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Intake of high levels of vitamin A and polyunsaturated fatty acids during different developmental periods modifies the expression of morphogenesis genes in European sea bass (Dicentrarchus labrax)

Laure A.N. Villeneuve[1]*, Enric Gisbert[2], Jacques Moriceau[1], Chantal L. Cahu[1] and José L. Zambonino Infante[1]

[1]UMR1067, Nutrition Aquaculture Génomique, IFREMER, 29280 Plouzané, France.
[2]Centre d'Aqüicultura, Institut de Recerca i Tecnologia Agroalimentaries (IRTA), Aptat. Correus 200, 43540 Sant Carles de la Ràpita, Tarragona, Spain.

*: Corresponding author : To whom correspondence should be addressed; e-mail: villeneuvelan@yahoo.fr , tel: 33-298224090 fax: 33-298224366

Abstract: The effect of the feeding period on larval development was investigated in European sea bass larvae by considering the expression level of some genes involved in morphogenesis. Larvae were fed a control diet except during three different periods (period A: from 8 to 13 d post-hatching (dph); period B: from 13 to 18 dph; period C: from 18 to 23 dph) with two compound diets containing high levels of vitamin A or PUFA. European sea bass morphogenesis was affected by these two dietary nutrients during the early stages of development. The genes involved in morphogenesis could be modulated between 8 and 13 dph, and our results indicated that retinoids and fatty acids influenced two different molecular pathways that in turn implicated two different gene cascades, resulting in two different kinds of malformation. Hypervitaminosis A delayed development, reducing the number of vertebral segments and disturbing bone formation in the cephalic region. These malformations were correlated to an upregulation of retinoic acid receptor γ, retinoid X receptor (RXR) α and bone morphogenetic protein (BMP)4. An excess of PUFA accelerated the osteoblast differentiation process through the upregulation of RXRα and BMP4, leading to a supernumerary vertebra. These results suggest that the composition of diets devoted to marine fish larvae has a particularly determining effect before 13 dph on the subsequent development of larvae and juvenile fish.

Keywords: Sea bass larvae; PUFA; Vitamin A; Morphogenesis; Retinoid pathway

INTRODUCTION

During the first three weeks of life, marine fish larvae undergo major morphological and functional changes to acquire their adult features. From hatching until 7 days post-hatching (dph), the feeding of European sea bass larvae is endogenous. It then becomes mixed until resorption of the vitellus: this generally occurs around 13 dph. At this date, the secretory function of the exocrine pancreas is not fully operational: it only becomes efficient around 18-20 dph. The maturational process of the pancreas is also characterized by a strong decrease in amylase activity between 13 and 20 dph (Zambonino Infante & Cahu, 2001). The maturation of intestinal cells is characterized by a decrease in cytosolic enzyme activities between 18 and 25 dph, while the activities of the brush border membrane enzymes increased. During this period of intense functional changes, a huge morphological transformation of the larvae occurs, in particular with the development of the neurocranium and the jaw, the segmentation of the vertebra that becomes visible around 20 dph, and the settlement of the adult fins between day 27 and day 40 (Barnabé *et al.* 1976).

During the first weeks of development, the maturation processes of the gastrointestinal tract can be influenced by nutritional conditions. Several studies have recently demonstrated that the morphogenesis of marine fish larvae could be perturbed by inappropriate dietary levels of vitamin A (retinol; Haga *et al.* 2002; Villeneuve *et al.* 2005a) or polyunsaturated fatty acids (PUFAs; Cahu et al. 2003). Moreover, the induced skeletal malformations depended on the nutrient. In the case of hypervitaminosis A in European sea bass, Villeneuve *et al.* (2005a) showed that a high percentage of malformations were localized in the cephalic region and that this correlated with an abnormal increase in the level of expression of a nuclear receptor involved in the retinoid pathway, RARγ (Retinoic Acid Receptor). Another study (Villeneuve *et al.* 2005b) revealed that high dietary levels of marine phospholipids containing high levels of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) induced deformities affecting the vertebral column that correlated with the decrease in RXRα

(Retinoid X Receptor), another retinoid pathway receptor capable of constituting heterodimers with fatty acid receptors, PPAR (Peroxisome Proliferator-Activated Receptors). Taken together, these results indicated that the cellular retinoid pathway mainly involved RARs while the fatty acid pathway implicated the RXRs. Furthermore, these two signaling pathways act on different morphological areas through the action of the nuclear receptors.

Vitamin A and its active derivatives the retinoids play a key role in morphogenesis and cellular differentiation processes during vertebrate development (Ross *et al.* 2000). The major active derivative of vitamin A is retinoic acid, **which exists in two active forms: 9-*cis* and all-*trans* retinoic acid.** Its actions involve two different kinds of nuclear receptor: the RARs and the RXRs, **which usually exist under three subfamilies (α, β and γ**; Ross *et al.* 2000). **The RXRs bind to 9-*cis* retinoic acid whereas RARs bind both to all-*trans* and 9-*cis* retinoic acid (Ross *et al.* 2000)**. These receptors can form dimers either together, or with other receptors, such as thyroid hormone, vitamin D or fatty acid receptors (the PPARs, Yu *et al.* 1991). These dimers have the ability to regulate the expression of their target genes. The retinoid pathway can act on more than 500 genes (Balmer & Blomhoff, 2002) including the Hox genes **involved in tissue development and differentiation** (**Krumlauf, 1994**; Suzuki *et al.* 1999), Bone Morphogenetic Proteins (BMPs; Thompson *et al.* 2003) or Insulin-like Growth Factor (IGF; Gabbitas & Canalis, 1997). **BMPs and IGF are implicated in skeletal patterning, bone development and limb morphogenesis. BMPs are multifunctional regulators of vertebrate development: they regulate cell proliferation, differentiation, morphogenesis and apoptosis (Hogan, 1996). IGF-I is a mitotic agent which acts as a growth-promoter and as a differentiation factor in bone, muscle and cartilage (Zizola et *al.* 2002)**. Many studies have demonstrated that inadequate dietary levels of vitamin A lead to the apparition of skeletal malformations (Haga *et al.* 2002, 2003; Villeneuve *et al.* 2005a).

In fish nutrition, lipids are the major energy source (Sargent *et al.* 1999) and as fish are unable to synthesize *de novo* n-3 PUFA, they have to find them in their food. It has been

recently demonstrated that these PUFA, and more particularly EPA and DHA, are more efficiently used by marine fish when present in the phospholipidic fraction of the diet (Gisbert *et al.* 2005). Fatty acids are natural ligands for PPARs which play a key role in lipid metabolism and energy balance. As mentioned above, PPARs and RXRs can dimerize to regulate the target genes involved in these two signaling pathways (Keller *et al.* 1993). This underlines the key role of RXRs in several pathways and, as a consequence, any perturbation of the expression of the RXRs may have repercussions on these pathways.

Many genes involved in development processes, including Hox genes, have been intensively studied and some of them are expressed in defined spatio-temporal windows (Krumlauf, 1994). **Nevertheless, few studies have investigated interactions between exogenous nutrition and the genes involved in morphogenesis. It has been shown that an excess of vitamin A can have negative effects on fish morphogenesis, but in this case, the excess of vitamin A was present in tank water (balneation) but not directly in the diet (Suzuki *et al*., 2000).** We previously demonstrated that diets containing high levels of vitamin A or phospholipids disturbed developmental processes when ingested by European sea bass larvae between day 7 and day 40. In the present study, our goal was to determine whether these effects appeared when larvae were fed these diets at a particular developmental stage. To check this, we considered three different periods of larval life.

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 *Ifremer* (Centre de Brest). Fish were acclimated and divided into twenty one 35 L-cylindroconical fibreglass tanks (2100 larvae/tank) at a initial density of 60 larvae/L. Tanks were supplied with running sea water, which had been previously passed through first a sand filter, followed by a tungsten heater and a degassing column packed with

plastic rings. Throughout the experiment, water temperature and salinity were kept at 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). The 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 Animal (NRC, 1985).

Four days after hatch (dph), larvae were divided into 7 experimental groups (3 replicates per group) that were fed three isonitrogenous and isolipidic experimental compound microdiets from the onset of exogenous feeding at 7 dph. The control diet contained 0.08 g/kg of vitamin A (Sigma R3250, 500000 UI/g) and 175 g/kg of soybean lecithin, whereas the RA diet contained 32 g/kg of retinol (Table 1). Although the phospholipid content of the experimental diets was similar, diets differed in their respective sources of this nutrient; marine phospholipid in the MP diet and soybean lecithin in the RA diet. The composition of the control diet was very similar to the one previously used and that had supported good growth and survival in European sea bass larvae (Villeneuve et al., 2004) and in this study, it has been used as the control diet. In order to evaluate the ontogenetic effect of the diet on larval morphogenesis, fish were fed RA and MP diets at three different developmental periods (Fig. 1): between 8 and 13 dph (abbreviated as RA-A and MP-A), 13 and 18 dph (abbreviated as RA-B and MP-B) and 18-23 dph (abbreviated as RA-C and MP-C), while apart from the above-mentioned intervals, larvae were fed the control diet. The control group was fed the control diet throughout the experimental time. Microdiets were prepared as previously described (Cahu, 2003) and pellet size was 200-400µm. Throughout the experimental period, larvae were continuously fed in large excess for 24 hours per day using automatic belt feeders. Food ingestion was monitored by observing larvae digestive tracts under a binocular microscope, dietary microparticles being visible by transparency.

Table 1
Composition of the experimental compound diets.

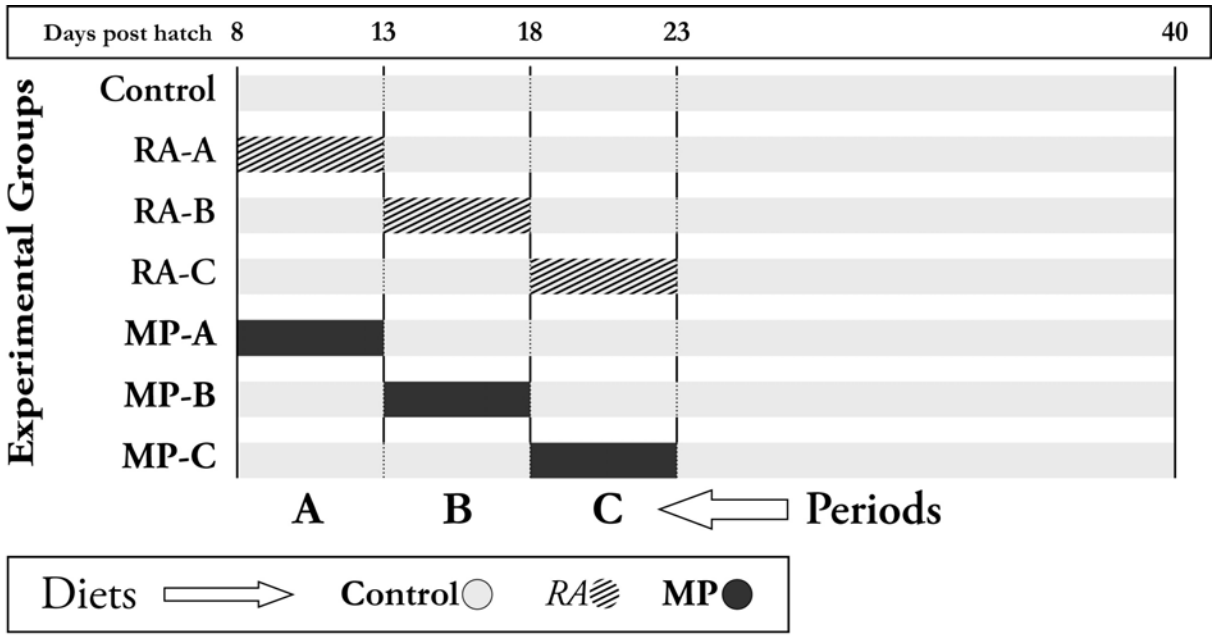
Ingredients	**C**	**RA**	**MP**
Fish meal[1]	555	555	555
Hydrolyzed fish meal (CPSP)[1]	140	140	140
Soybean lecithin[1]	175	175	0
Marine lecithin[1]	0	0	175
Vitamin mixture (without RA)[2]	80	80	80
Mineral mixture[3]	40	40	40
Betaine	10	10	10
Retinol (*all-trans*)	0.08	32	0.08
Analysed composition		%	
Proteins (Nx6.25)	56	56	59
Lipids	19	19	17
Containing:			
Phospholipids	13	13	13
EPA + DHA	1.1	1.1	4.8
Ash	15	15	15
Dry matter	94	94	94
Energy (J/kg)	1651	1651	1626

[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 lecithin (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, KIO_4 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.

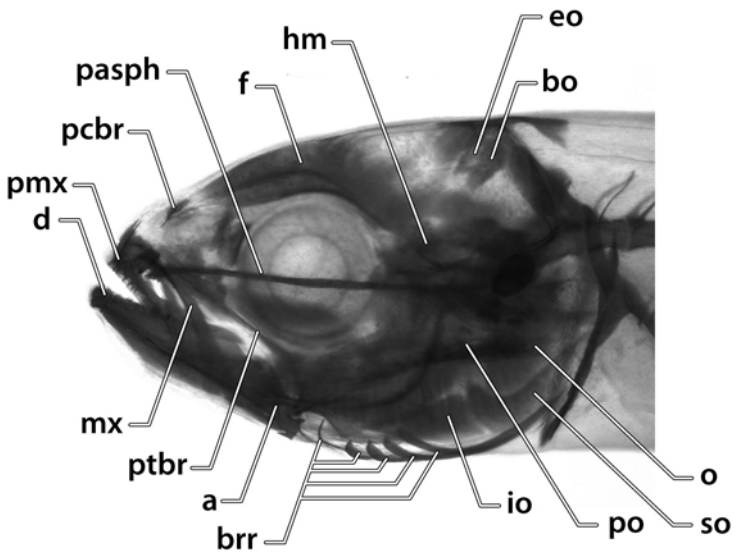
Figure 1. Schema of the distribution of control, RA and MP diets.



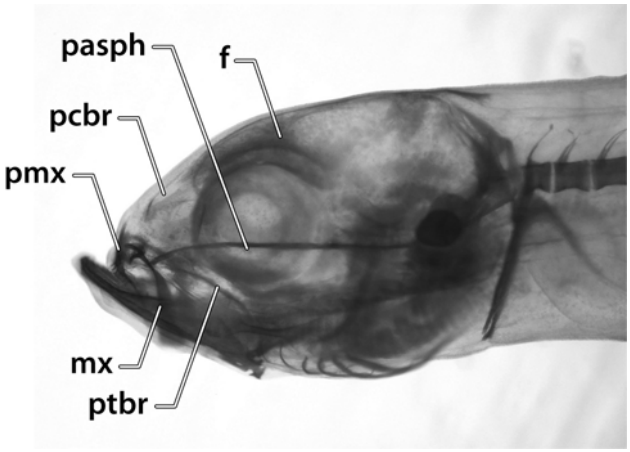
Sampling. To evaluate larval growth, ten specimens were randomly sampled from each experimental tank (30 larvae per experimental diet) at 25 and 37 dph. They were killed with an overdose of anaesthetic (tricaine methanesulfonate, MS 222) and their wet body weight was measured to the nearest 0.1 mg. Larvae (20-50 larvae, depending on wet body weight) were sampled from each tank at 25 dph and kept at –20°C pending assays of pancreatic (trypsin, EC 3.4.21.4 and amylase, EC 3.2.1.1) and brush border intestinal enzymes (alkaline phosphatase, EC 3.1.3.1), which are indicators of the maturation level of the digestive tract. Fifty larvae were collected for mRNA studies from all experimental tanks at 13, 16, 25 and 37 dph, and total RNA was immediately extracted. In all cases, sampling procedures were performed as previously described (Cahu *et al.* 2003). The incidence of body skeletal malformations (splachnocranium, neurocranium and vertebral column deformities) was counted at 37 dph (100 larvae per experimental diet). A complete description of each malformation typology has been previously described in Villeneuve *et al.* (2004) **and head**

abnormalities are summarized in Figure 2. Repetitive sampling made it impossible to calculate larval survival.

Figure 2. Dietary induced malformations in the cranium of European sea bass larvae (age: 40 days post hatch). (a) Normal larvae, (b) deformed larvae (abnormal structures are indicated). Specimens were stained with Alizarin red to reveal calcified structures. Abbreviations: *a*, angular; *bbr*, branchiostegal rays; *bo*, basioccipital ; *d*, dentary; *eo*, exoccipital ; *f*, frontal; *hm*, hyomandibula ; *io*, interoperculum; *mx*, maxilla; *o*, operculum; *pasph*, parasphenoid; *pcbr*, lamina paracerebralis; *pmx*, premaxilla; *po*, preoperculum; *ptbr*, pterygoid process; *so*, suboperculum.



b



Analytical methods. Larvae were dissected under a binocular microscope on a glass cutting board kept on ice (0ºC). Individuals were cut into 4 parts: head, pancreatic segment (PS), intestinal segment (IS) and tail, in order to limit the assay of enzymes to specific segments. This dissection inevitably produced a crude mixture of organs in each segment. The pancreatic segment contained the pancreas, liver, heart, muscle and spine. The intestinal segments **were homogenized in 30 volumes (v/w) of mannitol (50 mM) Tris-HCl (2 mM), pH = 7,** and contained the intestine, muscle and neural spine (Zambonino Infante *et al*. 1997). Once dissected, the pancreatic segments were homogenized in 5 volumes (v/w) of ice-cold distilled water. Trypsin and amylase activities were measured using Na-benzoyl-DL-arginine-p-nitroaniline and starch as substrates (Holm *et al.* 1988; Métais & Bieth, 1968), respectively, in both pancreatic and intestinal segments. Purified brush border membranes from the intestinal segment were obtained according to a method developed for intestinal scrapping (Crane *et al.* 1979). Alkaline phosphatase, an enzyme of the intestinal brush border membrane, was quantified using p-nitrophenylphosphate as substrate (Bessey *et al.* 1946). The degree of purification of the brush border membrane, considering alkaline phosphatase as a marker for cell membrane fraction, was close to that reported in the literature (i.e. 11.0; Crane *et al.* 1979). Pancreatic and intestinal specific enzyme activities were expressed as μmol of substrate hydrolyzed per min and mg of protein (U/mg protein), with protein being determined using the Bradford method (Bradford, 1976). Secretions of trypsin and amylase were calculated as a ratio of activity in the intestinal segment related to total activity (PS+IS), considering that enzyme activity in PS can be used as an index of the synthesis function of pancreas and in IS as an index of pancreatic secretory function (Péres *et al.* 1998).

Protein concentration in the 3 diets was assayed following the Dumas method (Nitrogen Analyser 2000, Fisons Instruments, Nx6.25, Carlo Erba, Milan, Italy).

Total lipids in diets were determined according to a slightly modified version of Folch's procedure (Folch *et al.* 1957), chloroform being replaced by dichloromethane. The separation

of neutral lipids and phospholipids was carried out according to the procedure described by Juaneda & Rocquelin (1985). Fatty acids were saponified by a 2 M KOH-methanol solution and then esterified in a 0.7 M HCl-methanol solution. Fatty acid methyl esters (FAME) were separated by gas chromatography, in an Auto-system Perkin-Elmer with a flame ionization detector, BPX 70 capillary column (25 m x 0.22 mm i.d. x 0.25 μm film thickness) and split-splitless injector, with helium as the carrier gas. The injector and detector temperatures were 220ºC and 260°C, respectively. The initial oven temperature was 50°C, but was increased to 180°C by increments of 15°C/min, maintained for 5 min, and finally increased to 220°C by increments of 3°C/min. Data acquisition and handling were carried out by connecting the gas chromatograph to a Nelson computer. The individual FAME were identified by comparing the retention times of authentic standard mixtures and the results of individual FA compositions were expressed as percentages of total identified FAME in the PL and NL fractions.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Different cDNA fragments from genes coding for retinoic acid receptors and for signaling molecules known for interacting with retinoic acid were purified in European sea bass larvae by RT-PCR, cloned (Cahu *et al.* 2003), 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 (BMP-4; AJ 567451) and Insulin-like Growth Factor-I (IGF-I; AJ 579342). Glyceraldehyde-3-phosphate deshydrogenase (GAPDH; AJ 567450) was chosen as house-keeping gene.

Real-time RT-PCR. cDNA samples were treated with DNase 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), 7.5 μL QuantiTect

SYBR Green PCR Master Mix 2X (QIAGEN GmbH, Germany). For each target gene, forward and reverse primers (Villeneuve *et al.* 2004) were chosen from the sequences previously cloned. Thermal cycling was initiated with incubation at 95°C for 13.5 min in order to activate HotStarTaq^{TM} DNA Polymerase. After this initial step, 45 cycles of PCR were performed. Each PCR cycle consisted of heating for 30 sec at 95°C for denaturing, then for 1 min at 60°C 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 these products were sequenced by MilleGen (Labège, France). Standard curves were established for each gene by plotting CT values against the $\log_{10}$ of 5 different dilutions (in triplicate) of cDNA sample solutions. Real-time PCR efficiency were determined for each gene from the slopes given by BIORAD software, applying the equation $E=10^{[-1/slope]}$. We calculated the relative expression ratio of each gene using REST© software (http://www.wzw.tum.de/gene-quantification/). The relative expression ratio for a considered gene is based on the PCR efficiency (E) and CT of a sample versus the control, and expressed in comparison to the reference gene (GAPDH), according to Pfaffl's mathematical model (Pfaff, 2001):

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

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 the activation and proliferation of cells (Gause & Adamovicz, 1994).

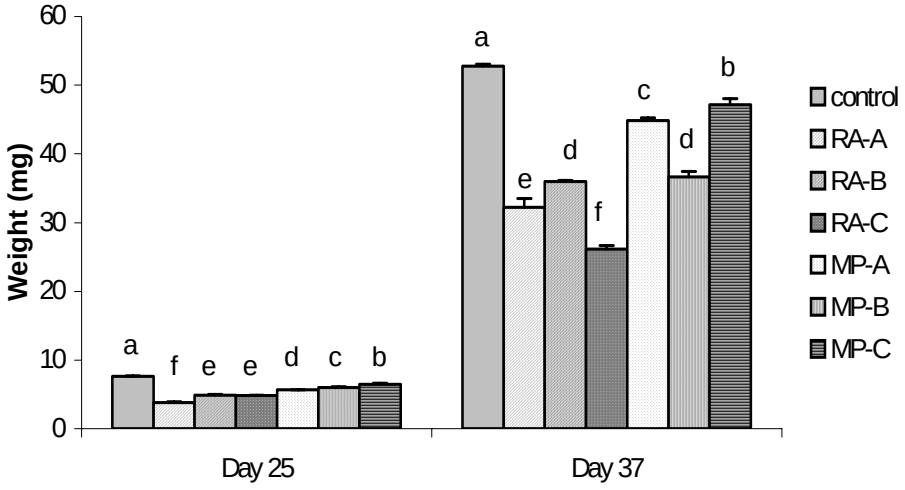
Statistical analyses. Results are given as means $\pm$ SD. All data were checked for variance homogeneity using Barlett's test (Dagnelie, 1975). Growth, enzymatic specific activity and $\arcsin(x^{1/2})$-transformed trypsin and amylase secretions were compared by means of a one-way ANOVA followed by 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).

RESULTS

Growth. At 25 dph, larvae fed the control diet exhibited the best growth (Fig. 3). Whatever the feeding period, the weight of larvae fed the MP diet was significantly greater than that of larvae fed RA diets. Globally, the more the larvae were fed diets in the early stages, the less they grew (MP-C > MP-B > MP-A).

Figure 3. Growth at 25 and 37 dph of European sea bass larvae fed isonitrogenous and isolipidic diets containing a high level of vitamin A (RA diet), a high level of marine phospholipids (MP diet) or the control diet, according to the feeding periods. Means ± S.D. (n = 3) with different superscript letters for the same day being significantly different ($P < 0.05$).



At 37 dph, the control group still exhibited the best growth (52.8 ± 0.26 mg) and fish fed MP diets were still heavier than those fed RA diets over the same periods. In the experimental groups fed RA diets, the best growth was observed in the RA-B group (36 ± 0.17 mg) and the lowest in the RA-C group (26.1 ± 0.52 mg), while the RA-A group exhibited intermediate growth (32.2 ± 1.26 mg; P < 0.05). In the MP groups, the greatest growth was obtained in the MP-C group (47.2 ± 0.9 mg), the least in the MP-B group (36.6 ±

0.76 mg), with intermediate growth being observed in the MP-A group (44.9 ± 0.35 mg; $P < 0.05$).

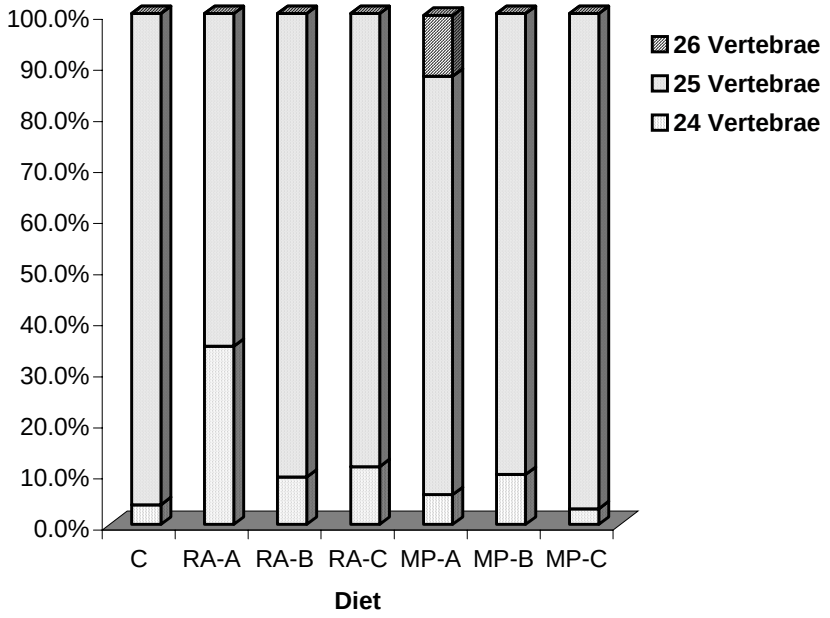
Skeletal malformations. The number of vertebrae most frequently observed in European sea bass larvae is 25. The six experimental groups fed on RA or MP diets contained individuals with 26 or 24 vertebrae instead of 25.

In the MP-A group, 3% of the larvae exhibited 23 vertebrae but this was not significantly different ($P = 0.413$) from findings for other groups.

RA diet ($F = 21.12$, $P = 0.001$) and feeding period ($F = 8.76$, $P = 0.006$) effects were evidenced in fish with 24 vertebrae. The RA diet induced the loss of a vertebra during period A. No significant difference was observed when this diet was applied during periods B and C (Fig. 4).

MP diet ($F = 17.79$, $P = 0.002$) and feeding period ($F = 17.25$, $P = 0.0006$) effects were also observed in larvae with 26 vertebrae. The number of vertebrae significantly increased in association with the MP diet ($P < 0.005$) and this effect was only apparent when the diet was ingested during period A ($P \leq 0.0001$; Fig. 4).

Figure 4. Percentage of vertebrae number in European sea bass larvae at 37 days post hatch.



The number of fish exhibiting jaw malformations depended on diet ($F = 16.96$, $P = 0.003$) but not on the feeding period. At 40 dph, 4 to 8% of larvae exhibited jaw deformities in the control and MP groups. The rate of these deformities was significantly higher in the experimental groups fed on RA diets ($P < 0.05$) regardless of the period.

The rate of malformations in the vertebral column region (scoliosis, lordosis, vertebrae fusion…) depended both on the diet ($F = 40.83$, $P = 0.0001$) and on the feeding period ($F = 15.54$, $P = 0.001$). This rate was significantly higher in fish fed on the MP diet ($P = 0.0003$) during periods A ($P < 0.02$) and B ($P < 0.009$), respectively affecting 20% and 29% of the total number of fish at day 40. At the same age, only 6% to 19% of larvae fed on either the control or RA diet exhibited vertebral deformities.

Enzymatic activities and secretion rate. Specific activities and secretion rates were determined at 25 dph.

The highest specific activity of alkaline phosphatase was measured in the control group (1192 ± 170.2 mU/mg prot; Fig. 5). There was no statistically difference between the

control, RA-B, RA-C and MP-B groups. This specific activity was 64% lower in the RA-A group (429 ± 5.7 mU/mg prot) and 57% lower in the MP-A group (512 ± 88.4 mU/mg prot).

The specific activity of amylase did not significantly differ between the control, RA-C and MP-C groups. This activity was significantly higher (Fig. 6) in the other four experimental groups.

Figure 5. Specific activity of alkaline phosphatase of the intestinal brush border in European sea bass at 25 days post hatch. Values shown are means ± S.D. (n = 3) and different superscript letters for the same day designate significant differences ($P < 0.05$).

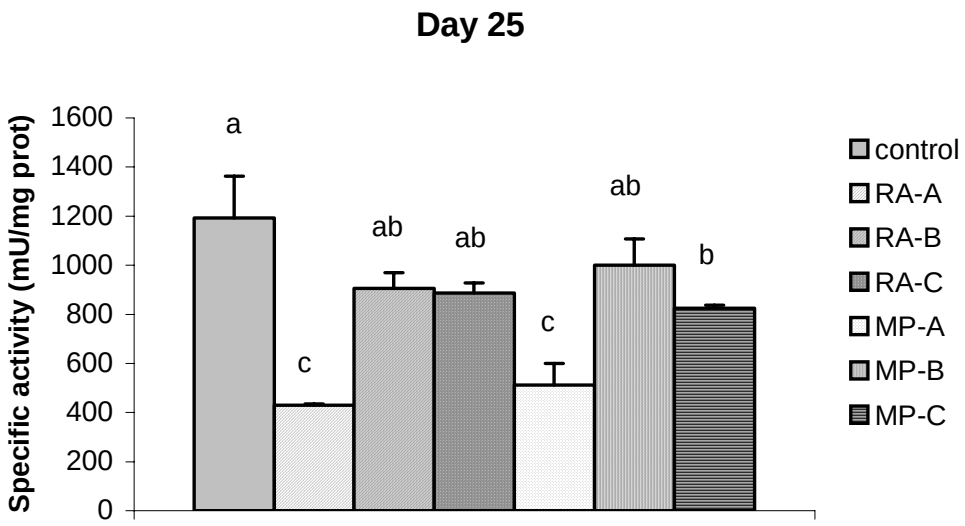
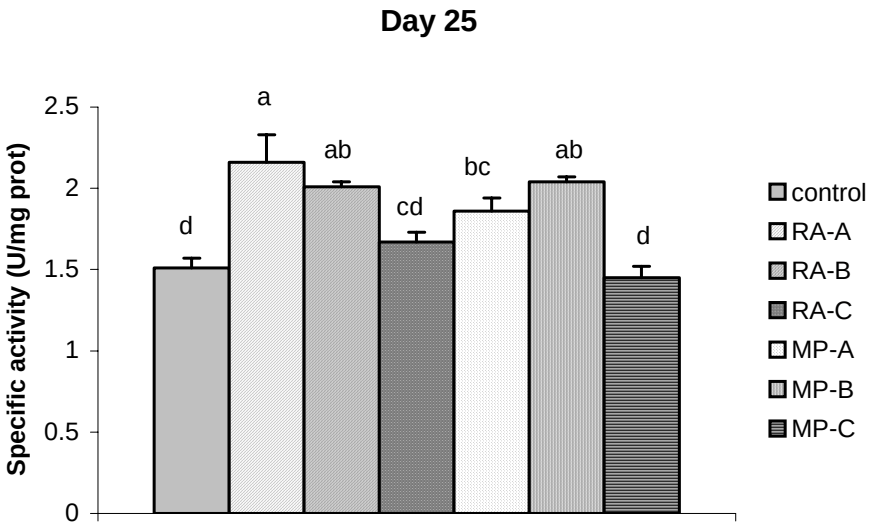
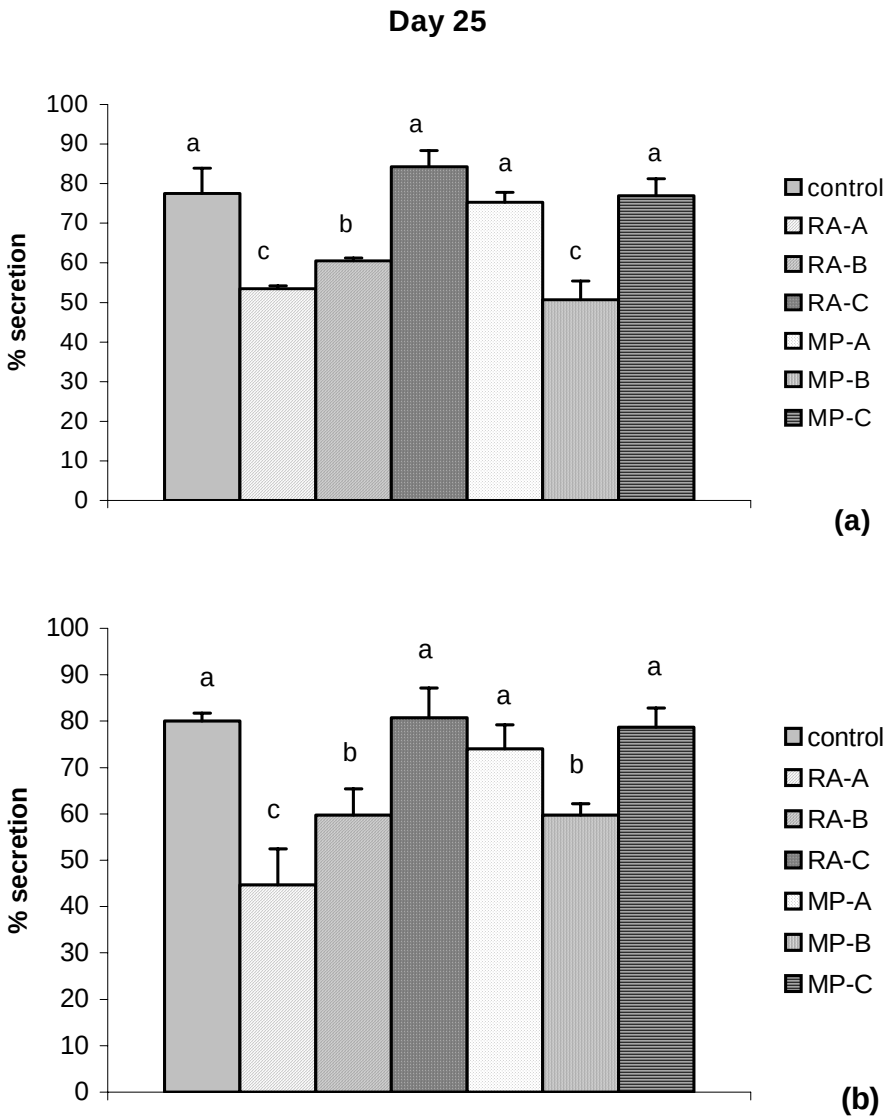


Figure 6. Specific activity of amylase in European sea bass at 25 days post hatch. Values shown are means ± S.D. (n = 3) and different superscript letters for the same day designate significant differences (P < 0.05)



The highest values for amylase secretion rate were observed in the control group (Fig. 7a) and in the MP-A, MP-C and RA-C groups (77.5 ± 6.36, 75.3 ± 2.52, 77 ± 4.24 and 84.3 ± 4.04 %, respectively). The lowest values were measured in fish fed on MP-B and RA-A (50.7 ± 4.73 and 53.5 ± 0.71%, P < 0.05; respectively). The RA-B group exhibited an intermediate value. The trypsin secretion profile was comparable to that of amylase (Fig. 7b).

Figure 7. Secretion rate of amylase (a) and trypsin (b) in European sea bass larvae at 25 days post hatch. Values shown are means ± S.D. (n = 3) and different superscript letters for the same day designate significant differences ($P < 0.05$).



Gene expression. Variations in gene transcription are reported in table 2 and significant variations during periods A and B are emphasized in Figure 8. This graph shows that the variations in RXRα transcription observed at 18 dph (regardless of the feeding period) and in BMP4 transcription at 13 dph for period A were linked to the developmental stage of the larvae while the other variations were induced by the diets.

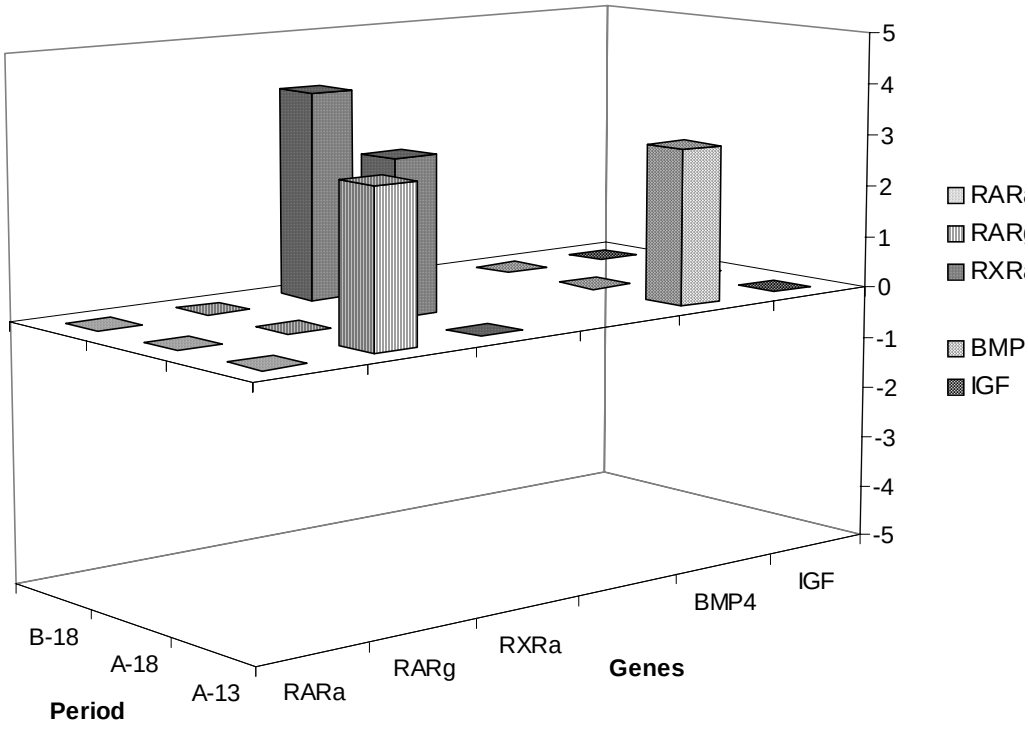
At 13 dph, in the RA-A group, RARγ and BMP4 transcriptions were significantly higher (transcription factors: x2.77, $P < 0.03$ and x2.84, $P < 0.003$, respectively) than in the control group. BMP4 transcription was also higher in the MP-A group (transcription factor: x2.34, $P < 0.03$) while RARγ transcription was not modified ($P = 0.08$).

Table 2. Variations in gene transcription levels during periods A, B and C in larvae fed RA or MP diets, in comparison to the control group. Up and down regulations correspond to positive and negative values respectively. Significant variations are in bold.

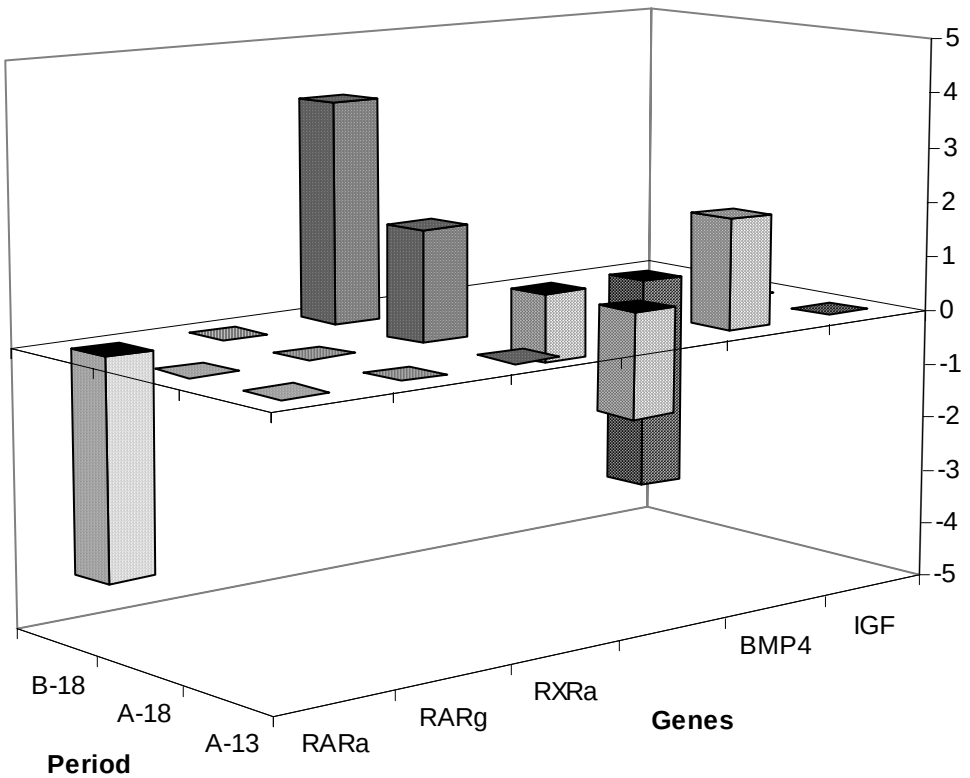
		Period A		Period B		Period C	
Day 13	RARα - RA	+ 1.49	P=0.40				
	- MP	- 1.14	P=0.73				
	RARγ - RA	**+ 2.77**	**P<0.03**				
	- MP	+ 2.17	P=0.08				
	RXRα - RA	+ 1.19	P=0.6				
	- MP	+ 1.36	P=0.33				
	BMP4 - RA	**+ 2.84**	**P<0.003**				
	- MP	**+ 2.34**	**P<0.03**				
	IGF-I - RA	- 2.18	P=0.89				
	- MP	- 2.06	P=0.9				
Day 18	RARα - RA	+1.06	P=0.70	-1.04	P=094		
	- MP	-1.55	P=0.23	**-3.51**	**P<0.001**		
	RARγ - RA	-1.04	P=0.83	+1.49	P=0.42		
	- MP	-1.19	P=0.74	-1.66	P=0.29		
	RXRα - RA	**+2.88**	**P<0.001**	**+3.46**	**P<0.05**		
	- MP	**+1.85**	**P<0.001**	+1.19	P=0.79		
	BMP4 - RA	+1.06	P=0.6	+1.14	P=0.32		
	- MP	**-1.81**	**P<0.001**	**-1.26**	**P<0.001**		
	IGF-I - RA	-1.07	P=0.68	+1.43	P=0.22		
	- MP	-1.54	P=0.35	**-3.43**	**P<0.001**		
Day 23	RARα - RA	+1.37	P=0.09	-1.09	P=069	+1.47	P=0.29
	- MP	+1.06	P=0.7	+1.35	P=0.4	+1.49	P=0.26
	RARγ - RA	+1.16	P=0.65	**-1.96**	**P<0.001**	+1.20	P=0.45
	- MP	+1.13	P=0.6	-1.19	P=0.56	**+1.96**	**P<0.03**
	RXRα - RA	+1.12	P=0.29	**-1.94**	**P<0.001**	+1.21	P=0.75
	- MP	-1.19	P=0.4	-1.21	P=0.5	**-2.31**	**P<0.03**
	BMP4 - RA	**+1.50**	**P<0.001**	**-2.64**	**P<0.001**	+1.12	P=0.61
	- MP	-1.22	P=0.27	**-2.06**	**P<0.001**	-1.16	P=0.68
	IGF-I - RA	+1.20	P=0.64	-1.19	P=0.74	+1.48	P=0.32
	- MP	-1.13	P=0.58	-1.36	P=0.29	-1.07	P=0.72
Day 40	RARα - RA	**+3.39**	**P<0.002**	**+2.99**	**P<0.001**	**+3.82**	**P<0.001**
	- MP	**+2.42**	**P<0.001**	**+7.89**	**P<0.001**	+1.47	P=0.36
	RARγ - RA	**+3.25**	**P<0.001**	+1.03	P=0.88	+1.95	P=0.17
	- MP	+1.00	P=0.93	**+3.15**	**P<0.001**	-1.01	P=0.9
	RXRα - RA	+1.34	P=0.22	-1.11	P=0.81	+1.59	P=0.23
	- MP	-1.13	P=0.34	**+3.58**	**P<0.001**	+1.13	P=0.56
	BMP4 - RA	**+2.08**	**P<0.02**	+1.21	P=0.48	+1.73	P=0.09
	- MP	-1.09	P=0.73	**+3.44**	**P<0.001**	-1.03	P=0.84
	IGF-I - RA	+1.26	P=0.41	+1.15	P=0.54	+1.34	P=0.3
	- MP	-1.19	P=0.40	+2.48	P=0.1	-1.33	P=0.4

Figure 8. 3D representations of significant up and down regulations of gene transcription reported in Table 2 in larvae fed RA (7A) and MP (7B), during the period A (d13 and d18) and B (d18).

Figure 8
Effect of RA diet



Effect of MP diet



At 18 dph, RXRα transcription was positively regulated in the RA-A and MP-A groups (respective transcription factors: x2.88 and x1.85, $P < 0.001$). In the RA-B group, RXRα transcription was 3.5 times greater than in the control group. In fish fed the MP diet during period A, BMP4 transcription was 1.8 times lower ($P < 0.001$). In fish fed on MP during period C, the transcription levels for RARα, BMP4 and IGF-I were significantly lower (respective transcription factors: 1/3.51, 1/1.26 and 1/3.43, $P < 0.001$).

At 23 dph, in the RA-A experimental group, BMP4 transcription was 1.5 times higher while, in the RA-B group, RARγ, RXRα and BMP4 transcription was respectively 1.96, 1.94 and 2.64 times ($P < 0.001$) lower than in the control group. In fish fed MP-B, BMP4 transcription was 2.1 times less ($P < 0.001$). In fish fed MP-C, RARγ transcription increased (transcription factor: x1.96, $P < 0.03$) while RXRα transcription decreased (transcription factor: 2.3, $P < 0.03$). At this date, the MP diet had no noticeable effect when ingested during period A.

At the end of the rearing period (40 dph), RARα, RARγ and BMP4 transcription was significantly higher in group RA-A than in the control (respective transcription factors: x3.39, $P < 0.002$; x3.25, $P < 0.001$ and x2.08, $P < 0.02$). 2.42, 2.99, 3.82 and 7.89 fold increases in RARα transcription were respectively observed for the MP-A ($P < 0.001$), RA-B ($P < 0.001$), RA-C ($P < 0.001$) and MP-B ($P < 0.001$) groups with respect to the control. In fish fed MP-B, transcription of RARγ, RXRα and BMP4 was also enhanced (respective transcription factors: x3.15, x3.58 and x3.44, $P < 0.001$). At this date, there was no variation in gene expression in the MP-C group with respect to the control.

DISCUSSION

At present, there is very little information available about the effect of nutrients on morphogenesis processes in fish. Studies on mammals indicate that some nutrients act on several key genes that govern morphogenesis, such as Hox genes (Krumlauf, 1994), during

specific windows of time. Based on these data, we may assume that a comparable action occurs in fish at specific periods. In mammals, studying these windows is very difficult since developmental processes occur *in-utero.* In sea bass larvae, these developmental processes still continue after hatching and this particularity facilitates studies concerning the effects of nutrition on morphogenesis and makes this a very interesting species for developmental studies.

At 23 dph, the control group exhibited the best growth. In MP groups, the lowest growth was observed when the MP diet was ingested, during period A. We had previously demonstrated (Villeneuve et al., 2005b) that a diet containing a high percentage of EPA and DHA (4.8%) in the phospholipid fraction did not allow optimal larval growth. The present data confirm this result and also indicate that the earlier this MP diet is ingested, the greater its negative affects upon larval growth. In the case of a hypervitaminosis A condition, sea bass larvae growth was globally more depressed in comparison with the two other larvae groups, but this reduction in growth was more pronounced during (8-13 dph) period A. At the end of the experiment (day 40), global growth followed a similar pattern to that observed at day 23, except for surprisingly poor growth in the RA-C experimental group. This result was certainly not only due to nutritional conditions, and might have been related to other parameters that were not monitored in this work.

During the first weeks of life, the digestion processes in marine fish larvae switch from a primary to an adult mode of digestion (Zambonino Infante & Cahu, 2001). Some changes occur in the specific activities of pancreatic enzymes, with a particularly marked decrease in amylase with age. Moreover, the pancreatic secretion function becomes progressively efficient. The maturation of intestinal cells is characterized by a reduction in cytosolic enzyme activities and a sharp increase in brush border membrane enzyme activities. Alkaline phosphatase is considered to serve as a marker for the maturation of the brush border of enterocytes: the greater its activity, , the better the level of intestinal maturation

(Zambonino Infante & Cahu, 2001). Maturation of the gastrointestinal tract is normally achieved around 25 dph in European sea bass (Zambonino Infante & Cahu, 2001). We consequently measured specific enzymatic activities or pancreatic secretion level at this date. In the present work, a higher level of alkaline phosphatase specific activity was observed in the control group, indicating that these fish exhibited the best development; in the others groups, the overall levels of alkaline phosphatase activity were in line with general growth rates. A proper development of control and MP-C larvae was also supported by the data of trypsin and amylase pancreatic secretion at day 25, and the low level of amylase activity. In the other experimental groups, amylase specific activity was greater, suggesting a delay in the maturation of the digestive tract. **Previous works have shown that unbalanced diets induced a delay in digestive maturation (Ribeiro *et al.* 2002). This delay in maturation was always associated with a reduction in larvae growth and survival. These effects were mainly linked to poor digestion and metabolic use of these diets.**

We observed an effect of the diet and feeding period on European sea bass morphogenesis and especially on their number of vertebrae. Other authors had previously reported that the number of vertebrae in fish could be influenced by factors other than nutrition, such as triploidy in trout (Kacem *et al.* 2004) and by temperature in halibut (Lewis *et al.* 2004). Compared to the control group, a significant percentage of larvae exhibited the loss of one vertebra (n = 24) when fed the RA diet during period A, or the development of a supernumerary vertebra (n = 26) with the MP diet during the same period. In both cases, RXRα expression increased at day 18. We have previously demonstrated that, under optimal nutritional conditions, this nuclear receptor expression was high during the early stages of development and then decreased as the differentiation processes came to an end (Villeneuve *et al.* 2004). The present data also indicated that RXRα was highly expressed during early stages, as its expression was as high in the RA-A and MP-A groups as in the control before day 18. Afterwards, its expression was higher in the RA-A and MP-A groups than in fish fed

the control diet. This indicated that these two diets stimulated its transcription when they were distributed between 8 and 13 dph.

In fish fed diets containing an excess of retinol (RA diet), we observed an increase in RARγ transcription at day 13, which was probably the result of direct stimulation by retinoic acid. The literature indicates that RARs and RXRs can form heterodimers and that these dimers can control the transcription levels of the receptors involved in them (Balmer & Blomhoff, 2002; Ross *et al*. 2000). We can therefore hypothesise that the increased number of RARγ receptors at day 13 probably stimulated RXRα expression at day 18. Furthermore, we demonstrated in a previous study that in a case of hypervitaminosis A, RARγ was the main RAR isoform involved in the apparition of skeletal malformations that affected the cephalic region of sea bass larvae (Villeneuve *et al.* 2005a). The RA-A group exhibited deformities localised in the cephalic region (as mentioned above) but also in the vertebral column (loss of one vertebra). At day 13, BMP4 expression was stimulated and mention is made in the literature of how a synergy between BMP4 action and the retinoid pathway (Glozak & Rogers, 1998) can lead to cellular apoptosis. This apoptosis process had been observed in embryonal carcinoma cells: in the presence of BMP4, the specific activation of RARα and RARγ (via the presence of 9-cis retinoic acid in the culture medium), is sufficient to cause cell death (Glozak & Rogers, 1998). It has also been demonstrated that BMPs and retinoid signalling pathways could cooperate to induce the differentiation of marrow preadipocytes into osteoblasts (Skillington *et al.* 2002). These two types of cell probably resulted from a common precursor, located in bone marrow that can differentiate *in vitro* between adipocytes and osteoblasts (Skillington *et al.* 2002). Indeed, under conditions leading to bone loss (for example osteoporosis), the decreased osteoblast count correlated with increased adipocyte differentiation, suggesting that adipocytes are generated at the expense of osteoblasts.

Osteoblastic differentiation is characterised by stimulation of the expression of alkaline phosphatase (Skillington *et al.* 2002). In our study, we did not observe any increase in

alkaline phosphatase-specific activity, almost certainly because of the delayed development of larvae fed RA-A. This delay was revealed by their poor intestinal maturation and their high amylase specific activity which should have decreased under normal conditions (Cahu & Zambonino Infante, 2001). Taking these results together, we can hypothesise that the increase in BMP4 and RARγ expression reduced the number of osteoblasts available for bone formation and that this loss of bone cells was counterbalanced by preadipocyte differentiation into osteoblast induced by the co-operation between retinoic acid and BMP4. These events certainly interfered with the normal process governing differentiation of bone tissue and actively delayed it. Our hypothesis is supported by the persistence of high levels of RXRα expression and the fact that this receptor is necessary for achieving osteoblast differentiation. Nevertheless, the reduced number of osteoblasts may have led, on one hand, to the loss of a vertebra and, on the other, to malformations affecting the cephalic region. It is interesting to note that RXRα transcription continued to be influenced in fish fed on the RA diet during period B, but, in this case, it only affected the process associated with morphogenesis of the head. This suggests that, during this window of development, the genes targeted by RXRα, and involved in the formation of the vertebral column, did not receive any other influences. This also revealed that the morphogenesis mechanisms implicated in its formation were successfully carried out.

With respect to larvae fed on the MP diet, the increased RXRα expression at day 18 could not be correlated with a stimulation of RARγ since the MP and control diets had the same vitamin A level. The MP diet contained a high level of marine phospholipids with an elevated percentage of PUFA, and particularly of EPA and DHA (4.8%). European sea bass larvae digest this kind of phospholipid very efficiently (Cahu et al. 2003) leading to an increased PUFA body content. These fatty acids are natural ligands for PPARs and we assume that the excess of PUFA in the larvae stimulated PPAR expression. PPARs dimerize with RXRs and that this binding may be responsible for the high levels of RXRα transcription

observed. Moreover, DHA is known to be a RXR ligand (Mata de Urquiza et al. 2000). Thus, the binding of DHA with RXRα could also be responsible for the increased transcription of RXRα in fish fed on the MP diet. At day 13, the stimulation of BMP4 expression could have amplified the osteoblast differentiation process leading to the apparition of a supernumerary vertebra. At 18 dph, the low level of BMP4 transcription suggested that osteoblast maturation had been achieved, since it had been previously reported that BMP4 expression decreases as bone matures (Solheim, 1998). The pancreas development markers seemed to confirm this hypothesis: the elevated secretion rate (trypsin and amylase) revealed that the exocrine pancreas maturation process in the MP-A group was at a comparable level to that observed in the control group, while it was delayed in the RA-A and MP-B groups (no BMP4 stimulation was observed in these groups). Moreover, amylase specific activity was significantly lower in the MP-A than RA-A group.

Villeneuve et al. (2004) previously described the normal expression profile for the genes studied during the development of sea bass larvae reared under optimal nutritional conditions. They reported a global increase in RARs expression and a decrease in RXRα transcription during larval development. In the present study, at 23 and 40 dph, the expression levels of both RARs were greater in the experimental groups than in the control group while expression levels for RXRα and BMP4 were lower than in the reference group. These results suggested that normal expression tendencies, even though emphasised with respect to the control group, were again observed from day 23 but that they were also still influenced by nutritional parameters. These transcription modulations for the genes implicated in the retinoid signalling pathway had no effect on the malformation rate as the morphogenesis and cellular differentiation processes could no longer be influenced at these periods.

In summary, European sea bass morphogenesis is very sensitive to vitamin A and phospholipid dietary levels during the early stages of development. The genes involved in these processes can be modulated between 8 and 13 dph and our results indicated that retinoid

and fatty acid pathways implicated two different gene cascades. Inadequate dietary levels of vitamin A and PUFA led to different kinds of skeletal malformations. On one hand, hypervitaminosis A delayed development, reducing the number of vertebral segments and disturbing bone formation in the cephalic region, while on the other, an excess of PUFA accelerated osteoblast differentiation, leading to a supernumerary vertebra. These results suggest that, from a nutritional point of view, it is necessary to distinguish two larval developmental periods, those before and after 13 dph, since the composition of their diet could have a significant impact on sea bass morphogenesis before this date.

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