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)

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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.

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

During the first 3 weeks of life, marine fish larvae undergo major morphological and functional changes to acquire their adult features. From hatching until 7 d 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, only becoming 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 activity between 18 and 25 dph, whereas the activity of the brush border membrane enzymes increases. During this period of intense functional changes, a huge morphological transformation of the larvae occurs, in particular in the development of the neurocranium and the jaw, the segmentation of the vertebrae 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 PUFA (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 this correlated with an abnormal increase in the level of expression of a nuclear receptor involved in the retinoid pathway, the retinoic acid receptor (RAR) γ. Another study (Villeneuve et al. 2005b) revealed that high dietary levels of marine phospholipids containing high levels of EPA and DHA induced deformities affecting the vertebral column that correlated with the decrease in retinoid X receptor (RXR) α, another retinoid pathway receptor capable of constituting heterodimers with fatty acid receptors, PPAR. Taken together, these results indicated that the cellular retinoid
pathway mainly involved RAR, whereas the fatty acid pathway implicated the RXR. Furthermore, these two signalling pathways acted 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 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 – RAR and RXR – which usually exist in three subfamilies (α, β and γ; Ross et al. 2000). RXR bind to 9-cis retinoic acid, whereas RAR 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 (PPAR; 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 involved in tissue development and differentiation (Krumlauf, 1994; Suzuki et al. 1999), bone morphogenetic protein (BMP; Thompson et al. 2003) and insulin-like growth factor (Gabbitas & Canalis, 1997). BMP and insulin-like growth factor are implicated in skeletal patterning, bone development and limb morphogenesis. BMP are multifunctional regulators of vertebrate development: they regulate cell proliferation, differentiation, morphogenesis and apoptosis (Hogan, 1996). Insulin-like growth factor -1 is a mitotic agent that 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 appearance 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 recently been demonstrated that these PUFA, particularly EPA and DHA, are more efficiently used by marine fish when present in the phospholipid fraction of the diet (Gisbert et al. 2005). Fatty acids are natural ligands for PPAR, which play a key role in lipid metabolism and energy balance. As mentioned earlier, PPAR and RXR can dimerize to regulate the target genes involved in these two signalling pathways (Keller et al. 1993). This underlines the key role of RXR in several pathways, and, as a consequence, any perturbation of the expression of the RXR 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 the tank water (balneation) but not directly in the diet (Suzuki et al. 2000). We previously demonstrated that diets containing a high level 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 Ecoloserie 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 litre-cylindroconical fibreglass tanks (2100 larva/tank) at an initial density of 60 larvae/l. Tanks were supplied with running sea water, which had been previously passed through 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 g/l, respectively, and the oxygen level was maintained above 6 mg/l by setting the water exchange of the tank at 30 %/h (flow rate 0.18 l/min). The photoperiod was 24 h light/0 h dark, and maximum light intensity was 9 W/m² at the water surface. All animal procedures and handling were conducted in compliance with the Guide for the Care and Use of Laboratory Animal (National Research Council, 1985).

Four days after hatching, larvae were divided into seven experimental groups (three 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 vitamin A (Sigma R3250, 500 000 IU/g; Sigma, St Louis, MO, USA) and 175 g/kg soyabean lecithin, whereas the high-vitamin-A (RA) diet contained 32 g/kg retinol (Table 1). Although the phospholipid content of the experimental diets was similar, the diets differed in their respective sources of this nutrient: marine phospholipid (MP) in the MP diet and soyabean 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). In order to evaluate the ontogenetic effect of the diet on larval morphogenesis, fish were fed the RA and MP diets at three different developmental periods (Fig. 1): 8–13 dph (abbreviated as RA-A and MP-A), 13–18 dph (RA-B and MP-B) and 18–23 dph (RA-C and MP-C). Apart from the afore-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 et al. 2003), and the pellet size was 200–400 μm. Throughout the experimental period, larvae were continuously fed in large excess for 24 h/d using automatic belt-feeders. Food ingestion was monitored by observing larval digestive tracts under a binocular microscope, dietary microparticles being visible as transparency.

Sampling

To evaluate larval growth, ten specimens were randomly sampled from each experimental tank (thirty larvae per experimental diet) at 25 and 37 dph. They were killed with an overdose of anaesthetic (tricaine methanesulfonate, Sigma A5040), 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 (splanchocranium, neurocranium and vertebral column deformities) was counted at 37 dph (100 larvae per tank). For details of diets and procedures, see p. 678.

### Analytical methods

Larvae were dissected under a binocular microscope on a glass cutting-board kept on ice (0°C). Individuals were cut into four parts – head, pancreatic segment, intestinal segment 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) mannitol (50 mM) Tris-HCl (2 m M), 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) ice-cold distilled water.

Trypsin and amylase activities were measured using Nα-benzoyl-DL-arginine-p-nitroanilide and starch, respectively, as substrate (Métais & Bieth, 1968; Holm et al. 1988) in both the pancreatic and intestinal segments. Purified brush border membranes from the intestinal segment were obtained according to a method developed for intestinal scraping (Cranе 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 the 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 substrate hydrolysed per min and per mg protein (U/mg protein), 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 (pancreatic segment plus intestinal segment), considering that enzyme activity in the pancreatic segment can be used as an index of the synthetic function of pancreas, and in the intestinal segment as an index of pancreatic secretory function (Péres et al. 1998).

The protein concentration of the three diets was assessed following the Dumas method (Nitrogen Analyser 2000, Fisons Instruments, N × 6-25; Carlo Erba, Milan, Italy).

Total lipids in the 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 Juana & 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 were separated by GC, in an Auto-system Perkin-Elmer (Wellesley, MA, USA) with a flame ionization detector, BPX 70 capillary column (25 m × 0·22 mm internal diameter × 0·25 µm film thickness) and split-splitless injector, with He as the carrier gas. The injector and detector temperatures were 220°C and 260°C, respectively. The initial oven temperature was 50°C, but this 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 (Perkin-Elmer). The individual fatty acid methyl esters were identified by comparing the retention times of authentic standards and the results of individual fatty acid compositions were expressed as percentages of the total identified fatty acid methyl esters in the phospholipid and neutral lipid fractions.

RT-PCR analysis

Different complementary DNA (cDNA) fragments from genes coding for RAR and for signalling molecules known to interact 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: RXRα; accession number AJ 567907, RARα; AJ 496189, RARγ; AJ 496181, BMP-4; AJ 567451 and insulin-like growth factor-I (AJ 579342). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AJ 567450) was chosen as the housekeeping gene.

Real-time RT-PCR

cDNA samples were treated with DNase, and real-time PCR was performed using the iCycler iQ (Bio-Rad Laboratories Inc., Hercules, CA, USA). Quantitative PCR analyses for each gene were performed in a total volume of 15 µl containing 5 µl cDNA (10⁻³ dilution), 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, Hilden 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 15·5 min in order to activate HotStarTaq DNA polymerase (Qiagen). After this initial step, forty-five cycles of PCR were performed. Each PCR cycle consisted of heating for 30 s at 95°C for denaturing, and then for 1 min at 60°C for annealing and extension. Cycle threshold (CT) 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 the 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 log₁₀ of five different dilutions (in triplicate) of cDNA sample solutions. Real-time PCR efficiency was determined for each gene from the slopes given by BIORAD software, applying the equation

$$E = \frac{1}{\text{slope}}$$

We calculated the relative expression ratio of each gene using REST software (http://www.gene-quantification.info/). The relative expression ratio for a considered gene is based on the PCR efficiency (E) and CT of a sample compared with the control, and expressed in comparison to the reference gene (GAPDH), according to Pfaffl’s mathematical model (Pfaff, 2001):

$$\text{Ratio} = \frac{\text{expression}_{\text{gene}}}{\text{expression}_{\text{control}} \times \frac{\text{CT}_{\text{control}}}{\text{CT}_{\text{sample}}}}$$

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

Statistical analyses

Results are given as means and standard deviations. All data were checked for variance homogeneity using Bartlett’s test (Dagnelie, 1975). Growth, enzymatic specific activity and arcanin(x¹/²)-transformed trypsin and amylase secretions were compared by means of one-way ANOVA followed by a 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 (Pfaff et al. 2002) using REST software. Two thousand random allocations were performed and differences were considered to be significant 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 the RA diet. Globally, the more the larvae were fed the diet in the early stages, the less they grew (MP-C > MP-B > MP-A).
At 37 dph, the control group still exhibited the best growth (52.8 (SD 0.26) mg), and fish fed MP diets were still heavier than those fed RA diets over the same period. In the experimental groups fed RA diets, the best growth was observed in the RA-B group (36 (SD 0.17) mg) and the lowest growth in the RA-C group (26.1 (SD 0.52) mg), the RA-A group exhibiting intermediate growth (32.2 (SD 1.26) mg; P < 0.05). In the MP groups, the greatest growth was obtained in the MP-C group (47.2 (SD 0.9) mg) and the least in the MP-B group (36.6 (SD 0.76) mg), intermediate growth being observed in the MP-A group (44.9 (SD 0.35) mg; P < 0.05).

Skeletal malformations. The number of vertebrae most frequently observed in European sea bass larvae is twenty-five. The six experimental groups fed on the RA or MP diets contained individuals with twenty-six or twenty-four vertebrae instead.

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

The RA diet (F 21.12, P = 0.001) and feeding period (F 8.76, P = 0.006) effects were evidenced in fish with twenty-four 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 twenty-six vertebrae. The number of vertebrae significantly increased in association with the MP diet (P < 0.005), and this effect was apparent only when the diet was ingested during period A (P ≤ 0.0001; Fig. 4).

The number of fish exhibiting jaw malformations depended on the diet (F 40.83, P = 0.0001) and the feeding period (F 15.54, P = 0.001). This rate was significantly higher in fish fed 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 on day 40. At the same age, only 6–19% of larvae fed on either the control or the 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 (SD 170.2) mU/mg protein; Fig. 5). There was no statistically difference between

Fig. 3. Growth at 25 and 37 days post-hatching 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 and standard deviations (n = 3). abcMean values with unlike superscript letters for the same day were significantly different (P < 0.05). Fish were fed the high vitamin A (RA) diet and marine phospholipids (MP) diet at three different developmental periods: RA-A, MP-A, 8–13 d post hatching (dph); RA-B, MP-B, 13–18 dph; RA-C, MP-C, 18–23 dph. For details of diets and procedures, see p. 678. control; RA-A; RA-B; RA-C; MP-A; MP-B; MP-C.

Fig. 4. Percentage of vertebral number in European sea bass larvae at 37 d post-hatching. Fish were fed the high vitamin A (RA) diet and marine phospholipids (MP) diet at three different developmental periods: RA-A, MP-A, 8–13 d post hatching (dph); RA-B, MP-B, 13–18 dph; RA-C, MP-C, 18–23 dph. For details of diets and procedures, see p. 678. 26 Vertebrae; 25 Vertebrae; 24 Vertebrae. Con, control.

Fig. 5. Specific activity of alkaline phosphatase of the intestinal brush border in European sea bass at 25 d post-hatching. Means and standard deviations (n = 3). abcMean values with unlike different superscript letters for the same day were significantly different (P < 0.05). Fish were fed the high vitamin A (RA) diet and marine phospholipids (MP) diet at three different developmental periods: RA-A, MP-A, 8–13 d post hatching (dph); RA-B, MP-B, 13–18 dph; RA-C, MP-C, 18–23 dph. For details of diets and procedures, see p. 678. control; RA-A; RA-B; RA-C; MP-A; MP-B; MP-C.
the control, RA-B, RA-C and MP-B groups. This specific activity was 64% lower in the RA-A group (429 (sd 5.7) mU/mg protein) and 57% lower in the MP-A group (512 (sd 88.4) mU/mg protein).

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.

The highest values for amylase activity were measured in fish fed on MP-B and RA-A (50.7 (sd 4.73) % and 53.5 (sd 0.71 %), respectively; P < 0.05). The RA-A group exhibited an intermediate value. The trypsin secretion profile was comparable to that of amylase (Fig. 7b).

Gene expression. Variations in gene transcription are reported in Table 2. Significant variations during periods A and B are emphasized in Fig. 8, which 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, whereas the other variations were induced by the diets.

At 13 dph in the RA-A group, RARγ and BMP4 transcriptions were significantly higher (transcription factor 2.77-fold (P < 0.03) and 2.84-fold (P < 0.0003), respectively) than in the control group. BMP4 transcription was also higher in the MP-A group (transcription factor 2.34-fold; P < 0.03), whereas RARγ transcription was not modified (P = 0.08).

At 18 dph, RXRα transcription was positively regulated in the RA-A and MP-A groups (respective transcription factors 2.88-fold and 1.85-fold; P < 0.0001). 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 insulin-like growth factor 1 were significantly lower during period C, the transcription levels for RARα, BMP4 and insulin-like growth factor 1 were significantly lower (respective transcription factors 1/3.51, 1/1.26 and 1/3.43; P < 0.001). At 23 dph, BMP4 transcription was 1.5 times higher, whereas 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 1.96-fold; P < 0.03), whereas 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 controls (respective transcription factors 3.39-fold (P < 0.002); 3.25-fold (P < 0.001) and 2.08-fold (P < 0.001)) lower 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 1.96-fold; P < 0.03), whereas 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.
Influence of feeding period on morphogenesis

Table 2. Variations in gene transcription level during periods A (8–13 d post-hatching; dph), B (13–18 dph) and C (18–23 dph) in larvae fed high-vitamin-A (RA) or marine phospholipid (MP) diets, in comparison to the control group

<table>
<thead>
<tr>
<th>Day 13</th>
<th>Period A</th>
<th>Period B</th>
<th>Period C</th>
</tr>
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<tbody>
<tr>
<td>RARα - RA</td>
<td>+1.49</td>
<td>P&lt;0.001</td>
<td>+1.06</td>
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<tr>
<td>MP</td>
<td>-1.14</td>
<td>P&lt;0.001</td>
<td>-1.55</td>
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<tr>
<td>RARγ - RA</td>
<td>+2.77*</td>
<td>P&lt;0.001</td>
<td>+1.04</td>
</tr>
<tr>
<td>MP</td>
<td>+2.17</td>
<td>P&lt;0.001</td>
<td>+1.36</td>
</tr>
<tr>
<td>RXRα - RA</td>
<td>+1.19</td>
<td>P=0.06</td>
<td>+2.84*</td>
</tr>
<tr>
<td>MP</td>
<td>+1.36</td>
<td>P&lt;0.001</td>
<td>+2.34*</td>
</tr>
<tr>
<td>BMP4 - RA</td>
<td>-2.18</td>
<td>P&lt;0.89</td>
<td>IGFI - RA</td>
</tr>
<tr>
<td>BMP4 - RA</td>
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<td>P=0.9</td>
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<table>
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<th>Period C</th>
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<td>MP</td>
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<td>+1.04</td>
<td>P=0.83</td>
<td>+1.49</td>
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<td>MP</td>
<td>-1.19</td>
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<tr>
<td>RXRα - RA</td>
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<td>P&lt;0.001*</td>
<td>+3.46*</td>
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<tr>
<td>MP</td>
<td>+1.85*</td>
<td>P&lt;0.001*</td>
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<td>BMP4 - RA</td>
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<td>P=0.6</td>
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<tr>
<td>MP</td>
<td>-1.81*</td>
<td>P&lt;0.001*</td>
<td>-1.26*</td>
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<td>IGFI - RA</td>
<td>-1.07</td>
<td>P=0.68</td>
<td>+1.43</td>
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<td>MP</td>
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<td>P=0.35</td>
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<th>Day 23</th>
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<tr>
<td>RARα - RA</td>
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<td>-1.09</td>
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<td>MP</td>
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<td>RARγ - RA</td>
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<td>MP</td>
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<td>P=0.6</td>
<td>-1.19</td>
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<td>P=0.29</td>
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<td>P=0.4</td>
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</tr>
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<td>P&lt;0.001*</td>
<td>-2.64*</td>
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<td>MP</td>
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<td>P=0.27</td>
<td>2.06*</td>
</tr>
<tr>
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<td>P=0.64</td>
<td>-1.19</td>
</tr>
<tr>
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<td>-1.13</td>
<td>P=0.58</td>
<td>-1.36</td>
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<th>Day 40</th>
<th>Period A</th>
<th>Period B</th>
<th>Period C</th>
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RAR, retinoic acid receptor; RXR, retinoid X receptor; BMP, bone morphogenetic protein; IGFI, insulin-like growth factor. +, −, positive and negative values.
* Significant variations.

Discussion

There is currently 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 as developmental processes occur in utero. In sea bass larvae, these developmental processes still continue after hatching, and this particularity facilitates studies of the effects of nutrition on morphogenesis, which makes this a very interesting species for developmental studies.

At 23 dph, the control group exhibited the best growth. In the 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 hypervitaminosis A, sea bass larvae growth was globally more depressed in comparison with the two other larval groups, but this reduction in growth was more pronounced during period A (8–13 dph). 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 due to factors other than 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 secretory 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 other 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 for 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 maturation of the digestive tract. Previous works have shown that unbalanced diets induce a delay in digestive maturation (Ribeiro et al. 2002). This delay in maturation was always associated with a reduction in larval growth and survival, effects linked mainly to the poor digestion and metabolic use of these diets.

We observed an effect of the diet and feeding period on European sea bass morphogenesis, especially on the number
of vertebrae. Other authors have previously reported that the number of vertebrae in fish can be influenced by factors other than nutrition, such as triploidy in trout (Kacem et al. 2004) and temperature with halibut (Lewis et al. 2004). Compared with the control group, a significant percentage of larvae exhibited the loss of one vertebra (to twenty-four vertebrae) when fed the RA diet during period A, or the development of a supernumerary vertebra (to twenty-six vertebrae) 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, expression of this nuclear receptor 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 the early stages of development, as its expression was as high in the RA-A and MP-A groups as in the controls before day 18. Afterwards, its expression was higher in the RA-A and MP-A groups than in the 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 RAR and RXR can form heterodimers and that these dimers can control the levels of transcription the receptors involved in them (Ross et al. 2000; Balmer & Blomhoff, 2002). We can therefore hypothesize 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 appearance of skeletal malformations that affected the cephalic region of sea bass larvae (Villeneuve et al. 2005a). The RA-A group exhibited deformities localized to the cephalic region (as mentioned earlier) as well as to the vertebral column (the 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 BMP and retinoid signalling pathways could cooperate to induce the differentiation of marrow pre-adipocytes 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 characterized by stimulation of the expression of alkaline phosphatase (Skillington et al. 2002). In the present 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 hypothesize 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 pre-adipocyte differentiation into osteoblasts induced by the cooperation between retinoic acid and BMP4. These events certainly interfered with the normal process governing the 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 the RA diet during period B, but in this case it affected only 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, were not subject to any other influences. It also revealed that the mechanisms of morphogenesis implicated in its formation were successfully carried out.

With regard to larvae fed the MP diet, the increased RXRα expression at day 18 could not be correlated with a stimulation of RARγ as the MP and control diets had the same vitamin A level. The MP diet contained a high level of MP with an elevated percentage of PUFA, particularly EPA and DHA (48%). European sea bass larvae digest this kind of phospholipid very efficiently (Cahu et al. 2003), leading to an increased body PUFA content. These fatty acids are natural ligands for PPAR, and we assume that the excess of PUFA in the larvae stimulated PPAR expression. PPAR dimerize with RXR, and this binding may be responsible for the high levels of RXRα transcription observed. Moreover, DHA is known to be an 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 the MP diet. At day 13, the stimulation of BMP4 expression could have amplified the osteoblast differentiation process, leading to the appearance of a supernumerary vertebra. At 18 dph, the low level of BMP4 transcription suggested that osteoblast maturation had been achieved, as it has been previously reported that BMP4 expression decreases as bone matures (Solheim, 1998). Pancreatic development markers seemed to confirm this hypothesis: the elevated secretion rate (of trypsin and amylase) revealed that the process of maturation in the exocrine pancreas in the MP-A group was comparable with that observed in the control group, whereas it was delayed in the RA-A and MP-B groups (no BMP4 stimulation being observed in these groups). Moreover, amylase-specific activity was significantly lower in the MP-A than the RA-A group.

Villeneuve et al. (2004) previously described the normal expression profile of the genes studied during the development of sea bass larvae reared under optimal nutritional conditions. They reported a global increase in RAR expression and a decrease in RXRα expression during larval development. In the present study, at 23 and 40 dph, the expression levels of both RAR were greater in the experimental groups than...
in the control group, whereas the expression levels of RXRα and BMP4 were lower than in the reference group. These results suggested that normal expression tendencies, even though emphasized with regard to the control group, were again observed from day 23 and 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 points.

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 the present 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 malformation. On one hand, hypervitaminosis A delayed development, reducing the number of vertebral segments and disturbing bone formation in the cephalic region, whereas 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 – before and after 13 dph – as the composition of the diet could have a significant impact on sea bass morphogenesis before this date.

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