Dietary levels of all-*trans* retinol affect retinoid nuclear receptor expression and skeletal development in European sea bass larvae

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European sea bass larvae were fed different dietary vitamin A levels. Growth, skeletal development and the expression of genes involved in larval morphogenesis were evaluated. From 7 to 42 d post-hatching, larvae were fed five isoproteic and isolipidic compound diets with graded levels of retinyl acetate (RA; RA0, RA10, RA50, RA250 and RA1000, containing 0, 10, 50, 250 and 1000 mg RA/kg DM, respectively), resulting in an incorporation of 12, 13, 31, 62 and 196 mg all-*trans* retinol/kg DM. Larvae fed extreme levels of RA had weights 19 % and 27 % lower than those of the RA50 group. The RA1000 diet induced a fall in growth with an increase of circulating and storage retinol forms in larvae, revealing hypervitaminosis. High levels of RA affected maturation of the pancreas and intestine. These data indicated that the optimal RA level was close to 31 mg/kg DM. Inappropriate levels of dietary RA resulted in an alteration of head organisation characterised by the abnormal development of the splanchnocranium and neurocranium, and scoliotic fish. Of the larvae fed RA1000, 78·8 % exhibited skeletal abnormalities, whereas the RA50 group presented with 25 % malformations. A linear correlation between vitamin A level and malformation percentage was observed and mainly associated with an upregulation of retinoic acid receptor-γ expression in the RA1000 group during the 2 first weeks after hatching. The expression of retinoid X receptor-α decreased during normal larval development when that of the retinoic acid receptors increased. This work highlights the involvement of retinoid pathways in the appearance of dietary-induced skeletal malformations during post-hatching development in sea bass.

Retinol: European sea bass: Skeletal malformations: Retinoic acid nuclear receptors (RAR, RXR): Growth factors (BMP-4, IGF-1)

During the first weeks of life, marine fish larvae undergo significant morphological and physiological modifications to acquire all the adult features by the end of the larval period. Several factors can interfere with the normal development of larvae and affect their quality, which can be defined as the capacity to continue a normal development until the juvenile and adult stages (Cahu *et al.* 2003*b*). Skeletal deformities of fish larvae can be due to many parameters in hatcheries, for example the hydrodynamics in the tanks, inadequate light intensity, water temperature and salinity, and an unbalanced diet (Cahu *et al.* 2003*b*). The ingestion of an inadequate diet may delay or prevent the genetically programmed sequence of body development, including skeletal patterning and intestinal maturation (Dedi *et al.* 1997; Zambonino Infante *et al.* 1997; Reifen *et al.* 1998; Uni *et al.* 2000; Cahu *et al.* 2003*a,b*; Haga *et al.* 2003). Up until now, owing to the absence of a compound diet (Cahu *et al.* 2003*a*), marine fish larvae were fed at mouth opening with live prey, which did not allow accurate experiments on their nutritional requirements to be conducted. The recent development of a balanced compound diet has made it possible to study the influence of the nature, molecular form and level of dietary nutrients.

There is a close relationship between larval nutrition at first feeding and skeletal abnormalities (Zambonino Infante *et al.* 1997, 1999; Suzuki *et al.* 2000). It is well known that genes involved in body patterning can be regulated by the nature and molecular form of the nutrients (Krumlauf, 1994; Suzuki *et al.* 1998, 2000; Balmer & Blomhoff, 2002; Haga *et al.* 2003). Generally, marine fish larvae hatch much earlier in their development than other vertebrates, suggesting that the spatiotemporal sequences of the skeletal development in teleosts are quite different from those of higher vertebrates (Haga *et al.* 2002). Therefore, marine fish species let us study the molecular processes influenced by nutrition and involved in the appearance of skeletal malformations in the early stages of teleost development. In order to study the relationship between skeletal malformations and dietary nutrients, we have chosen a nutrient, vitamin A, which is known to have teratogenic effects in vertebrates at inappropriate dietary levels. This work on vitamin A may provide an understanding of some of the cellular mechanisms influenced by nutrition and involved in the onset of deformities in marine fish, and allow the expansion of this study to other nutrients also implicated in skeletal abnormalities.

Abbreviations: BMP, bone morphogenetic protein; dph, days post-hatching; IGF, insulin-like growth factor; IS, intestinal segment; PS, pancreatic segment; RA, retinyl acetate; RAR, retinoic acid receptor; RXR, retinoid X receptor.
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Data on dietary vitamin A requirements for marine fish are scarce (Gouillou-Coustans & Guillaume, 2001). Retinoids, which are vitamin A-related compounds, regulate a wide variety of biological functions, including cell differentiation, proliferation and morphogenesis (Haga *et al.* 2002). The biological activity of vitamin A is mediated mainly by its active metabolite, retinoic acid. There are two active forms of retinoic acid, 9-*cis* and all-*trans* retinoic acid, which are obtained by the dehydrogenation of vitamin A (Gouillou-Coustans & Guillaume, 2001). Several studies have shown that an excess of dietary vitamin A results in abnormalities during embryogenesis in mammals (Cohlan, 1953), birds (Lee *et al.* 2001), amphibians (Minucci *et al.* 1996) and fish (Dedi *et al.* 1997; Ornsrud *et al.* 2002). The biological effects of retinoids (vitamin A metabolites) are exerted mainly through the activation of two groups of nuclear receptor, retinoic acid receptors (RAR) and retinoid X receptors (RXR), which usually exist under three sub-families (α, β and γ; Ross *et al.* 2000). The RXR bind to 9-*cis* retinoic acid, whereas the RAR bind both to all-*trans* and 9-*cis* retinoic acid (Ross *et al.* 2000). These receptors can dimerise together, and RXR can also heterodimerise with other types of nuclear receptor (Yu *et al.* 1991). These heterodimers can bind to the response elements of fatty acids, vitamin D and thyroid hormones, or to specific retinoic acid response elements localised on target genes, and thereby suppress or enhance the transcription of more than 500 genes either directly or indirectly (Balmer & Blomhoff, 2002).

Retinoic acid is essential for initiating the expression of Hox or sonic hedgehog (shh) gene families that are involved in tissue development and differentiation (Joore *et al.* 1994; Krumlauf, 1994; Conlon, 1995; Haga *et al.* 2002). Skeletal patterning, bone development and limb morphogenesis are also regulated by other gene families, including bone morphogenetic proteins (BMP) and insulin-like growth factors (IGF). BMP are multifunctional regulators of vertebrate development, regulating cell proliferation, determination, differentiation, morphogenesis and apoptosis (Hogan, 1996). IGF-1 is a mitogenic agent acting as not only a growth-promoting, but also a differentiation factor in bone, muscle and cartilage (Zizola *et al.* 2002). Both an excess and a deficiency of vitamin A, which disrupt the retinoid-signalling pathway including the above-mentioned gene families, lead to abnormal development of the heart, bone, craniofacial structures, central nervous system, pigment cells and axis formation in vertebrate embryos (Helms *et al.* 1997; Ross *et al.* 2000; Suzuki *et al.* 2000; Gouillou-Coustans & Guillaume, 2001; Haga *et al.* 2002). The signalling pathways described above are well known in higher vertebrates, but it is still unclear whether they are functional in marine fish after hatching and whether they can be modulated by nutrition.

The aims of the present study were (1) to evaluate the effect of different vitamin A dietary levels on fish development, in terms of optimal growth, intestinal maturation and incidence of skeletal malformations, (2) to provide an insight into dietary larval requirements, and (3) to study the influence of vitamin A on gene expression during European sea bass larval morphogenesis.

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), where they were acclimated and divided into fifteen cylindroconical fibreglass tanks (35 litres) at a initial density of sixty larvae per litre. Tanks were supplied with running sea water, which had been previously filtered through a sand filter and 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 up to 30 % per h (flow rate 0·18 l/min). The photoperiod was 24 light:0 dark, and light intensity was 9 W/m^2 maximum 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)).

At 4 d post-hatching (dph), larvae were divided into five experimental groups (three tanks per group) that were fed from 7 dph with experimental compound microdiets (Table 1) containing graded level of all-*trans* retinyl acetate (RA; 500 000 USP units/g; Sigma-Aldrich, St Louis, MD, USA). Diets containing 0, 10, 50, 250 and 1000 mg of added RA/kg DM were designated RA0, RA10, RA50, RA250 and RA1000, respectively. For comparative purposes, the RA50 diet was considered as the control. The size of the microdiet was 200–400 μm. During the experimental period, larvae were continuously fed in large excess for 24 h daily using a belt feeder. At the end of the trial (42 dph), fish fed with the same experimental diet were pooled and transferred to a 700 litre square fibreglass tank (one tank per diet) and reared until 80 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).

Table 1. Composition of the experimental compound microdiet.

Ingredients*	g/kg DM
Fish meal	579
Hydrolysed fish meal	140
Soya lecithin	151
Vitamin mixture† (without retinyl acetate)	80
Mineral mixture‡	40
Betaine	10
Proximate composition	**%**
Proteins (N × 6·25)	53·0
Lipids	18·1
Ash	11·5
Humidity	10·0
Energy (kJ/kg)§	15·67

* All dietary ingredients were commercially obtained: fish meal (La Lorientaise, Lorient, France), hydrolysed fish meal (CPSP G, Soluble Fish Protein Concentrate, Sopropêche, Boulogne sur Mer, France), soya lecithin (*Ets Louis François,* St Maur des Fossés, France).

† Composition / kg vitamin mixture: choline concentrate 50 % 200 g, vitamin E (500 UI/g) 10 g, vitamin D_3 (500 000 UI/g) 500 mg, vitamin B_3 1 g, vitamin B_5 2 g, vitamin B_1 100 mg, vitamin B_2 400 mg, vitamin B_6 300 mg, vitamin C 20 g, vitamin B_9 100 mg, vitamin concentrate B_{12} (1 g/kg) 1 g, biotin 1 g, vitamin K_3 1 g, meso-inositol 30 g, cellulose 732 1 g.

‡ Composition / kg 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 3 g, $CaCO_3$ 215 g, $MgSO_4$ $7H_2O$ 124 g, NaF 1 g.

§ Calculated as: total carbohydrate × 16·7 J/kg, fat × 37·7 J/kg, protein × 16·7 J/kg.

Sampling

To evaluate growth, ten specimens were randomly sampled from each experimental tank (n 30 per experimental diet) at 28, 37 and 42 dph, and killed with an overdose of tricaine methanesulphonate (MS 222), their body weight being measured to the nearest 0·1 mg. To evaluate the level of maturation of the digestive system, larvae (n 20–50, depending on body weight) were sampled from each tank and kept at −20°C until analysis. Samples for measuring brush border intestinal enzymes were taken at 28 and 42 dph, whereas pancreatic enzyme activities were measured only at 42 dph. Fifty larvae were collected for messenger RNA studies from the RA0, RA50 and RA1000 experimental tanks at 10, 15, 21 and 42 dph, and the total RNA was immediately extracted from total-body homogenate. The same number of fish were sampled at 2, 10, 15, 21, 37 and 42 dph and frozen at −80°C in order to evaluate retinol (the active transport form) and retinyl palmitate (the storage form of retinol) in fish fed different levels of RA. In all cases, sampling procedures were performed as previously described (Cahu et al. 2003a). The incidence of body skeletal malformations (splanchnocranium, neurocranium and vertebral column deformities) was counted at 87 dph (n 80 per experimental diet) (Fig. 1). At the end of the rearing period (42 dph), larval survival was calculated as the percentage of surviving individuals in relation to the initial number of larvae. From 15 dph onwards, samplings accounted for the determination of larval survival; indeed, the critical stage of development corresponding to resorption of the yolk sac and a high mortality rate has been overcome after 15 dph (Barnabé et al. 1976).

Analytical methods

Larvae were dissected under a binocular microscope at 0°C as previously described (Cahu et al. 2003a). Individuals were cut into four 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 PS contained, besides the pancreas, the live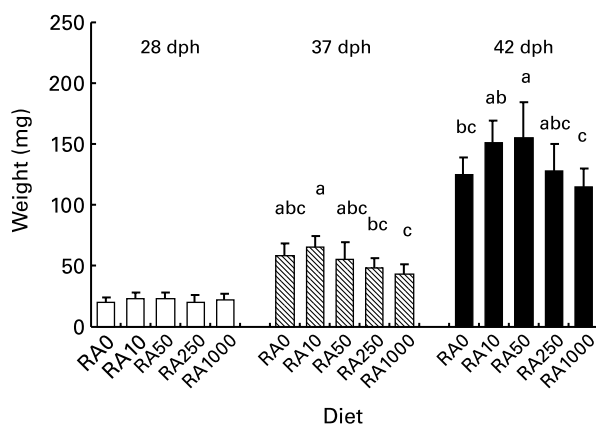r, heart, muscle and spine. The IS contained the intestine, muscle and spine. Once dissected, PS were homogenized in five volumes (v/w) of ice-cold distilled water. Trypsin (EC 3.4.21.4) activity was measured using Na-benzoyl-DL-argi-nine-p-nitroaniline as substrate (Holm et al. 1988) in both PS and IS. Purified brush border membranes from the IS were obtained according to a method developed for intestinal scraping (Crane et al. 1979). The degree of purification of the brush border membrane, considering alkaline phosphatase and aminopeptidase N as markers of cell membrane fraction, was close to that reported in the literature (Crane et al. 1979), i.e. 13·5-fold and 10-fold, respectively. Alkaline phosphatase (EC 3.1.3.1), aminopeptidase N (EC 3.4.11.2) and maltase (EC 3.2.1.20), three enzymes of the intestinal brush border membrane, were quantified using p-nitrophenylphosphate, L-leucine-p-nitroaniline and D(+)-maltose, respectively, as substrates (Bessey et al. 1946; Maroux et al. 1973; Dahlqvist, 1970). Specific enzyme activities were expressed as μmol of substrate hydrolysed per min and per mg protein (U/mg protein), and protein was determined using the Bradford method (Bradford, 1976). The ratio of the trypsin activity in the IS related to total activity (PS + IS) was calculated, considering that enzyme activity in the PS can be used as an index of the synthetic function of pancreas, and that in the IS can be used as an index of pancreatic secretory function (Zambonino Infante et al. 1997). This ratio reflected the trypsin secretion rate.

Retinol and retinyl palmitate were extracted from non-dissected larvae that were homogenised with a PBS solution by means of a Polytron MR 2100 (Kinematica AG, Luzern, Switzerland). Then, 1 ml homogenate was transferred into a dark brown tube, and 1 ml ethanol (containing 50 μg/l 2,6-di-tert-butyl-4-methylphenol), the internal standard and 2 ml hexane (containing 50 μg/l 2,6-di-tert-butyl-4-methylphenol) were added (Grolier et al. 1995). After mixing the solution for 1 min, the organic layer was separated by centrifugation (5 min at 2000 g). After that, the organic layer was collected in another dark brown tube, which was flushed with N_2 gas until the residue was completely dry. This operation was repeated twice, and the residue was collected and dissolved in methanol for retinol and retinyl palmitate determination by HPLC. The HPLC system consisted of a Waters 600E (Waters, Milford, MA, USA) apparatus with UV detection at 325 nm (Waters 486, Tunable Absorvance Detector; Waters) and a C18 novapack 30 cm column (Waters). Retinol and retinyl palmitate were quantified using internal and external standards (retinyl acetate; Sigma-Aldrich).

RT-PCR analysis

Different complementary DNA fragments of genes coding for RAR and for signalling molecules known for interacting with retinoic acid were purified in European sea bass larvae by RT-PCR, cloned, 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 IGF-1 (AJ 579342). Glyceraldehyde-3-phosphate deshydrogenase (AJ 567450) was chosen as the house-keeping gene.

Real-time RT-PCR

Complementary DNA 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

Fig. 1. Growth of European sea bass larvae fed isonitrogenous diets containing different all-trans retinol levels. Values shown are means for three samples with their standard errors shown by vertical bars, and different superscript letters for the same day designate significant differences (P<0·05).

15 μl containing 5 μl complementary DNA (dilution 10^{-3}), 1·5 μl fluorescein (100 nmol/l; Bio-Rad Laboratories Inc.), 1 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 (Table 2) were chosen in the sequences previously cloned. Thermal cycling was initiated with incubation at 95°C for 13·5 min for activation of HotStarTaq DNA polymerase (Qiagen). After this initial step, 45 cycles of PCR were performed. Each PCR cycle consisted of heating at 95°C for 30 s for denaturing and then at 60°C for 1 min for annealing and extension. 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 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 the cycle threshold values against the $\log_{10}$ of five different dilutions (in triplicate) of complementary DNA sample solutions. The efficiency of real-time PCR efficiency (E) determined for each gene from the given slopes in Bio-Rad software, according to 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 the cycle threshold (CT) of a sample (RA0 or RA1000) *v.* the control (RA50), and expressed in comparison to the reference gene (glyceraldehyde-3-phosphate deshydrogenase; GAPDH), according to Pfaffl's mathematical model (Pfaffl, 2001):

$$\text{ratio} = [(E_{\text{gene}})^{\Delta CT_{\text{gene}}(\text{control}-\text{sample})}]/[(E_{\text{GAPDH}})^{\Delta CT_{\text{GAPDH}}(\text{control}-\text{sample})}]$$

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

Statistical analyses

Results are given as means together with their standard deviations. All data were checked for variance homogeneity using Barlett's test (Dagnelie, 1975). Growth, enzymatic specific activity and arcsin($x^{1/2}$)-transformed trypsin secretion ratio were compared by means of a one-way ANOVA followed by the Student Newman Keuls multiple range test when significant differences were detected ($P<0.05$). Statistical differences in gene expression between control and samples were evaluated in group means by 2000 randomisation tests with a pair-wise reallocation (Pfaffl *et al.* 2002) using REST software, and differences were considered significant at $P=0.05$.

Results

Growth and survival

At the beginning of the exogenous feeding period (9 dph), larval body weight was 0·8 (0·05) mg. No statistically significant differences were observed between fish fed with diets containing different levels of retinol up to 30 dph (22·0 (1·53) mg; $P>0.05$). At day 37, the highest body weight was observed for larvae fed RA0, RA10 and RA50, whereas growth was significantly lower in larvae fed RA1000 ($P<0.05$) (Fig. 1). At the end of the larval rearing period (42 dph), intermediate dietary levels of RA (RA50) resulted in higher weight values, in contrast to larvae fed extreme concentrations of retinol (RA0, RA1000) ($P<0.05$).

At 42 dph, fish fed RA50 and RA10 exhibited the highest survival percentages ($P<0.05$), i.e. 62 (5·7) % and 68 (7·7) % respectively; survival in fish fed with RA0, RA250 and RA1000 was not significantly different (40 (6·8) %, 41 (7·2) % and 46 (7·8) %, respectively).

Retinol and retinol palmitate diet and larval content

Analysis of the retinol content of the tested diets showed that the real incorporated level of all-*trans* retinol was 12, 13, 31, 62 and 196 mg/kg DM for the RA0, RA10, RA50, RA250 and RA1000 diets, respectively.

At the end of the rearing period (42 dph), no statistically significant differences in retinol body content were detected between fish fed RA0, RA10, RA50 and RA250 (18·2 (SD 1·93) μg/100 g). The above-mentioned values were significantly lower than those obtained with RA1000 (26·4 (SD 6·90) μg/100 g; $P<0.05$) (Fig. 2(a)).

The diets were consumed as soon as the microparticles reached the tank water. The accumulation of retinyl palmitate in developing larvae was dependent on the level of retinol administered by the experimental diets, reaching a plateau at 15 dph (Fig. 2(b)). A linear correlation was established at 42 dph between dietary RA concentration and the level of retinyl palmitate found in the larvae ($y = 0.455x + 23.685$; $R^2 = 0.987$, $P<0.0006$). At 42 dph, no statistically significant differences were detected between the levels of retinyl palmitate in larvae fed diets containing 0, 10, 50 and 250 mg retinyl acetate/kg DM ($P>0.05$). The values of retinyl palmitate in larvae fed RA1000 were significantly higher than those measured in the rest of the dietary groups (109·4 (SD 32·20) *v.* 37·2 (SD 14·02) μg/100 g; $P<0.05$).

Skeletal malformations

The incidence of skeletal malformations in European sea bass juveniles (age 87 dph, mean weight 4·4 (0·41) g) are presented in Figs. 3(a) and 4. Among the four types of malformation

Table 2. Nucleotide sequences of the PCR primers used to assay gene expression by real-time quantitative PCR.

| Gene	Forward primer	Reverse primer	PCR product length (nucleotides)
Glyceraldehyde-3-phosphate deshydrogenase	GAGGTCAAGGTTGAGGGTGA	CCAGTGGACTCAACCACGTA	125
Retinoid X receptor-α	CTGGTAGAGTGGGCCAAGAG	GTTCTGTGAGCACCCTGTCA	223
Retinoic acid receptor-α	CGCTAAACCGAACCCAGA	CTTCTCGGCCTGTTCCAA	170
Retinoic acid receptor-γ	GCAAAGCTCACCAAGAGACC	GCGTAGTGAAGCCTGGTAGC	180
Bone morphogenetic protein-4	CTGCTCTCTTCCGCTGAACT	GGCTCACATCAAAGCTCTCC	205
Insulin-like growth factor-1	GTCTTGGCAGGTGCACAGTA	ACACGCTGCAGTTTGTGTGT	157
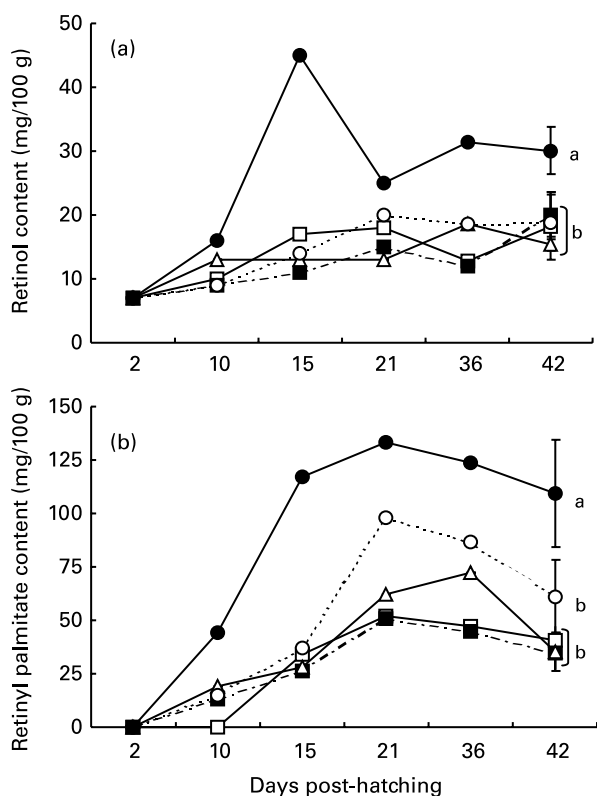
Fig. 2. Levels of retinol (a) and retinyl palmitate (b) in European sea bass larvae fed isonitrogenous diets containing different all-*trans* retinol levels (□, RA0; ■, RA10; △, RA50; ○, RA250; ●, RA1000) at different stages of development. Only means for three samples with standard deviation shown by vertical bars are presented for larvae aged 42 days post-hatching. Different superscript letters for the same day designate significant differences ($P < 0.05$). RA, retinyl acetate.
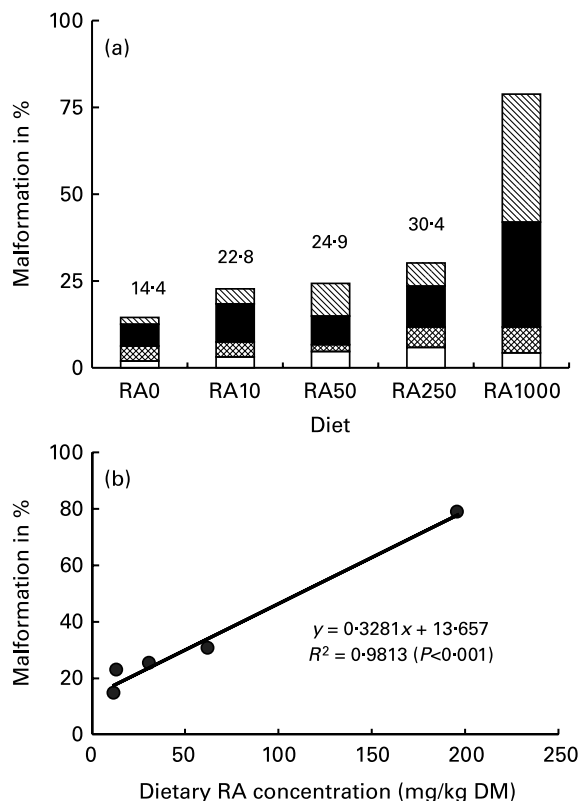

Fig. 3. Incidence of skeletal malformations (a) and linear correlation between dietary levels of all-*trans* retinol and percentage of malformation (b) in European sea bass larvae fed isonitrogenous diets containing different doses of all-*trans* retinol. (□) Vertebral column, (▨) Operculum, (■) Maxilla, (▨) Neurocranium.

observed, underdevelopment of the neurocranium and splanchnocranium (especially the maxillary bones and operculum) showed the highest incidence in all experimental groups, especially in fish with the highest level of retinyl palmitate (RA1000). A significant linear relationship was shown between the retinyl palmitate level in the 42 dph larvae and the incidence of malformations in the fish (Fig. 3(b)).

The correlation coefficients between retinyl palmitate concentrations in the larvae and the incidence of skeletal malformations in the splanchnocranium, neurocranium and vertebral column are presented in Table 3. The presence of deformities in the neurocranium and splanchnocranium (maxilla and operculum) was strongly correlated to the level of retinyl palmitate in the larvae, whereas no statistically significant correlation was observed between the incidence of deformities in the vertebral column and the retinyl palmitate level.

Enzyme activities

The level of RA in the diet induced statistically significant differences in the trypsin secretion ratio at 42 dph ($P < 0.05$). The highest trypsin secretion ratio (54·6 (2·99) %) was observed in fish fed RA50, whereas the lowest values were those of fish fed RA1000 (40·7 (2·89) %; $P < 0.05$). Intermediate trypsin secretion ratios were observed for larvae fed RA0 (47·5 (1·47) %), RA10 (46·8 (5·71) %) and RA250 (46·2 (4·26) %).

The specific activity of intestinal brush border enzymes was also affected by the dietary content of RA (Fig. 5). At 28 dph, the alkaline phosphatase specific activity was 40 % higher in fish fed RA50 and RA250 (1254 (SD 153·1) and 1262 (SD 85·7) mU/mg protein, respectively) than in those fed RA0 (549 (SD 47·1) mU/mg protein), whereas intermediate specific activities were detected in fish fed RA10 and RA1000 (829 (SD 153·1) and 884 (SD 94·3) mU/mg protein, respectively). At the end of the larval rearing period, no statistically significant differences were observed between fish fed different levels of retinyl acetate ($P > 0.05$; Fig. 5(a)).

Aminopeptidase and maltase specific activities followed the same trend as that of alkaline phosphatase. At 28 dph, the aminopeptidase and maltase specific activity in fish fed RA0 was 50 % lower than those reared with RA50 (140 (SD 41·9) v. 284 (SD 33·4) mU/mg protein and 102 (SD 24·7) v. 207 (SD 35·6) mU/mg protein, respectively ($P < 0.05$). The rest of the experimental groups showed intermediate enzymatic activities with respect to the control group (Fig. 5(b), (c)). At 42 dph, no statistically significant differences in enzymatic specific activity were observed between fish fed different diets ($P > 0.05$).

Gene expression

Effect of development on gene expression in RA50 diet. Gene expression at 42 dph was compared with that at 10 dph in fish fed RA50 (Fig. 6). At 42 dph, the two sub-types of RAR studied were significantly upregulated by a factor of 7·27 ($P < 0.05$) and 35·56 ($P < 0.05$), respectively, whereas RXRα was downregulated
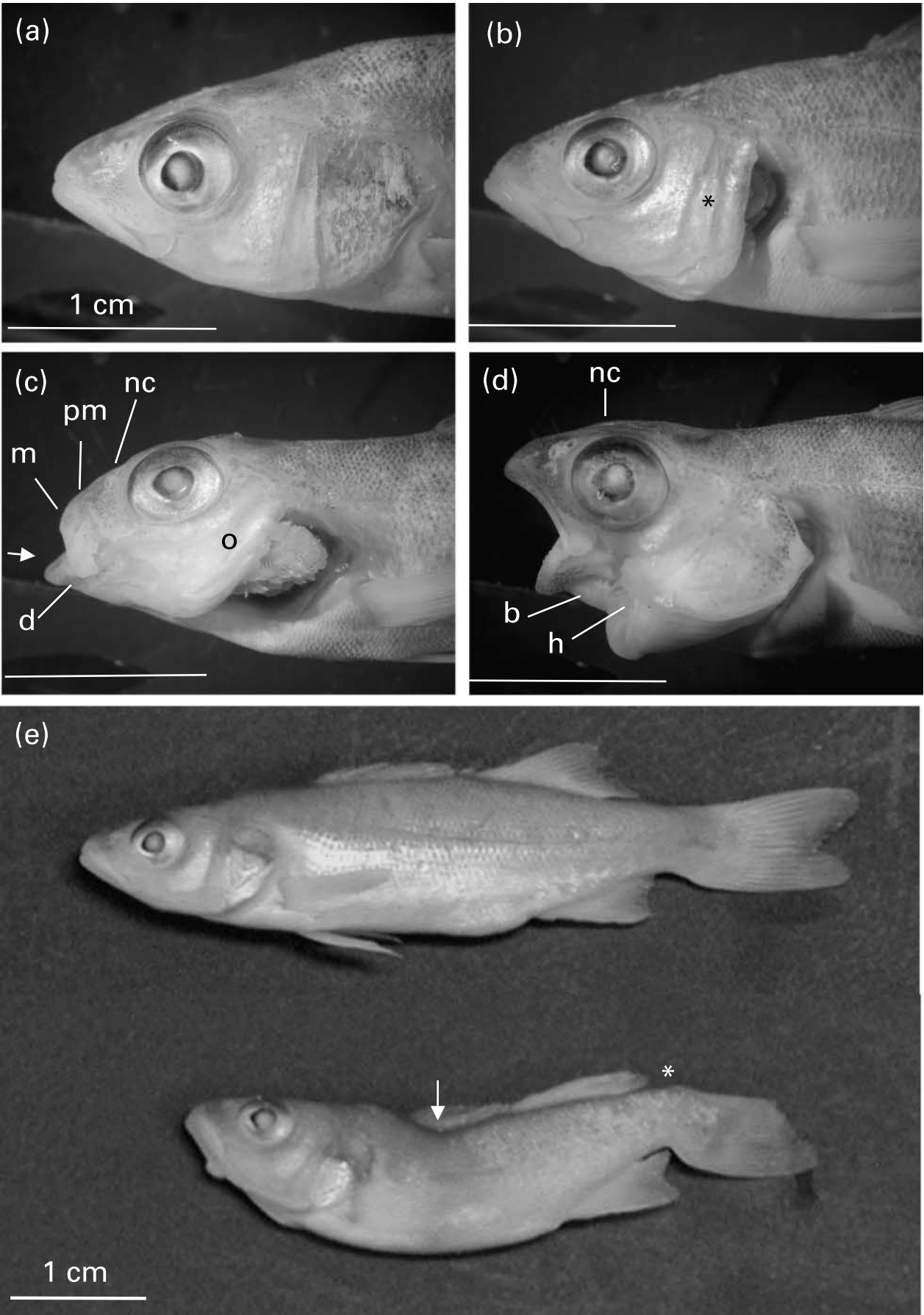
Table 3. Correlation (Pearson product moment correlation) between the levels of retinyl acetate (RA) in the diet and the incidence of different skeletal deformities in European sea bass (*Dicentrarchus labrax*) juveniles (87 days post-hatching).

Variable	Dietary RA	
	r	*P*
Dietary RA	1·000	0·000
Neurocranium	0·955	0·003
Splanchnocranium	0·951	0·005
Operculum	0·992	0·004
Vertebral column	0·386	0·579

Fig. 5. Specific activity of alkaline phosphatase (a), aminopeptidase N (b) and maltase (c) of the intestinal brush border in European sea bass at 28 and 42 days post-hatching (dph). Values shown are means for three samples with standard deviations shown by vertical bars and different superscript letters for the same day designate significant differences ($P<0.05$). RA, retinyl acetate.

35·44-fold ($P<0.05$). BMP-4 and IGF expression remained at the same level during this period.

Effect of experimental diets on gene expression. At 15 dph, IGF-1 was significantly downregulated in fish fed RA0 (downregulation factor 5·60; $P<0.01$) (Fig. 7(a)) compared with the control group, whereas it was significantly upregulated at 21 dph (upregulation factor 5·8; $P<0.05$). At 21 dph, a higher RXRα expression was also noted (upregulation factor 4·4; $P<0.05$) with respect to the control group. At 42 dph, the only significant difference was observed for RARγ, which was downregulated 3·08-fold compared with the RA50 group ($P<0.001$). It is interesting to note that RXRα expression was five times higher in the RA0 compared with the control group, even though this was not significant ($P<0.07$).

In fish fed RA1000 (Fig. 7(b)), the most striking gene regulation was observed at 10 dph for RARγ, which was upregulated by a factor of 82·49 compared with the control group. Other genes were significantly upregulated in larvae fed RA1000 compared with RA50 at 15 dph, (RARα by 4·4; $P<0.05$), 21 dph (IGF-1 by 7·06; $P<0.05$) and 42 dph (RXRα by 8·26; $P<0.05$).

Discussion

Recommendations on vitamin requirements for marine fish larvae are generally based on data obtained for the juveniles of cold freshwater species, such as salmonids (National Research Council, 1993). The vitamin requirements of salmonids were, however, overestimated owing to the water-soluble nature of the diets used in early studies on these requirements (Kaushik *et al.* 1998). Quantitative data on the dietary vitamin requirements for marine fish larvae are scarce (Gouillou-Coustans & Guillaume, 2001).

For many years, experiments on larval nutrition were conducted with dietary formulations close to those used for the juvenile stages, but the diets leading to good performance in juveniles induced high mortality and very poor growth in larvae (Cahu *et al.* 2003a). The lack of a compound diet for the early fish larval stages has been the main limiting factor in establishing their nutritional requirements. In current rearing practice, marine fish larvae are generally fed live prey, rotifers and *Artemia*.
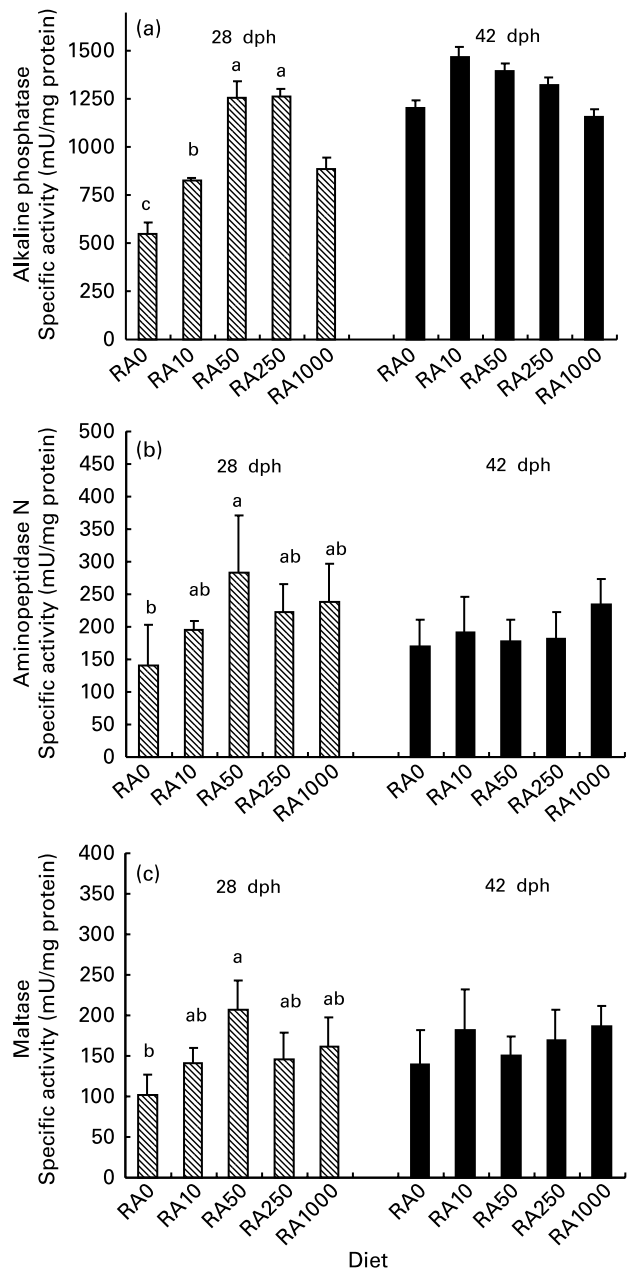
These feeding strategies make it difficult to perform accurate nutritional studies because of the variability of the nutrient content, especially vitamins, in live prey. However, the recent development of a balanced compound diet for first feeding

Fig. 4. Retinol dietary-induced malformations in European sea bass juveniles. (a) Head of a normal fish. (b) Fish with a partially undeveloped operculum (*). (c) Deformed head with reduced neurocranium (nc) and severe anomalies in the splanchnocranium (pm, premaxilla; m, maxilla d, dentary; o, operculum) resulting in mouth prognosis (→). (d) Deformed basibranchial (b) and hypohyal (h) cartilages of the splanchnocranium and undeveloped neurocranium (nc) in an abnormal fish head. (e) Normal (upper image) and scoliotic (lower image) European sea bass juvenile. Lordosis and kyphosis are indicated by ↓ an arrow and *, respectively.
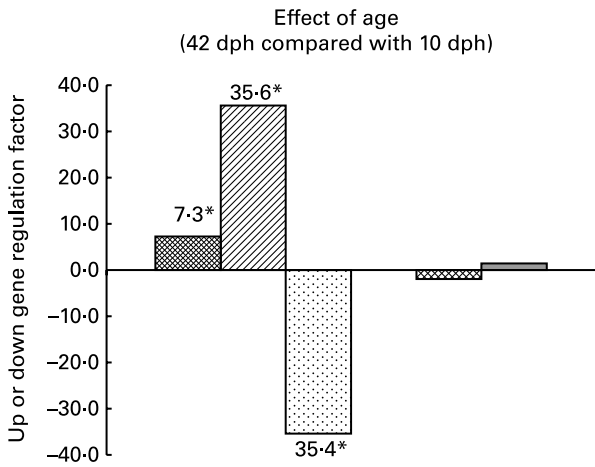
Fig. 6. Influence of age (between 10 and 42 days post-hatching (dph)) in European sea bass larvae control group fed retinyl acetate (RA) 50. Gene expressions of retinoid acid receptor-α (■), retinoid acid receptor-γ (▨), retinoid X receptor-α (▦), bone morphogenetic protein-4 (▨) and insulin-like growth factor-1 (▦) at 42 dph were normalised to the values obtained at 10 dph in the control group. The numbers indicate the relative gene expression ratio. Mean values were significantly different between 42 and 10 dph *$P < 0.05$.

marine fish larvae (Cahu *et al.* 2003*a*) provided an opportunity to conduct nutritional experiments and evaluate the effect of dietary molecules on early fish development. Compound diets contain fish meal that mainly provides proteins and also significant amounts of some nutrients such as fatty acids, minerals and
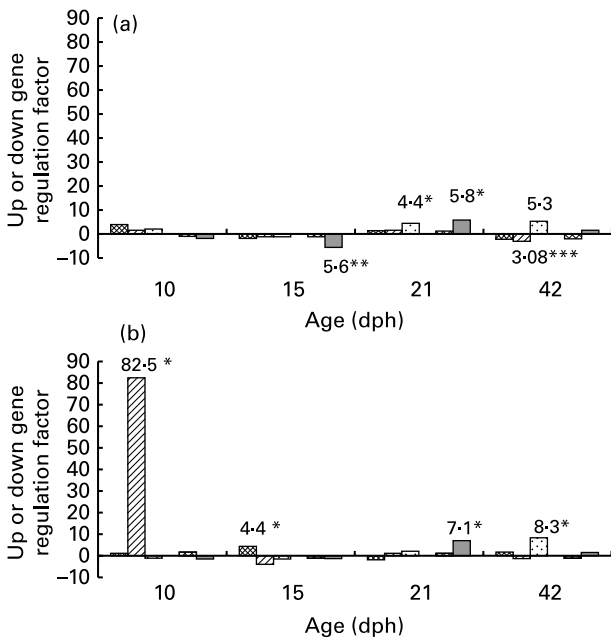

Fig. 7. Effect of retinyl acetate (RA) 0 (a) and RA1000 (b) diets in European sea bass larvae. Expressions of retinoid acid receptor-α (▨), retinoid acid receptor-γ (▨), retinoid X receptor-α (▧), bone morphogenetic protein-4 (▫) and insulin-like growth factor-1 (▦) at 10, 15, 21 and 42 days post-hatching (dph) were normalised by their corresponding values in RA50 group at the same dates. The numbers indicate the relative gene expression ratio. Mean values were significantly different within the same developmental stage compared with the RA50 group: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

vitamins such as vitamin A. Therefore, the real dosage of these nutrients in the experimental diets is always slightly different from the theoretical formulation.

At the end of the experiment, fish weight gain was affected by the level of dietary retinol. Under our experimental conditions, the group of larvae fed the diet containing the intermediate level of retinol (RA50) was considered to be the control group, since the dietary formulation, especially the vitamin composition, was similar to that previously described (Cahu *et al.* 2003*a*) as assuring the optimal growth and survival of animals. In comparison, larvae fed extreme levels of retinol (RA0 and RA1000 diets) were 19 % and 27 % lower in final weight with respect to the control diet group. As previously shown in Japanese flounder (Dedi *et al.* 1997; Haga *et al.* 2003) and Atlantic salmon (Ornsrud *et al.* 2002), high dietary doses of vitamin A led to lower growth. In our case, the growth reduction was particularly observed with the RA1000 diet and was associated with an increase in both circulating and accumulation forms of retinol, which probably reveals a hypervitaminosis in larvae. Low levels of dietary retinol also reduced larval growth in Japanese flounder (Dedi *et al.* 1997). In our study, the final body weight of European sea bass fed RA0 was lower than that of the control group.

A complete absence of retinol in the diet would have been lethal, but this had not been observed because of the incorporation in experimental diets of fish meal containing a low amount of different vitamins. Survival followed a similar trend to growth and was affected both by low and high dietary levels of all-*trans* retinol. It should be pointed out that a survival percentage higher than 50 % (obtained in the control and RA10 groups) is generally considered as very good in seabass larva-rearing (Cahu & Zambonino Infante, 2001); the survival percentage obtained in the control group, in addition to the growth data, ensured that valid physiological and nutritional observations could be drawn from this study.

Previous studies have shown that the pancreatic and intestinal enzyme activity provides a reliable marker of larval fish development (Péres *et al.* 1998; Zambonino Infante & Cahu, 1999). Under the experimental conditions described here, an excess of dietary all-*trans* retinol (RA1000) resulted in low trypsin secretion ratio; this effect cannot be attributed to the different nature or quantity of dietary proteins, as shown by several authors (Cahu & Zambonino Infante, 2001), since all the diets tested had the same protein sources and levels. A low trypsin secretion ratio at the end of the larval period might indicate a delay in the maturational process of the pancreas (Cahu & Zambonino Infante, 2001). It is well known that pancreatic differentiation is controlled by thyroid hormones, whose receptors interact with those of retinoids (Kobayashi *et al.* 2002). It can be suggested that high levels of vitamin A might induce a disruption of this signalling pathway, affecting the maturation of the pancreas and consequently its secretory functions.

European sea bass larvae normally achieve complete development of their digestive tract around the fourth week of life, with the onset of the brush border membrane digestion of enterocytes concurrent with the decline of cytosolic digestion. This maturational process is well known in mammals (Henning, 1987) and has been also described in fish (Zambonino Infante *et al.* 1997; Zambonino Infante & Cahu, 1999). Vitamin A interferes with the normal development of chicken and rat intestinal mucosa as it influences enterocyte proliferation and maturation (Reifen *et al.* 1998; Uni *et al.* 2000) and decreases brush border enzyme-specific activity (Uni *et al.* 2000). In our study, fish fed RA0 exhibited the lowest specific activities in

the three brush border enzymes studied at 28 dph. This reflected the lower stage of development of larvae fed RA0 compared with those from other groups. The maturation of intestinal brush border enzymes normally takes place between 20 and 30 dph in European sea bass, and this maturation process is generally completed by the end of the larval period (42 dph; Zambonino Infante & Cahu, 1999).

Retinoids are essential for normal embryonic development through their direct or indirect action on gene families such as Hox (Joore *et al.* 1994; Krumlauf, 1994), shh (Helms *et al.* 1997), BMP (Yates *et al.* 2002) or IGF (Allan *et al.* 2003) that are involved in morphogenesis. Any perturbation of these pathways interferes with normal body patterning, leading to abnormalities such as albinism and jaw or vertebral column deformities (Haga *et al.* 2002, 2003). Retinoids exert their teratogenic effects via their receptors, and a major involvement of RAR, especially RARγ, has previously been demonstrated in the appearance of malformations, compared with RXR (Ross *et al.* 2000). Nevertheless, RXR are essential in transducing the retinoid signal for a large number of retinoic acid-dependent processes because the RAR/RXR heterodimers are the functional units (Ross *et al.* 2000).

In the present study, fish fed RA50 (control group) exhibited a decrease in RXRα expression between 10 and 42 dph, probably reflecting the need for such receptors during differentiation processes in the early development of European sea bass. Afterwards, their synthesis slowed down as the cells underwent less of a differentiation process. In fish fed RA0, the presence of maternal retinol in the yolk sac could have balanced the lack of dietary vitamin A during the early stages of development and could have been responsible for the absence of significant difference in the gene expression pattern compared with the RA50 group. Up to 21 dph, no difference in RARα and RARγ expression was observed. RXRα was upregulated at 21 dph (by a factor of 6·75), but, as mentioned earlier, its role in the teratogenic retinoic acid signal is not major compared with that of RARγ. Taken together, these data may explain the very low percentage of malformed fish in this experimental group. The low level of dietary vitamin A (RA0) affected RARγ expression only between 21 and 42 dph, which seemed to be too late to perturb the morphogenesis as European sea bass already have adult features at this age.

Since RA0 larvae exhibited significant variations in IGF-1 expression at 15 dph (by a factor of $-5\cdot6$) and 21 dph (by a factor of 8·9) compared with the reference group, the downregulation at 15 dph may be linked to a vitamin A deficiency. Indeed, the same IGF-1 downregulation had previously been observed in the Japanese quail (Fu *et al.* 2001), correlated to an increase in the expression of the IGF-1 receptor, which may maximise the effect of this signalling molecule. We did not study the IGF-1 receptor gene, but we can hypothesise that the IGF-1 downregulation observed in the present study was most likely linked to an increase in the IGF-1 receptor at day 15. Such a regulation of IGF-1 could exert positive feedback in a second phase and lead to the IGF-1 upregulation at 21 dph.

Another hypothesis could also explain the down/upregulation of IGF-1. This gene belongs to the family of genes that are potentially inducible by retinoic acid (Gabbitas & Canalis, 1997) as it has a high probability of possessing a retinoic acid response element in its promoter (Balmer & Blomhoff, 2002). The increased expression of IGF-1 at day 21, in the absence of its inductor, may play a conservative role by preventing a significant decrease in IGF-1, at least over a short period. Such preventive mechanisms have been observed in young animals suffering from malnutrition and have been particularly studied for digestive enzymes in mammals (Raul *et al.* 1982) and in fish (Cahu & Zambonino Infante, 1994). This increase, taken together with the abnormal expression of RXR at 21 and 42 dph, may reflect a disorder of cellular function. The lack of vitamin A may have been partially balanced by the maternal retinol of the yolk sac during the early stages of development and by an insufficient endogenous production of retinoic acid after the resorption of the vitelus as the larvae showed retarded growth.

Using RARγ-null mutant mice, it has been shown that this RAR sub-type may be required for the teratogenic retinoic acid signal in the generation of the trunk but not for the craniofacial skeletal abnormalities. Moreover, RARγ-null mice were resistant to high doses of retinoic acid, indicating that RARγ may play a major role in mediating retinoid toxicity (Ross *et al.* 2000). These findings must be considered with those of the present study: at 10 dph, fish fed RA1000 exhibited an RARγ expression 82·5 times higher than that of the reference group, as well as the highest percentage of malformations. As these malformations mainly affected the maxilla and the neurocranium, we may assume that the genes involved in fish and controlled by RAR are different from those in mice, or that the vertebra were too differentiated to be perturbed. Several studies demonstrated the implication of shh and hoxd-4 (Suzuki *et al.* 1998), which possess retinoic acid responsive elements, in malformations affecting pharyngeal arch development in Japanese flounder treated with retinoic acid (Suzuki *et al.* 2000). Haga *et al.* (2002) showed that the expression of RAR, without any distinction between sub-families, increased after 3 days of treatment with retinoic acid and was linked to jaw deformities, whereas the expression of RXR did not seem to be affected. Our study showed that it was in fact RARγ sub-family expression that was increasing. Moreover, we noted a significant increase in RXRα expression at 42 dph compared with the RA50 group, suggesting that fish fed RA1000, like larvae fed RA0, exhibit functional disorders. Retinoic acid is thought to induce abnormalities by shifting the border of expression of Hox genes and eventually causing a transformation of anterior cell fates to more posterior cell fates (Suzuki *et al.* 1998).

These skeletal abnormalities may also involve IGF-1 and BMP-4. It has been demonstrated that retinoic acid can enhance IGF-1 expression and affect the development of limbs (Qin *et al.* 2002), whereas BMP-2 and BMP-4 are thought to be mediators of the osteogenic actions of growth hormone and IGF-1 (Li *et al.* 1998) and to be responsible for bone and tooth formation. Retinoic acid can also cooperate with BMPs during osteoblast differentiation even though BMP-4 does not have any retinoic acid response elements in its promoting region (Balmer & Blomhoff, 2002). This may explain why we did not observe any significant variation in BMP-4 expression during our experiment. The linear correlation between vitamin A content in larvae and the malformation percentages observed in this study, coupled with data from other authors (Conlon, 1995; Suzuki *et al.* 1998, 2000; Haga *et al.* 2002, 2003), might indicate that disruption of the signalling pathway of retinoid nuclear receptors results in a perturbation of cell differentiation and development, finally leading to skeletal malformations.

In fish, several studies have demonstrated that retinoids can affect the expression of shh, hoxd-4 and RAR genes in Japanese flounder, resulting in abnormal development of the pharyngeal skeleton and vertebral column (Suzuki *et al.* 1998, 2000; Haga *et al.* 2002, 2003). Similarly, the present data showed that

unbalanced levels of dietary all-*trans* retinol resulted in an alteration of head organisation in European sea bass juveniles, which was characterised by abnormal development of the operculum, premaxillary, maxillary, dentary and neurocranium, and basibranchial and hypohyal cartilages. Malformations were not only observed in the cephalic region, since a moderate number of fish also showed scoliosis in their vertebral column. The possible involvement of Hoxd-4 and shh genes in the development of the pharyngeal skeleton and vertebral column in sea bass larvae deserves to be studied.

It is interesting to note that experiments with marine fish larvae allow skeletal malformations to be induced via a nutritional pathway. Other species used as biological models, for example zebrafish (Joore *et al.* 1994), hatch at a later developmental stage than marine fish larvae (Barnabé *et al.* 1976; Kimmel *et al.* 1995). Therefore, abnormalities induced by retinoic acid have so far been observed in experiments conducted under non-nutritional conditions during embryogenesis, for example by implanting beads soaked in retinoic acid in chicken embryos (Lee *et al.* 2001), by bathing *Xenopus* embryos in a medium containing dissolved retinoic acid (Minucci *et al.* 1996) or by rearing Japanese flounder embryos in water containing controlled concentrations of retinoic acid (Suzuki *et al.* 1998, 2000; Haga *et al.* 2003).

Results related to body weight gain, maturation of the digestive system and incidence of malformations indicate that the optimal dietary level of vitamin A for European sea bass larvae was around 31 mg/kg DM (RA50 diet), which is much higher than the requirement of 0·75 mg/kg DM reported for salmonid juveniles (National Research Council, 1993). These results seemed to indicate that vitamin A requirements depend on the stage of development (Gouillou-Coustans *et al.* 1998), since the specific growth rate of larvae is higher than that of older fish, and differentiation and morphogenetic processes are very intense during this period to achieve the juvenile/adult phenotype.

In conclusion, the optimal dietary retinol level for a harmonious development of sea bass larvae was close to 31 mg/kg DM. The observed linear correlation between vitamin A level in larvae and malformation percentage was linked to a modification in RXRα, RARα and RARγ expression patterns, whereas BMP-4 and IGF-1 expressions were not affected by dietary retinol doses. This demonstrates the influence of nutrition on the retinoid pathways that play an important role in body patterning and the induction of skeletal malformations during post-hatching development in European sea bass.

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