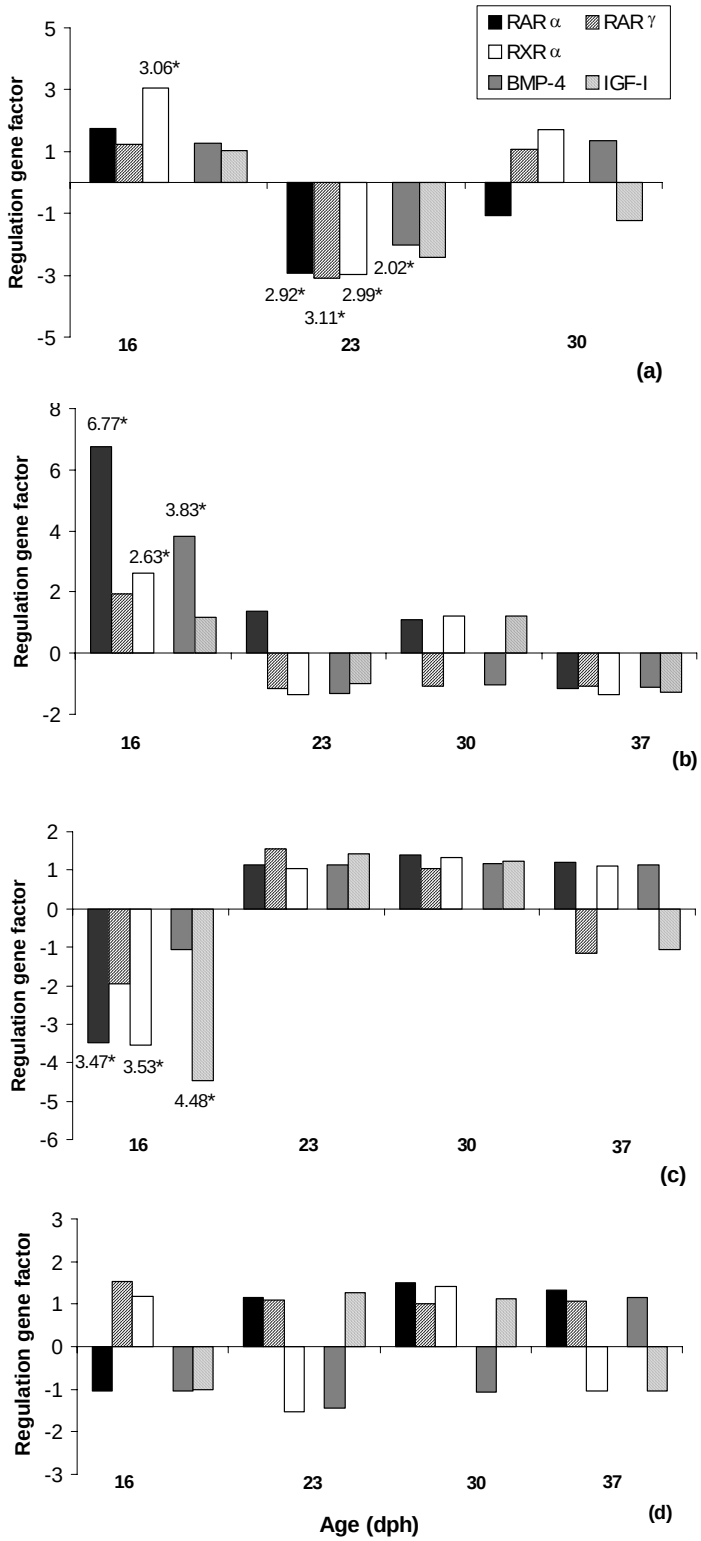
Effect of

Only da oup, were considered **reared in standard n d in this experiment** lied genes, except RAR found ($P >$ 0.05).

Effect of experimental diets on gene expression)

At 16 dph, fish fed the NL3 (Fig. 3a) diet exhibited a significant up regulation of RXRα expression (up-regulation factor: 3.06, $P < 0.001$) compared to that observed in the control group (PL1), while, at 23 dph, RARα, RARγ, RXRα and BMP4 were significantly down regulated by factors of 2.9, 3.1, 3.0 and 2.0 ($P < 0.001$), respectively. At 16 dph, larvae fed the NL1 diet (Fig. 3b) showed a significant up regulation of RARα (up-regulation factor: 6.8, $P < 0.001$), RXRα (up-regulation factor: 2.6, $P < 0.01$) and BMP4 (up-regulation factor: 3.8, $P < 0.001$) expression compared to the control group, while no other difference was observed after this age. Similarly, significant differences were only observed at 16 dph in fish fed the PL5 diet (Fig. 3**c**) compared to the PL1 group but RARα ($P < 0.01$), RXRα ($P <$ 0.001) and IGF-I ($P < 0.001$) were 3.5, 3.5 and 4.5 times down regulated, respectively. The PL3 diet (Fig. 3**d**) did not seem to have any effect on larval gene expression compared to the PL1 diet.

Figure 3. Regulation gene factor for RXRα, RARα, BMP4, RARγ and IGF-I in European sea bass larvae fed NL3 (a), NL1 (b), PL5 (c) and PL3 (d) diets. Plotted data are means ± S.D. (n = 4). Values with an asterisk denote the existence of statistically significant differences between fish fed experimental diets and the control group (PL1).



DISCUSSION

For many years, the development of a dry microdiet for first feeding marine fish larvae, sustaining good growth, survival and harmonious development, has been the major objective of many laboratories. In this sense, the determination of dietary requirements has shown that different nutrients directly influence larval growth and skeletal development. Recently, it has been shown that the dietary phospholipid level and phospholipid:neutral lipid value were influential for European sea bass development (Cahu *et al.* 2003*b*). The effect of different dietary levels of PUFAs and their form of supply on the phospholipid and neutral lipid fraction on European sea bass larvae still remained to be determined. Moreover, we found interesting to assess the possible interaction between PUFA pathway and retinoid pathway which are involved in morphogenic processes.

Good growth and survival has been obtained with a compound microdiet containing 11% of phospholipids (Cahu *et al.* 2003*b*). This value was used in the current study to formulate experimental diets with an appropriate level of phospholipids (PL5, PL3, PL1 and NL1 diets). However, although the NL3 diet only contained 8% of phospholipids, this percentage was still acceptable in terms of growth and larval survival rates (Cahu *et al.* 2003*b*). The tested diets, differing by the source and concentration of PUFAs, greatly affected both growth and survival of European sea bass larvae. The diet containing a moderate level of EPA and DHA in the phospholipid fraction (PL3) induced the best growth and survival, while the diet with a similar level of EPA and DHA in the neutral lipid fraction (NL3) was lethal for sea bass larvae. A higher percentage of EPA and DHA in the PL fraction (PL5) negatively affected growth, survival and the incidence of skeletal malformations in larvae. Observed differences in growth performance between larvae fed low rates of dietary EPA and DHA depended on the supply form of both essential fatty acids: when they were in the PL fraction (PL1) good growth was observed, while growth was lower when they were in the NL fraction. These results **strengthen the fact** that PL fraction is more efficient than NL fraction to supply n-3 PUFA in developing larvae.

Little is known about the optimal DHA/EPA ratios for first feeding marine larvae. Lipid composition of eggs/yolk reserves has been suggested as an indicator for determining the nutritional requirements of newly hatched larvae. Typically a dietary DHA/EPA ratio of 2:1 is found in marine species and thus, it has been suggested as adequate for larval nutrition (Sargent *et al.* 1997). Total DHA/EPA ratios in PL diets were very similar (1.9:1, 2.2:1 and 2.4:1 in PL1, PL3 and PL5 diets, respectively) and slightly higher than those of NL diets (1.8:1 in NL1 and NL3 diets). It has been suggested that marine fish larval diets with ratios of DHA/EPA less than or equal to 1 are suboptimal, either by not providing sufficient DHA or by providing an undesirable excess of EPA (Sargent *et al.* 1997). Therefore, the observed differences in growth and survival of European sea bass in the present study could not be attributed to inappropriate DHA/EPA ratios. Here, the DHA/EPA ratio that promoted the highest growth rate and survival in European sea bass fell in the range of recommended values for gilthead sea bream and red drum, which are 2:1 and >2.5:1, respectively (Brinkmeyer & Holt, 1998 ; Rodriguez *et al.* 1998), indicating similar essential fatty acid requirements for this group of species.

Our results demonstrated the existence of a direct relation between the amount of n-3 PUFA in phospholipids and the effect on the normal development of the skeleton during the larval stage. An effect of oxidation of PUFAs could be evoked (Sargent *et al.* 2002) but it is unlikely that the observed skeletal abnormalities resulted from an oxidation of PUFAs in the microparticles since the formulation of experimental diets fells within the range of recommended NRC levels for dietary antioxidants, such as vitamin E (α-tocopherol) and ascorbic acid (National Research Council, 1993).

Then, a direct effect of n-3 PUFAs on skeletal development must be considered. A previous study (Villeneuve *et al.* in press) showed that skeletal malformations induced in European sea bass larvae by a nutritional factor, vitamin A, could be related to the expression of genes involved in their development, such as RARα, RARγ, RXRα. In this study, European sea bass larvae fed the NL3 diet exhibited a significant up-regulation of RXRα expression at 16 dph compared to the control group. This diet contained a high proportion of neutral lipids, which are less efficient for fish larvae than phospholipids. It has been previously demonstrated that several PUFAs can activate RXRα expression, while the strongest up-regulation was obtained with DHA in rats (Mata de Urquiza *et al.* 2000). Moreover, a direct activation of the transcription of RXRα by several fatty acids has been experimentally shown (Steineger *et al.* 1998), which supports the idea that under present experimental conditions, the high level of cellular PUFAs present in the neutral lipid fraction in the NL3 group directly stimulated the transcription level of RXRα. Fish fed NL1 also exhibited a significant up-

regulation of RXRα at 16 dph. It is well known that RXRα preferentially binds the α isoform of RARs (Ross *et al.* 2000 ; Egea *et al.* 2001), so the increase of RXRα could led to the up-regulation of RARα and these two kinds of receptors might have formed active dimers. The up-regulation of RARα was not revealed in NL3 group and this might be due to their poor nutritional status. Several studies have demonstrated that the retinoic acid pathway and BMP4 can synergize to induce apoptosis (Glozak & Rogers, 1998). BMP4 does not seem to have a RARE in its nucleotidic sequence (Balmer & Blomhoff, 2002), but its activation by retinoic acid would require a gene-specific activator, which is still unknown (Thompson *et al.* 2003). This hypothesis suggests that, in the present study, a possible apoptosis induced by lipids in an inappropriate form interfered with the normal differentiation processes of the larvae, leading to a high rate of malformations in fish fed NL1 and the death of specimens in the NL3 experimental group. In the NL3 group, fish were close to death and this might explain the significant down-regulation of the studied RARs, RXRα and BMP4 at the age of 23 dph.

Concerning larvae fed PL5, we noted that the diet exerted an antagonist effect on gene expression at 16 dph in comparison to the NL1 group. Instead of an up-regulation, we observed a down-regulation of RARα, RXRα and IGF-I. It is interesting to note that the RARα expression pattern also followed the one of RXRα. The PL5 diet contained a high concentration of *n*-3 PUFA, mainly in the phospholipid fraction and, as fish fed this diet exhibited a retardation of growth and not an optimal rate of survival, we might assume that this concentration of dietary *n*-3 PUFA was excessive. Thus**, it can be hypothesized that** under the present experimental conditions, the high rate of dietary PUFAs certainly activated the expression of PPARs (Kliewer *et al.* 1997). PPARs are known to interact with RXRs and the heterodimerization of PPARs with RXR indicates that the retinoid and peroxisome proliferator signaling pathways converge through the direct interaction of their respective nuclear receptors (Bonilla *et al.* 2000) and they might have activated the expression of target genes. **Assay on PPAR is necessary for further speculations and to confirm our present results.**

Under stress conditions, glucocorticoids stimulate RXRα transcription level (Steineger *et al.* 1998). They also stimulate PPARs expression and PPARs regulate genes involved in the activation of fatty acids to mobilize fatty acids and maintain energy homeostasis (Lemberger *et al.* 1996). Considering these results **reported by the literature**, we might assume that *n*-3 PUFAs present in the PL5 diet highly stimulated PPARs transcription, creating a state of nutritional stress. This hypothesis was reinforced by histological observations (data reported **in Gisbert *et al.* 2005**) showing an important accumulation of lipids in the enterocytes. As the proportion of intracellular *n*-3 HUFA was very high, the rate of PPARs transcription might have been elevated, leading to an excessive number of PPARs in the cell and consequently, an excess of PPAR/RXRα heterodimers. Thus, a negative feedback of these molecules on the RXRα gene might explain its lower transcription level in larvae aged 16 dph fed on PL5. Furthermore, this repression of RXRα was observed concurrently with the down-regulation of its partner RARα. IGF-I has a high probability to possess a RARE in its promoting region (Balmer & Blomhoff, 2002) and several studies have demonstrated that retinoic acid influences its transcription level (Gabbitas & Canalis, 1997 ; Fu *et al.* 2001). Thus, we might suppose that RXRα down-regulation indirectly led to IGF-I down-regulation through RARα repression, resulting in a high percentage of skeletal deformities (43.3 %), which mainly affected the vertebral axis of fish. It is interesting to note that the malformations observed in the present study mainly affected the vertebral column, while in a previous study (Villeneuve *et al.* in press), skeletal deformities induced by vitamin A were preferentially localized in the cephalic region. This might indicate that peroxisome proliferator and retinoid pathways involve two different cascades of morphogenetic genes. Further experiments would be necessary to study the expression pattern of PPARs and to assess a direct their implication in morphogenesis processes in sea bass larvae

In conclusion, the quantity and the form of EPA and DHA supply (NL or PL) in diets are determining for European sea bass larval development. Larvae used EPA and DHA more efficiently when present in the PL fraction: 1.1% EPA + DHA (corresponding to a total DHA/EPA ratio of 1.9:1) appeared as optimal since it induced a low level of malformations. Better growth and survival of larvae can be obtained with higher EPA + DHA levels but to the detriment of morphogenesis. An excess of these fatty acids (PL5 diet) induced severe skeletal malformations. High levels of skeletal deformities and a reduction in growth and survival were associated with a down-regulation in RXRα, RARα, RARγ and BMP4 expression. High levels of dietary triglycerides (NL3 diet) up-regulated the expression of RXRα at early stages of development (16 dph) disrupting the normal larval development and adversely influencing fish viability.

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