Effect of dietary vitamin A on Senegalese sole (Solea senegalensis) skeletogenesis and larval quality

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Abstract:

The effects of different levels of vitamin A (VA) in Senegalese sole larval performance and development were evaluated by means of a dietary dose–response experiment using enriched Artemia metanauplii as a carrier of this micronutrient. Larvae were fed from 6 to 27 days post hatch (dph) with enriched Artemia containing graded levels of total VA (1.3, 2.1, 4.5 and 12.9 µg VA mg\textsuperscript{-1} DW). The content of VA in live prey directly affected its accumulation in larvae and early juveniles. Retinyl palmitate accumulated during larval ontogeny, whereas retinol showed the opposite trend, decreasing from hatching until 41 dph and then remaining constant until the end of the study. In metamorphic larvae (10 and 15 dph), VA did not affect the number of thyroid follicles or the intensity of the immunoreactive staining of T\textsubscript{3} and T\textsubscript{4}. However, at older stages of development (post-metamorphic larvae: 20, 30, 41 and 48 dph), VA decreased the number of thyroid follicles but increased their mean size and enhanced T\textsubscript{3} and T\textsubscript{4} immunoreactive staining. A dietary excess of VA did not affect either larval performance in terms of growth and survival or the maturation of the digestive system. However, the most remarkable impact of this morphogenetic nutrient was detected during skeletal morphogenesis. Dietary VA accelerated the intramembranous ossification of vertebral centra, which led to the formation of a supranumerary haemal vertebra and a high incidence of fused and compressed vertebrae in fish fed 2.1, 4.5 and 12.9 mg VA mg\textsuperscript{-1} DW. In addition, VA also affected those structures from vertebrae and caudal fin formed by chondral ossification, leading to defects in their shape and fusions with adjacent skeletal elements. In particular, the caudal fin was the region most affected by the dietary treatments. In order of importance, the bones with more developmental anomalies were the modified neural and haemal spines, epural, hypurals and parahypural. The impact of systemic factors such as thyroidal hormones in skeletogenesis should not be neglected since present results revealed that an excess of dietary VA affected the levels of T\textsubscript{3} and T\textsubscript{4}, which might have affected bone formation and remodelling, leading to skeletal deformities.
1. Introduction

Since the nineties, Senegalese sole (Solea senegalensis Kaup, 1858) has been considered a promising flatfish species for diversifying European marine aquaculture (Dinis et al., 1999). Recently, as profit margins for the two main cultured Southern European fish species, gilthead sea bream and European sea bass, have decreased due to their overproduction, interest has increased in Senegalese sole farming in Mediterranean and Southern Atlantic waters. Some of the advantages of culturing Senegalese sole include its high market price, the natural spawning of wild broodstocks held in captivity and mass production of offspring, the rapid development of eggs and larvae, and the high growth rate exhibited by juveniles (see review in Dinis et al., 1999). However, several bottlenecks compromise the intensive culture of this flatfish species, such as the reproduction of F1 broodstock (Anguis and Cañavate, 2005), pathological outbreaks (Zarza et al., 2003), and the production of juveniles in proper quantity and quality to satisfy market demands (high incidence of pigmentary disorders and skeletal deformities) (Soares et al., 2002; Gavaia et al., 2002).

Skeletal deformities and pigmentary disorders are important factors affecting flatfish production costs and determining the fish external morphology, appearance, growth, survival rate, and final market price (Takeuchi et al., 1998; Gavaia et al., 2002; Hamre et al., 2005). The development of these abnormalities is linked to a poorly understood relationship between nutritional, environmental, and genetic factors. Among them, larval nutrition at first feeding is one of the key parameters that affect skeletogenesis and pigmentation processes during early development. In this regard, several studies have shown that nutrients are responsible for the appearance of...
skeletal deformities and pigmentation disorders when their level and/or form of supply in the diet are inappropriate or unbalanced (see review in Lall and Lewis-McCrea, 2007; Hamre et al., 2005). Several authors have indicated that colour abnormalities in Japanese flounder could be effectively reduced by feeding larvae with high doses of vitamin A (VA) (Estévez and Kanazawa, 1995; Dedi et al., 1997; Takeuchi et al., 1995; Haga et al., 2002; Tarui et al., 2006). However, larvae fed high levels of VA showed a high incidence of skeletal deformities (Estévez and Kanazawa, 1995; Dedi et al., 1997; Takeuchi et al., 1998; Martinez et al., 2007) due to the morphogenetic action of this nutrient, which is known to have teratogenic effects in vertebrates at inappropriate dietary levels (Ross et al., 2000). Thus, in a situation in which a given nutrient exerts positive and negative effects simultaneously on different quality parameters, it is very important to determine a safe level that assures a normal skeletal development (minimum incidence of skeletal deformities) while preventing pigmentary disorders (pseudoalbinism and/or ambicolouration). The rapid physiological changes that Senegalese sole larvae undergo throughout development, reaching a fully metamorphosed morphology at an age of 20 days at 20°C (Fernández-Díaz et al., 2001), make this species of particular interest for studying the dietary effects of vitamin A on skeletogenesis and metamorphosis.

The objective of the present study was to evaluate the effect of graded levels of dietary VA administered to Senegalese sole larvae during the Artemia feeding phase on larval performance (growth, survival, maturation of the digestive function, and metamorphosis success) and quality (incidence and typology of skeletal deformities).

2. Materials and methods

2.1 Larval rearing and experimental diets

Newly hatched larvae of Senegalese sole were obtained from Stolt Sea Farm SA (Cambre, La Coruña, Spain) and shipped by road to IRTA facilities. After their
acclimation, larvae were distributed (initial density: 50 larvae l⁻¹) in 12 cylindrical tanks (100 l) connected to a recirculation unit (Carbó et al., 2003). Water conditions were as follows: 18 ± 1 °C, 35 ppt salinity, pH between 7.8-8.2, and daily exchange of water (20%) in the recirculation system with gentle aeration and oxygenation (> 4 mg l⁻¹).

Photoperiod was 12L:12D, and light intensity was 500 lux at water surface.

Figure 1 shows the feeding protocol for Solea senegalensis used in the present study. In detail, larvae were fed from day 3 post hatch (dph) to 10 dph with rotifers (Brachionus plicatilis) enriched with Easy Selco™ (ES, INVE, Belgium) following manufacturer’s instructions. Rotifer density was 10 rotifers ml⁻¹ from 3 to 4 dph and gradually reduced to 5 rotifers ml⁻¹ at 10 dph. Rotifer density was adjusted twice a day in order to assure the optimal prey density. Enriched Artemia metanauplii (EG, INVE, Belgium) were offered to larvae from 6 to 37 dph at increasing densities from 0.5 to 12 metanauplii ml⁻¹. Artemia metanauplii density was adjusted four times per day (at 9, 12, 15 and 18 h) to assure the optimal prey density and nutritional VA value; adjustments were conducted according to Cañavate et al. (2006). The retention of VA in enriched Artemia metanauplii in larval rearing tanks during the first four hours of starvation post-enrichment did not change (Fernández, unpublished data). From 33 dph to the end of the experiment (48 dph), larvae were progressively weaned onto dry feed (Gemma Micro 150-300° Skretting, Spain).

The effect of VA in Senegalese sole skeletogenesis was evaluated by means of four different dietary regimes containing graded levels of VA and using enriched Artemia metanauplii as carrier; each regime was done in triplicate. As live preys (rotifers and Artemia nauplii) accumulate VA in different patterns (Giménez et al., 2007), we could not maintain the same levels of VA during the whole live prey-feeding period. Thus, we decided to focus our study only during the Artemia-feeding phase. The graded levels of VA in Artemia metanauplii were obtained by adding different amounts of retinyl palmitate (1,600,000 IU g⁻¹, Sigma-Aldrich, Spain) to a commercial enriching emulsion, Easy Selco™. Experimental emulsions were designed to contain
500 (D1), 1,000 (D2), 2,100 (D3) and 4,000 (D4) retinol equivalents g⁻¹ (Table 1). For comparative purposes, the emulsion containing 500 retinol equivalents g⁻¹ (1,666 IU VA g⁻¹) was considered as the control group (ES without retinyl palmitate). Both live preys were enriched as previously described in Fernández et al. (2008).

Different parameters were measured in order to evaluate the effects of increasing dietary VA levels on larval performance: retinoid content in enrichment emulsions, live prey and larvae; larval growth (in length and weight) and survival rate; metamorphosis (eye migration), bottom settlement and thyroid gland development (size and number of follicles); maturation of the digestive system; and incidence of pigmentation disorders and skeletal deformities. Larvae were sampled and sacrificed with an overdose of anaesthetic (Tricaine methanesulfonate, MS-222, Sigma) at different ages from 2 to 48 dph, depending on the parameter considered.

2.2 Biochemical analysis

The retinoid content of the enrichment emulsions, enriched Artemia metanauplii, and larvae was analyzed by HPLC, using a modified version of the method by Takeuchi et al. (1998). After sampling, live prey and larvae were washed with distilled water to remove salt and bacteria, and the samples were frozen at –80 °C until posterior analysis. Lipids were extracted with chloroform:methanol (C:M, 2:1) according to Folch’s method (Folch et al., 1957) and stored in C:M:BHT (2:1:0.01%) at 20 mg l⁻¹ and -20 °C until analysis. Lipid extracts were then evaporated and redissolved in methanol:acetone (1:1, v/v) prior to HPLC analysis. The HPLC system (Thermo Separation Products, San Jose, CA, USA) was equipped with a Lichrospher C-18 reversed-phase column (Merck, Darmstadt, Germany) and a UV–visible detector set at a wavelength of 325 nm. The mobile phase was a mixture (85:15, v/v) of methanol (98%) with 0.5% ammonium acetate and chloroform. The flow rate was 1.5 ml min⁻¹, and the elution time was 18 min. The concentration of each retinoid was calculated from calibration curves constructed with the peak area ratios of their external standards.
and an internal standard of retinol acetate added to the samples. All the reference retinoids were purchased from Sigma-Aldrich (Spain).

The specificity of the method for the different retinoid compounds is guaranteed by the retention times of the peaks in the standard injections and the lack of interfering peaks in the blank runs. The four point linear regressions of the peak area and the concentration ratios of the internal standard and each retinoid analysed had $r^2$ higher than 0.9886, and were considered linear in the range of the tested samples. The repeatability was assessed through the injection of five different standard solutions with a mixture of the retinoids analysed for each of the four levels used in the calibration curves. The coefficient of variation was in all cases below 5%. These standard analyses also allowed checking the % recovery of the assayed retinoids, which was found between 92 and 101%. No peak was considered below a signal/noise ratio of 10.

2.3 Larval growth and survival rate

At 2, 5, 10, 15, 20, 31, 41 and 48 dph, fifteen larvae from each tank were randomly sampled, rinsed with distilled water, and used for body size and dry weight determination. Larval standard length (SL) was measured with a digital camera connected to a binocular microscope Nikon SMZ 800 and an image analysis system (AnalySIS, Soft Imaging Systems, GmbH). Once larvae were measured in length, they were dried at 60 °C until their weight was constant. Samples were weighed with an analytic microbalance (Sartorius BP211D). Survival rate was calculated as the percentage of final surviving fish with respect to the initial number at the beginning of the trial minus those individuals removed for sampling.

2.4 Maturation of the digestive system

The specific enzyme activity of two intestinal brush border enzymes (alkaline phosphatase and aminopeptidase) and two pancreatic enzymes (trypsin and amylase)
was used to assess the degree of development and maturation of the digestive system of larvae fed graded levels of VA. Enzyme activity was measured at 15, 31, 41 and 48 dph (sampling size was 40, 30, 15 and 10 individuals per tank, respectively). Sampled fish were washed with distilled water and stored at –80 ºC prior to enzyme activity analysis. All fish were dissected to separate pancreatic and intestinal segments as described by Cahu and Zambonino-Infante (1994). Samples were homogenized (Ultra-Turrax D25 basic, IKA® - Werke) in five volumes (v/w) of ice-cold Milli-Q water and centrifuged at 3,300 g (3 min) at 4 ºC, and the supernatant was removed for pancreatic enzyme quantification. Intestinal brush border membranes for determination of intestinal enzymes were purified according to Crane et al. (1979).

Trypsin (E.C. 3.4.21.4) activity was assayed at 25 ºC using BAPNA (N-α-benzoyl-DL-arginine ρ-nitroanilide) as substrate (Holm et al., 1988). Amylase (E.C. 3.2.1.1) activity was measured using soluble starch (0.3%) dissolved in Na₂HPO₄ buffer pH 7.4 as substrate (Métais and Bieth, 1968). Alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37 ºC using 4-nitrophenyl phosphate (PNPP) as substrate (Bessey et al., 1946). Aminopeptidase N (E.C.3.4.11.2) was determined at 25 ºC according to Maroux et al. (1973) using sodium phosphate buffer 80 mM (pH = 7.0) and L-leucine ρ-nitroanilide as substrate (in 0.1 mM DMSO). Enzymatic activities were expressed as specific enzyme activity, in milliunits per milligram of protein (mU/mg protein), and soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were conducted in triplicate.

2.5 Metamorphosis and bottom settlement

Metamorphosis and settlement are two separate processes in flatfish development that might coincide in time depending on the species (Geffen et al., 2007). Thus, we used the term metamorphosis to define morphological and physiological development and the term settlement to define behavioural changes associated with the transition of
larvae from a planktonic to a benthonic way of life. Eye migration in Senegalese sole larvae is generally used as a measure of their metamorphosis progress. In this study, eye migration was evaluated at 10, 19, 20 and 30 dph ($n = 200$ larvae per dietary treatment) as in Fernández-Díaz et al. (2001). Data are presented as the relative amount of larvae at each stage of development at the same age. At the same sampling dates, digital photographs of the rearing tanks were taken in order to count the amount of swimming larvae in the water column and those at the bottom of the tank using image analysis software (AnalySIS).

The development of the thyroid gland (number and size of follicles) was evaluated in samples of 10, 15, 20, 30, 41 and 48 dph larvae ($n = 10$ larvae per rearing tank; $n = 30$ per dietary treatment). For histological purposes, larvae were processed according to standard histomorphological methods and stained with haematoxylin-eosin. Detection and semiquantification of thyroidal hormones, thyroxin ($T_4$) and triiodothyronine ($T_3$), was conducted according to Ortiz Delgado et al. (2006). At the end of the trial, three hundred and fifty specimens from each tank were examined to evaluate the effect of VA on juvenile pigmentation. Pigmentation in the ocular side was visually assessed by means of individual examination of all specimens, and pigmentary disorders were categorized according to the twelve categories described by Haga et al. (2002).

2.6 Skeletal deformities analysis, observations and measurements

To identify and quantify the skeletal deformities of larvae from the different dietary treatments, 50-60 larvae per tank were sampled at the end of the experiment and fixed in formaldehyde solution (10%) until double stained. Animals were stained for bone and cartilage in whole mount preparations using a modification of the method described by Klymkowsky and Hanken (1991).

After staining, fish were placed on their blind (left) side to observe meristic characters and skeletal abnormalities in the cranium, vertebral column, and caudal fin.
complex. Skeletal structures were identified and named according to Gavaia et al. (2002) and Wagemans and Vandewale (2001). The study focused on the mean number of vertebrae and the frequency of individuals with an abnormal number of vertebrae. Special attention was given to the deformities occurring in the cranial region, vertebral column, and caudal fin complex (hypurals, parahypural, epural, modified haemal spines and modified neural spine).

2.7 Statistical analysis

Results are given as mean and standard deviation. Data expressed as percentage (survival, incidence of skeletal deformities, eye migration success, pigmentation disorders, and larval bottom settlement) were previously arcsin(x^{1/2})-transformed. All data were checked for normality (Kolmogorov–Smirnov test) and homoscedasticity of variance (Bartlett's test) and then compared by means of One Way ANOVA (Zar, 1974). When significant differences were detected, the Tukey multiple-comparison test was used to detect differences among experimental groups. Correlation between different variables was evaluated with the Pearson Product Moment Correlation test. In all statistical analyses, the level of significant difference was set at $P < 0.05$. All the statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA).

3. Results

3.1 Retinoid content in experimental emulsions and live prey

Table 1 presents the total lipid and total VA content (retinol and retinyl palmitate) in experimental emulsions used for enriching Artemia metanauplii with graded levels of retinyl palmitate. No statistically significant differences were detected in the total lipid content of experimental emulsions containing different levels of VA (ANOVA, $P > 0.05$).
Total VA content in the emulsions increased with increasing levels of retinyl palmitate incorporated (ANOVA, \(P < 0.05\)). The retinoid content and total VA of enriched *Artemia* metanauplii is shown in Figure 2. The HPLC analysis revealed that the main retinoid found in enriched *Artemia* was retinyl palmitate (VA ester), representing between 67 to 76% of the total VA content. The retinyl palmitate concentration increased in enriched *Artemia* with increasing levels of this compound in the enriching emulsion (ANOVA, \(P < 0.05\)). The level of retinyl palmitate in live prey increased up to 5.1 times when we compared *Artemia* enriched with D1 and D4 (7.7 and 39.4 ng mg\(^{-1}\) DW, respectively). The content of retinol (VA alcohol) in enriched live prey followed a similar pattern. While the retinol content of *Artemia* enriched with D1 and D2 was not significantly different, its level increased from 3.4 to 15.3 ng mg\(^{-1}\) DW (4.5-fold increase) (ANOVA, \(P < 0.05\)) in D1- and D4-enriched *Artemia*, respectively. In contrast, the retinoic acid content in *Artemia* enriched with D4 was 16.8 times higher than in *Artemia* enriched with D1 and D2, in which it increased from 0.37 to 6.2 ng mg\(^{-1}\) DW, respectively. *Artemia* enriched with D3 showed intermediate levels of retinoic acid accumulation (1.0 ng mg\(^{-1}\) DW; 2.8-fold increase in relation to the control group) (ANOVA, \(P < 0.05\)). Retinal (aldehyde form of VA) was not detected in *Artemia* enriched with graded levels of VA.

### 3.2 Retinoid content in larvae

Figure 3 shows the retinoid (retinol and retinyl palmitate) content in Senegalese sole larvae fed different VA regimes between 2 and 48 dph. During the study, retinyl palmitate increased as a consequence of the level of this retinoid in *Artemia*, whereas retinol showed the opposite trend and decreased to 55% as compared to its content in 2-dph larvae.

At the end of the study, the accumulation of retinyl palmitate and retinol in early juveniles was linked to the level of total VA administered during the *Artemia* feeding phase \((r^2 = 0.97 \text{ and } 0.99, \text{ respectively}; \ P < 0.001, \text{ Pearson Product Moment}\)
Correlation test). However, only the values of retinyl palmitate and retinol body content in fish fed D4-enriched *Artemia* were significantly higher than the mean value from the rest of dietary groups (27.75 ± 2.68 vs. 22.61 ± 0.25 ng retinyl palmitate mg⁻¹ DW and 0.88 ± 0.07 vs. 0.70 ± 0.03 ng retinol mg⁻¹ DW; *P* < 0.05, ANOVA).

3.3 Larval growth and survival

At 10 dph, larvae fed D1-, D2-, and D3-enriched *Artemia* were significantly larger than larvae fed the diet containing the highest content of total VA (D4) (Fig. 4a; ANOVA, *P* < 0.05). However, no differences in larval size were detected at older ages (15, 20 and 31 dph) until 41 and 48 dph, coinciding with the weaning phase. At 41 and 48 dph, fish fed *Artemia* enriched with the control emulsion (D1) were larger than those from the rest of the dietary groups (Table 2; ANOVA, *P* < 0.05). Dry weight was not significantly affected by any of the dietary treatments at any sampling time of the experiment (Fig. 4b; ANOVA, *P* > 0.05). Different levels of total VA in enriched *Artemia* did not affect Senegalese sole larval survival at the end of the study (Table 2; ANOVA, *P* > 0.05).

3.4 Maturation of the digestive system

Figure 5 shows changes in the enzyme specific activity of selected pancreatic and intestinal enzymes from fish fed the control diet (D1). From 15 to 48 dph, the specific activity of amylase gradually decreased from 0.619 to 0.014 U mg protein⁻¹ (ANOVA, *P* < 0.05). A 2.8-fold decrease in trypsin specific activity was also observed between 15 (0.398 mU mg protein⁻¹) and 30 dph (0.145 mU mg protein⁻¹), remaining fairly constant until the end of the study. However, alkaline phosphatase specific activity was constant from 15 to 41 dph (4.02 U mg protein⁻¹) but showed a 2.2-fold increase at 48 dph (8.86 U mg protein⁻¹). In contrast, aminopeptidase-N specific activity remained constant throughout the studied period (mean value of 0.089 mU mg protein⁻¹). Different levels of VA did not affect the specific activity of pancreatic or intestinal enzymes at any sampling point considered (ANOVA, *P* > 0.05). At 41 and 48 dph, trypsin, alkaline
phosphatase and aminopeptidase-N specific activity tended to be lower in fish fed D3 and D4 in comparison to fish fed D1 and D2, although this reduction in enzyme activity was not statistically significant (data not shown).

3.5 Metamorphosis and bottom settlement

Results of thyroid gland development are presented in Table 3. In 10- and 15-dph metamorphic larvae, dietary VA levels affected the number of thyroid follicles, although not significantly (ANOVA, $P > 0.05$). The intensity of the immunoreactive staining of T$_3$ and T$_4$ hormones showed no differences between the above-mentioned larval ages (Table 4). At older stages of development (20, 30, 41 and 48 dph post-metamorphic larvae), the increase in dietary VA reduced the number of follicles while increasing their average size (ANOVA, $P < 0.05$). These changes in the development of the thyroid glands concurred with an increase in the immunoreactive staining of T$_3$ and T$_4$ hormones (Fig. 6).

Bottom settlement was a fast process in Senegalese sole larvae that coincided with metamorphosis (eye migration). At 20 dph all fish had settled to the bottom, and most of them had completed eye migration. The level of VA in enriched Artemia did only significantly affect the process of settlement in metamorphosing larvae at 9 dph, when fish fed D3 and D4 showed higher rates of benthic larvae (8.6 ± 2.9%) in contrast to those from D1 and D2 groups (6.1 ± 1.8%) (ANOVA, $P < 0.05$). No significant differences in the rate of benthic larvae were detected at older ages among different experimental groups (12 dph: 66.0 ± 8.8%; 14 dph: 89.5 ± 4.0%; 19 dph: 96.4 ± 1.4%; 20 dph: 100%; data shown as the mean value of all experimental groups). Eye migration results are shown in Figure 7. The onset of eye migration started earlier in fish fed D2, D3 and D4 than in the control group. At 10 dph, the D2, D3, and D4 groups showed a higher frequency of specimens in stage 1 than the control group (23.8 vs. 2.2%, respectively; ANOVA, $P < 0.05$). However, these differences were not evident at older ages (19, 20 and 30 dph). Also, no differences in the frequency of fish at further
stages of eye migration (stages 2-4) were detected among different dietary experimental groups (ANOVA, $P > 0.05$). At 30 dph, eye migration process was completed (stage 4) and any case of abnormal eye migration was recorded in any of the experimental groups.

At the end of the study, the rate of fish exhibiting pigmentation problems was the same for all the dietary groups (ANOVA, $P > 0.05$), with an average incidence of pseudoalbinism of $2.3 \pm 1.0\%$. Ambicolouration was not observed in any of the sampled fish fed different levels of VA.

3.6 Skeletal deformities: typology and frequencies

Dietary levels of VA directly affected skeletogenesis and the incidence of skeletal deformities in Senegalese sole (Figure 8a). The frequency of deformed specimens increased with the dietary dose of VA, as well as the incidence of fish with more than one deformity in their skeleton (ANOVA, $P < 0.05$). In particular, the incidence of deformities ranged from fish with only one small skeletal abnormality to fish displaying multiple deformities with different degrees of severity (Fig. 8b; Fig. 11).

Cranial deformities (26.7%) were only observed in fish fed D4 (Figure 8c). The structures mostly affected were those related with the opercular complex, especially the preopercular, interopercular, ceratohyal, and ceratobranchials 1-5. The incidence of cranial deformities in the D4 group was significantly correlated to the presence of deformed prehaemal vertebrae numbers 1-3 ($r^2 = 0.998$, $P = 0.002$). No skeletal deformities were observed in the jaw apparatus and neurocranium in any of the dietary treatments.

The vertebral column was composed of 45 vertebrae, divided in 8 prehaemal and 37 haemal vertebrae (including the urostyle). No significant differences were detected in the mean number of specimens with 44, 45 and 46 vertebrae (ANOVA, $P > 0.05$) among the different VA treatments. However, the incidence of a supranumerary
Vertebra was higher in fish fed D2, D3 and D4 than in those fed D1 (36.0, 38.0 and 44.4 vs. 28.0%, respectively; $P = 0.02$).

Figure 9 shows the incidence of deformities along the vertebral column axis. In all experimental groups, most of deformities affecting the axial skeleton where observed between the vertebra number 38 and the urostyle; whereas increasing dietary levels of VA increased the incidence of deformities in the prehaemal vertebrae.

Skeletal abnormalities in the vertebral column (prehaemal and haemal regions) increased with increasing levels of dietary VA in enriched Artemia ($r^2 = 0.981$, $P = 0.018$; Fig. 10a). Torsion of the first three prehaemal (cephalic) vertebrae (14%) was recorded only in fish fed the highest dose of VA (D4). This type of deformity consisted of a change in the morphology of the vertebral disk resulting in a realignment of the axial skeleton and a slight torsion of the basioccipital articulatory process (Fig. 11b).

The frequencies of deformities in prehaemal and haemal vertebral centrums (fusion and compression) were significantly affected by the level of VA in the diet (Fig. 10b, c), although prehaemal centrums were less affected than haemal ones (16.7 vs. 63.3% in fish fed D1). No significant differences were detected in the incidence of deformities affecting the prehaemal centrums among fish fed D1, D2 and D3 diets (18.4%; ANOVA, $P > 0.05$), whereas the incidence of deformities in haemal vertebrae significantly increased with the level of dietary VA (ANOVA, $P < 0.05$). However, the frequency of fish with abnormal prehaemal and haemal centrums significantly increased 3.2 (59.3%) and 1.5 times (97.3%), respectively, in fish fed D4 (ANOVA, $P < 0.05$), indicating that prehaemal centrums were more sensitive than haemal centrums to dietary levels of VA.

Vitamin A also significantly affected the incidence of deformed neural and haemal spines (Fig. 10d and e; ANOVA $P < 0.05$). Figures 11c and 11d show different typologies of deformities affecting vertebral spines. The frequency of both abnormal neural spines and haemal spines was similar between fish fed D1 and D2 (78.4 and 71.4%, respectively), whereas it progressively increased in fish fed D3 (86.7 and 80.7, respectively), whereas it progressively increased in fish fed D3 (86.7 and 80.7,
respectively) and D4 diets (98.7 and 92.7%, respectively), showing significant
differences (ANOVA, \( P < 0.05 \)). The incidence of deformed parapophyses increased
from 19.3% in fish fed D1 up to 50.7% in fish fed D4 (2.6-fold increase; Fig. 10f),
whereas those specimens fed D2 and D3 showed intermediate values of abnormal
parapophyses (35.0%).

The dietary VA level affected all the skeletal structures composing the caudal
fin complex, although the incidence of deformities varied depending on the structure
considered and the dose of VA (Fig. 12a). The most common deformity affecting the
parahypural and the hypurals (1-5) was the fusion of these structures with those
adjacent, which produced changes in their regular shape (Fig. 11e-h). The occurrence
of abnormal hypurals increased with high levels of dietary VA. The incidence of fish
with abnormal hypurals almost doubled, from 36.7% in fish fed D1 up to 66.0% in those
fed D4. Fish fed D2 and D3 showed intermediate values of abnormal hypurals (48.7%),
with no significant differences between them (ANOVA \( P < 0.05 \); Fig. 12c). The
incidence of abnormal parahypural was similar among fish fed Artemia enriched with
D1, D2, and D3 (13.3% average value for the three treatments), which was significantly
lower than in fish in the D4 group (41.3%; Fig. 12b; ANOVA, \( P < 0.05 \)). The incidence
of deformed (twisted) epural in fish fed D2 and D3 (31.0%) showed a 1.9-fold increase
in relation to fish from the control group (16.0%), whereas this rise was 3.7 times higher
in fish fed D4 (58.7%; Fig. 12d). No significant differences were detected in the
incidence of deformities affecting the modified neural spine between D1, D2, and D3
groups (40.4%; ANOVA, \( P > 0.05 \)), whereas in the D4 group the number of fish with
abnormal modified neural spine significantly increased 1.7 times (68.0%; Fig. 12e;
ANOVA, \( P < 0.05 \)). The frequency of abnormal modified haemal spines (1-2) tended to
increase with increasing levels of dietary VA (Fig. 12f), being 2.3 times higher in fish
fed D4 than in those fed D1. A significant increase of 1.4- and 1.9-fold was recorded in
fish fed D2 and D3, respectively (ANOVA \( P < 0.05 \)).
4. Discussion

The effects of different levels of VA in Senegalese sole larval development were studied by means of a dose-response experiment using enriched Artemia metanauplii as carrier. Although the use of microdiets in co-feeding rearing protocols for the early weaning of Senegalese sole has been greatly improved (Fernández-Díaz et al., 2006; Engrola et al., 2009), we decided to bioencapsulate VA in live prey because Senegalese sole larvae cannot be fed exclusively with microdiets. As previously shown (Giménez et al., 2007), total VA in Artemia metanauplii accumulated proportionally to the content of retinyl palmitate in the enriching emulsions. Although retinoic acid was absent in the original emulsion, its presence in the metanauplii enriched with the highest levels of VA (D3 and D4) indicated that live prey were able to metabolize different retinoids and oxidize retinol into retinoic acid. Since retinoic acid is a much more active VA metabolite than the other retinoids (Ross et al., 2000), interpreting the results from the dose-response experiment must take into consideration its presence in D3- and D4-enriched Artemia metanauplii. The retinoid content in live prey directly affected the accumulation of VA in the larvae and, especially, in early juveniles, as retinyl palmitate and retinol body contents clearly showed. Of the two forms of VA, retinyl palmitate was the dominant form accumulated in Senegalese sole tissues. Under our experimental conditions, retinyl palmitate accumulated during larval ontogeny, whereas retinol showed the opposite trend, decreasing from hatching until 41 dph and then remaining constant until the end of the study. Retinyl esters, the main form of retinoids in live prey, are hydrolyzed into retinol in the lumen of the larval digestive tract, absorbed by the enterocytes, re-esterified, and transported to the liver through the lymphatic system by chylomicrons. Once in the liver, the main site for VA body storage, retinyl esters are hydrolyzed and re-esterified again in retinyl palmitate, which is finally stored in hepatocytes (Hamre et al., 2005). Thus, the accumulation of
retinyl palmitate in Senegalese sole larvae would reflect the dose-dependent accumulation of this form of VA due to the experimental feeding treatments and the larval age. In contrast, ontogenetic changes in both VA metabolism and larval requirements might explain the decrease in retinol content during the experimental period, since this form of VA and retinal constitute the total VA content in eggs and newly hatched larvae, with their content decreasing with larval development and metamorphosis (Moren et al., 2004a).

In fish species, VA requirements for normal development and optimal growth present inter-specific differences. Thus, in Japanese flounder (Dedi et al. 1997; Haga et al. 2003), Atlantic salmon (Ørnsrud et al. 2002), European sea bass (Villeneuve et al., 2005, 2006), red sea bream (Hernández et al., 2006), and gilthead sea bream (Fernández et al., 2008), high dietary doses of VA during larval development lead to poor growth performance and survival. Surprisingly, we found that Senegalese sole larval survival and growth, in terms of body weight, were not affected by the dietary VA content, and differences in total length were only observed after the weaning. Therefore, high levels of VA were not toxic (hypervitaminosis A) in terms of final growth in weight and survival of the fish, and the smaller size of the fish might be a consequence of a higher incidence of deformities in the caudal region of their vertebral column (Haga et al., 2002). According to the National Research Council, the requirements of VA for juveniles of different fish species, such as rainbow trout, salmon, channel catfish and sea bream, ranged between 1,000 and 3,500 IU kg$^{-1}$ (NRC, 1993). In contrast, when considering different flatfish species, the safe level of VA in *Artemia* nauplii for preventing the development of skeletal abnormalities in Japanese flounder was less than 45,200 IU VA kg$^{-1}$ (Dedi et al., 1995). In summer flounder and Atlantic halibut juveniles fed microdiets containing different levels of VA, a diet containing less than 52,873 and 8,333 IU VA kg$^{-1}$ respectively has been described as the best for assuring a proper juvenile development (Lewis-McCrea and Lall, 2007; Moren et al., 2004b, respectively). Under present experimental conditions, Senegalese
sole larvae fed *Artemia* metanauplii enriched with a commercial emulsion containing 4,333 IU kg\(^{-1}\) showed a high incidence of skeletal abnormalities, which seems to indicate that this species is quite sensitive to low dietary levels of this nutrient. However, published results regarding the VA requirements in different fish species might be taken cautiously, since there might be differences depending on the stage of development of experimental fish (larva vs. juvenile), the type of retinoid compound included into the diet (retinyl esters, retinoic acid or carotenoids), the experimental design, the rearing conditions or the analytical method for VA quantification.

Thyroid hormones, VA, and fatty acids are all factors that have been shown to affect metamorphosis in flatfish by disrupting the normal pigmentation and eye migration patterns (see reviews by Hamre et al., 2005, 2007). Several authors have described hyperpigmentation (Martínez et al., 2002) or improved pigmentation (Estévez and Kanazawa, 1995; Takeuchi et al., 1995; Dedi et al., 1997; Haga et al. 2002) of flatfish larvae fed live prey enriched with VA, although in some studies high VA levels increased the frequency of skeletal deformities. Under the present conditions, dietary VA did not affect pigmentation patterns in Senegalese sole. This might indicate a species-specific sensitivity to a dietary excess of VA in the differentiation of pigmentary cells that may either differentiate into adult melanophores or disappear by apoptotic processes (see review in Bolker and Hill, 2000).

In addition, dietary levels of VA did not alter the process of settlement in metamorphosing Senegalese sole larvae, although they affected eye migration in early metamorphosis (10 dph). Thus, 10-dph larvae fed high levels of VA (D2, D3, and D4 groups) showed a precocious formation of the ocular channel and the initiation of eye migration. These differences were not observed in the latter stages. Senegalese sole presents a narrow size threshold for the onset of metamorphosis, resulting in a synchronised settling behaviour and a uniform post-settlement size distribution (Fernández-Diaz et al., 2001). Thus, the high frequency of larvae in early stages of metamorphosis at 10 dph might be associated with their larger size, since
metamorphosis in this species depends on larval size (see review in Geffen et al., 2007) and the levels of thyroid hormones (Ortiz Delgado et al., 2006; Klaren et al., 2008).

Pancreatic and intestinal enzyme activity provides a reliable marker of larval fish development (Zambonino Infante et al., 2008). In the present study, an excess of dietary VA did not affect the activity levels of these digestive enzymes in Senegalese sole larvae, which followed the general trend previously described for this species (Ribeiro et al., 1999). In contrast, gilthead sea bream (Fernández et al., 2008) and European sea bass (Villeneuve et al., 2005, 2006) larvae fed high doses of VA showed a delay in the maturation of their digestive function. Thus, we can hypothesize that the levels of VA tested in the present experiment are sublethal, since they did not affect the overall development of Senegalese sole larvae, neither in terms of larval survival, body weight, nor maturation of the digestive function. On the other hand, dietary VA levels affected dramatically the normal process of bone formation and skeletogenesis in Senegalese sole larvae.

Different studies have shown a high incidence of skeletal deformities in hatchery-reared early juveniles of Senegalese sole, ranging from 44% (Gavaia et al., 2002) to 80% (Engrola et al., 2009). In our study, fish fed the control diet also showed a high frequency of individuals with deformed skeletal structures. Furthermore, an increase of dietary VA resulted in a significant increase in deformities. The incidence of skeletal deformities reported in Senegalese sole reared under standard feeding protocols is higher than that observed in other commonly produced species in the Mediterranean area, like gilthead sea bream (Boglione et al., 2001; Fernández et al., 2008) or European sea bass (Villeneuve et al., 2005; Mazurais et al., 2008). Two different hypotheses might explain such a high incidence of skeletal deformities in Senegalese sole. The first considers that this flatfish species is more prone to develop skeletal disorders than other fish species under any rearing conditions. The second hypothesis postulates that since the skeletal deformities observed in Senegalese sole
were not lethal, higher final numbers of Senegalese sole specimens with deformities would be observed at the juvenile stage. Consequently, the observed incidence of deformities in Senegalese sole early juveniles was higher than in those species where deformities were lethal at early stages (Divanach et al., 1997; Koumoundouros et al., 1997; Boglione et al., 2001). Since both hypotheses are not mutually exclusive, determining which of the two models better explains the observations requires further developmental studies that would identify the most sensitive periods of morphogenesis and skeletogenesis to the development of deformities, as well as the timing of appearance of the deformities and their impact on larval survival.

The skeletal structures most affected by high dietary levels of VA in Senegalese sole were those from the vertebral column and caudal fin complex. Previously published studies found that several structures from the splanchnocranium, such as the premaxilla, maxilla and dentary bones, were the structures most severely affected by dietary VA (Haga et al., 2002, 2003; Villeneuve et al., 2005, 2006; Fernández et al., 2008). However, these skeletal structures did not show any changes in fish fed experimental diets in the present study. Since the diets with VA in excess were not offered to the larvae until 7 dph, when most of the pharyngeal skeleton was already ossified, the absence of changes in those skeletal structures is probably related to the timing of VA administration. In the present study, the opercular complex, in particular, the preopercular, interopercular, ceratohyal, and ceratobranchials 1-5, were mostly affected by the diet with the highest level of VA and retinoic acid (D4). The strong statistical correlation (Pearson Product Moment Correlation test) found between the deformed opercular structures and the cephalic vertebrae, suggests that the altered shapes of the opercular bones are a consequence of the torsion of the first three prehaemal (cephalic) vertebrae coupled with the restructuring processes of the cranial bones. These processes take place during eye migration and the completion of the typical asymmetrical body shape of this species; thus, the observed deformities seem to be more related to a disruption (acceleration) of the normal larval metamorphosis.
pattern, rather than dietary VA acting directly on the above-mentioned opercular elements.

Vitamin A impaired the development and number of vertebrae in Senegalese sole. Similarly to Japanese flounder (Haga et al., 2002) and gilthead sea bream (Fernández et al., 2008), high levels of dietary VA in Senegalese sole were responsible for a higher incidence of a supranumerary vertebra in the haemal region of the vertebral column of the fish. Contrastingly, in European sea bass an excess of VA resulted in the loss of one vertebra (Villeneuve et al., 2006). In Senegalese sole, since morphogenesis of the vertebral centrums follows a caudal direction (Gavaia et al., 2002), vertebrae from the haemal region are the last ones to differentiate and ossify by intramembranous ossification. The notochord is responsible for the proper morphogenesis of the vertebral centrums, and consequently this tissue plays an important role in inducing vertebral formation and maintaining vertebral morphogenesis (Witten et al., 2005). Thus, dietary VA levels might have disrupted the segmentation of the notochord and the normal process of morphogenesis in the vertebral centrums, leading to a change in the number of vertebrae, as Haga et al. (2009) recently demonstrated using transgenic zebrafish exposed to retinoic acid.

The impact of dietary VA on the incidence of skeletal deformities in different regions of the vertebral column was also affected by the timing of the intramembranous ossification. The first three prehaemal (cephalic) vertebrae, which are the first elements of the vertebral column to ossify (Gavaia et al., 2002), were the least affected in comparison to the rest of the prehaemal and haemal regions. Only deformed cephalic vertebrae were detected in fish fed D4-enriched Artemia containing high levels of retinyl palmitate and retinoic acid, whereas deformities affecting the rest of the prehaemal and all the haemal vertebrae were detected in all experimental groups, although at different prevalence rates. Therefore, the dose of VA and the timing of morphogenesis directly affect the incidence of skeletal disorders (Villeneuve et al., 2006; Mazurais et al., 2008). Skeletal deformities affecting prehaemal and haemal
vertebrae in Senegalese sole early juveniles included: compressed, deformed and fussed centrums; alterations of the intervertebral space; and deformed (twisted) parapophyses, neural and haemal spines, which were more frequent in the haemal vertebrae of fish fed high doses of dietary VA. According to Gavaia et al. (2002), who described the osteological development of the caudal complex and vertebral column in Senegalese sole for the first time, the development of both vertebral column and caudal fin complex begins at 12–13 dph (16-18 ºC). However, in the present study this development might have occurred earlier due to the slightly higher rearing temperatures. In this regard, the high incidence of deformities in the prehaemal and haemal regions of the vertebral column seemed to be related to an abnormally early differentiation pattern. Thus, a prolonged exposure to an excess of VA might have altered the normal process of morphogenesis in those skeletal elements formed either by chondral (neural and haemal spines) or by intramembranous (vertebral centrums) ossification. This would enhance the appearance of skeletal disorders, as previously described in Japanese flounder (Haga et al., 2002), Atlantic salmon (Ørnsrud et al., 2002), European sea bass (Villeneuve et al., 2005, 2006), red sea bream (Hernández et al., 2006), summer flounder (Martínez et al., 2007), and gilthead sea bream (Fernández et al., 2008). Compressed vertebrae and reductions in the intervertebral spaces might be associated with the presence of supranumerary vertebrae, as reported for gilthead sea bream (Fernández et al., 2008). However, in Senegalese sole the incidence of supranumerary vertebrae was not proportional to that of vertebral compressions and fusions. These findings suggest that these skeletal deformities might also be related to alterations in the areas of vertebral centrum growth and the failure of notochord cells to maintain proper vertebral development and growth, as described in Atlantic salmon (Witten et al., 2005).

The caudal fin complex was the most altered region of the Senegalese sole skeleton, although the incidence of deformities varied depending on the structure considered and the dose of VA. Although the deformities affected all the skeletal
elements composing the caudal fin, the most affected structures, in order of
importance, were the modified neural and haemal spines, epural, hypurals, and
parahypural. These results are in agreement with those observed in Japanese flounder
(Dedi et al., 1998) but differ from those reported in gilthead sea bream fed an excess of
VA, where the most affected caudal bones were the epurals, hypurals, parahypural,
nervous arch, and uroneurals (Fernández et al., 2008). The differences between both
flatfish species and gilthead sea bream might be due to species-specific patterns in the
morphogenesis of the caudal complex linked to metamorphosis and the acquisition of
asymmetry and benthic life. Considering previous descriptions of the osteological
development of the caudal fin complex (Gavaia et al., 1999; 2006) and the results
obtained in Japanese flounder and gilthead sea bream larvae fed graded levels of VA
(Dedi et al., 1998; Fernández et al., 2008, respectively), the differences in sensitivity to
dietary VA amongst caudal fin skeletal elements might be due to differences in their
ontogenetic development and the duration of VA exposure. The high incidence of
fusion between hypurals and parahypural has also been observed in Japanese
flounder early juveniles (Dedi et al., 1998). Thus, VA might have stimulated the
differentiation and proliferation of chondrocytes (hypertrophic differentiation) in the
above-mentioned structures, leading to their fusion due to their close proximity and
their almost simultaneous temporal development (Gavaia et al., 2002).

Up to this point, we have only considered the effect of VA on Senegalese sole
skeletogenesis by its direct action through retinoic acid in the skeletal tissue. However,
present results indicate that VA also affected the levels of thyroid hormones $T_3$ and $T_4$
in Senegalese sole larvae. Thyroid hormones are essential regulators of skeletal
development and bone maintenance (Wexler and Sharretts, 2007). During
development, thyroid hormones, especially $T_3$, are essential for the recruitment and
maturation of bone cells. In mammals, alterations in the thyroid status result in
acceleration of bone formation (by either chondral or intramembranous ossification),
growth abnormalities, bone loss, and increased fracture risk (Harvey et al., 2002). In
particular, excessive amounts of thyroid hormone induce increased activity of osteoblasts and osteoclasts leading to high bone turnover and loss of bone mineral density, as the activity of osteoclasts predominates over the activity of osteoblasts (Mikosch, 2005). The action of thyroid hormones on the development and health of the skeletal tissue is mediated by nuclear receptor proteins (TR), which are expressed in chondrocytes and osteoblasts. These proteins are members of the superfamily of hormone and orphan nuclear receptors and function as hormone-inducible transcription factors (Harvey et al., 2002). The TR proteins together with retinoid X receptors form heterodimers (RXR) that bind to specific T₃-response element sequences within target gene promoters and modulate their transcriptional regulation (Duncan Basset et al., 2007). Thus, there is a convergence of VA- and thyroid hormone receptor-mediated pathways on bone formation and remodelling. Although Senegalese sole has an acellular bone, the mechanisms of bone tissue formation and growth are quite conserved among vertebrates and also their signalling pathways (Witten and Huysseune, 2007), which implies that modifications in the thyroid hormone status might have a direct effect on skeletal morphogenesis. Disruption of these pathways by either dietary VA imbalances or changes in the levels of T₃ might affect the process of normal skeletogenesis, leading to skeletal deformities.

5. Conclusions

Under the present experimental conditions and independently of the feeding treatment, Senegalese sole exhibited high levels of skeletal abnormalities, particularly in the vertebrae and caudal fin complex. Therefore, even the control group (fish fed *Artemia* metanauplii enriched with a commercial emulsion) was exposed to a dietary dose of VA that might have altered the harmonious development of the axial and caudal skeleton. Thus, we need to conduct further research using emulsions with even lower levels of
VA (retinyl palmitate) to discriminate between the effects of this nutrient and other factors inducing skeletal disorders in Senegalese sole. In this regard, we need to evaluate the effect of other nutrients, such as essential fatty acids, minerals and vitamins (particularly liposoluble vitamins D, E, and K) (Lewis et al., 2007; Mazurais et al., 2008), genetic factors (Kacem et al., 2004), and/or unsuitable husbandry and rearing practices and rearing temperatures (Lewis et al., 2004; Blanksma et al., 2009; Sfakianakis et al., 2006), that might also have been affecting the skeletal development of Senegalese sole larvae. The inherent complexity of skeletogenesis is such that a holistic approach to discriminate and evaluate the relative importance of each of the above-mentioned factors is not possible, and consequently this question needs to be addressed in singular experiments.

Our studies on the effects of different dietary VA levels on Senegalese sole performance revealed that an excess of VA affected neither larval performance in terms of survival and growth nor the maturation of the digestive system. However, this morphogenetic nutrient had a remarkable impact in the skeleton morphogenesis. An excess of VA accelerated the intramembranous ossification of vertebral centrums, leading to a supranumerary haemal vertebra and a high incidence of fused and compressed vertebrae. In addition, VA also affected those structures from the vertebrae and caudal fin formed by chondral ossification, leading to defects in their shape and fusions with adjacent skeletal elements. However, we should not dismiss the impact of other systemic factors such as thyroidal hormones in skeletogenesis since in our studies an excess of dietary VA affected the levels of thyroid hormones (T$_3$ and T$_4$), which might have affected metamorphosis, bone formation and remodelling, leading to skeletal deformities.

Further studies are needed to identify the potential crosstalk between VA and thyroid hormones and their effects on the expression of different genes involved in Senegalese sole early morphogenesis and skeletogenesis.
Acknowledgments

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References


Figure captions

Figure 1. Feeding protocol of Senegalese sole. *Artemia* metanauplii were enriched with experimental emulsions containing 500 (D1), 1,000 (D2), 2,100 (D3) and 4,000 (D4) retinol equivalents g\(^{-1}\).

Figure 2. Retinoid (retinoic acid, retinol, and retinyl palmitate) and total vitamin A content (ng retinoid compound mg\(^{-1}\) DW) in *Artemia* metanauplii enriched with graded levels of VA [500 (D1), 1,000 (D2), 2,100 (D3) and 4,000 (D4) retinol equivalents g\(^{-1}\)]. For comparative purposes, the mean value of the total VA content in enriched live prey is included for each treatment. Different letters denote the existence of statistically significant differences among the content of different compounds depending on the treatment (ANOVA, *P* < 0.05).

Figure 3. Changes in body content of retinol and retinyl palmitate (ng retinyl palmitate mg DW\(^{-1}\)) of Senegalese sole larvae fed graded levels of vitamin A. Different indexed letters show significant differences between treatments (ANOVA, *P* < 0.05).

Figure 4. Growth in standard length (a) and dry weight (b) of Senegalese sole larvae fed *Artemia* enriched with graded levels of VA. At 10 dph, the asterisk denotes the existence of significant differences in standard length between groups (see text for details). The dotted line represents the onset of the weaning period. Different letters indicate statistically significant differences among dietary treatments (ANOVA, *P* < 0.05).

Figure 5. Changes in specific enzyme activity of trypsin (a), amylase (b), alkaline phosphatase (c), and aminopeptidase-N (d) in Senegalese sole fed the control diet.
Different letters denote the existence of statistically significant differences among different sampling points (ages).

Figure 6. Immunolocalization of $T_3$ and $T_4$ in Senegalese sole larvae fed different levels of vitamin A (haematoxylin and eosin/peroxidase staining). Thyroid follicles in a 15-dph larva from D1 (a) and D3 (b). Note the presence of a small follicle at the base of the aortic bulb (arrowhead); (c) and (d), thyroid follicles of a 20-dph larva exhibiting a weak $T_4$ immunoreactivity within the colloid (D1 treatment); (e) and (f), thyroid follicles of 30-dph larvae showing a moderate $T_3$ immunostaining (D1 treatment); (g), thyroid follicles of 41-dph larvae showing moderate $T_3$ immunoreactivity (D1 treatment). Note the increase of $T_3$ staining for the D3 treatment (h). Changes in the thyroid gland development at 48 dph when comparing D1 with D4 treatments: note the decrease in the number of follicles and the increase in their mean size, coupled with an increase of $T_4$ staining intensity on $S. senegalensis$ larvae from D4 treatment [(i) and (j), D1; (k) and (l)]. Scale bars represent 100 $\mu$m.

Figure 7. Metamorphosis stages of Senegalese sole larvae fed graded levels of vitamin A. Staging was established according to Fernández-Díaz et al. (2001). Different indexed letters show significant differences among treatments (ANOVA, $P < 0.05$).

Figure 8. Incidence of skeletal deformities affecting the head, vertebral column, and tail in Senegalese sole fed graded levels of vitamin A (a). Incidence of deformities considering the number of abnormal skeletal elements per fish (b). Cranial deformities in Senegalese sole fed the highest dose of VA showing the most affected skeletal elements of the opercular complex (c). Different indexed letters show significant differences among treatments (ANOVA, $P < 0.05$).
Figure 9. Incidence of deformities in prehaemal and haemal vertebrae along the vertebral axis in Senegalese sole larvae fed different levels of vitamin A. Feeding treatments: D1 (a), D2 (b), D3 (c), and D4 (d).

Figure 10. Incidence of skeletal deformities in the vertebral column of Senegalese sole fed graded levels of vitamin A. Total vertebral (prehaemal and haemal) deformities (a), deformed prehaemal (b) and haemal (c) centrums, abnormal neural (d) and haemal (e) spines, and parapophysis (f). Different indexed letters show significant differences among treatments (ANOVA, $P < 0.05$).

Figure 11. Examples of different typologies of skeletal deformities found in Senegalese sole under the present experimental conditions. (a) General view of a 30-dph metamorphic larva with a severe deformity in the vertebral column. (b) Torsion (T) of the first three prehaemal (cephalic) vertebrae resulting in deformed preopercular (Po), interopercular (Io) and ceratohyal (Ch). Note the space between the head and the abdominal region (arrow) as an indicator of the head’s torsion. (c) Ectopical structure connecting neural spines from two adjacent haemal vertebrae (arrow). (d) Compression of centrums of haemal vertebrae and haemal vertebra with deformed haemal prezigapophysis (Hprz) and poszigapophysis (Hpz). (e) Fusion of haemal vertebrae numbers 43 and 44 with fusion of their respective haemal spines (asterisk). (f) Deformities affecting the caudal fin: deformed urostyle, fused hypurals 4-3 and 2-1, and fusion of hypural 1 with the modified haemal spine. (g) Compression of haemal vertebrae numbers 41-44 and disappearance of the intervertebral space among them. (h) Fusion of hypurals 1-5 and compression of haemal vertebrae (note the absence of intervertebral spaces among vertebral centrums). Abbreviations: Ep: epural; Hy:
Figure 12. Incidence of deformities in the caudal fin complex in Senegalese sole fed graded levels of vitamin A. Percentage of specimens with at least one deformity in the caudal fin (a), parahypural (b), hypurals (c), epural (d), modified neural spine (e), and modified haemal spine (f). Different indexed letters show significant differences among treatments (ANOVA, $P < 0.05$).
Table 1. Total lipid and retinoid content (retinyl palmitate, retinol and total VA) in experimental Artemia enriching emulsions. Total lipid content is expressed as % DW and retinoid content in emulsions is expressed as μg mg^{-1} DW. Different letters within the same column show significant differences between emulsions (ANOVA, P < 0.05).

<table>
<thead>
<tr>
<th>Emulsion</th>
<th>Total lipids</th>
<th>Retinyl palmitate</th>
<th>Retinol</th>
<th>Total VA</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>84.3 ± 2.94</td>
<td>1.23 ± 0.010 a</td>
<td>0.0051 ± 0.0005 a</td>
<td>1.32 ± 0.030 a</td>
</tr>
<tr>
<td>D2</td>
<td>81.7 ± 3.31</td>
<td>2.07 ± 0.440 ab</td>
<td>0.0057 ± 0.0003 ab</td>
<td>2.09 ± 0.123 b</td>
</tr>
<tr>
<td>D3</td>
<td>87.8 ± 6.25</td>
<td>4.47 ± 0.830 b</td>
<td>0.0079 ± 0.0005 b</td>
<td>4.50 ± 0.249 c</td>
</tr>
<tr>
<td>D4</td>
<td>82.7 ± 3.01</td>
<td>12.87 ± 0.198 c</td>
<td>0.013 ± 0.002 c</td>
<td>12.91 ± 0.059 d</td>
</tr>
</tbody>
</table>
Table 2. Final larval size in standard length (SL) and dry weight (DW), and survival rate of Senegal sole larvae fed different levels of vitamin A. Values are mean ± standard deviation. Different letters within the same column show statistical significant differences.

<table>
<thead>
<tr>
<th>Dietary treatment</th>
<th>SL (mm)</th>
<th>DW (mg)</th>
<th>Survival (%)</th>
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<tbody>
<tr>
<td>D1</td>
<td>13.35 ± 0.09 a</td>
<td>7.42 ± 0.40</td>
<td>47.1 ± 4.0</td>
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<tr>
<td>D2</td>
<td>11.91 ± 0.10 c</td>
<td>5.29 ± 0.30</td>
<td>45.6 ± 2.9</td>
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<tr>
<td>D3</td>
<td>12.40 ± 0.10 b</td>
<td>6.13 ± 0.29</td>
<td>41.6 ± 0.7</td>
</tr>
<tr>
<td>D4</td>
<td>11.84 ± 0.10 c</td>
<td>6.30 ± 0.40</td>
<td>41.3 ± 5.1</td>
</tr>
</tbody>
</table>
Table 3. Differences in the number and size of thyriod follicles of Senegal sole larvae fed different levels of vitamina A. Different letters within the same age range (rows) show statistical significant differences among emulsions (ANOVA, P < 0.05).

<table>
<thead>
<tr>
<th>Days post-hatching</th>
<th>Number of follicles</th>
<th>Size (mean ± SD) µm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td>10 dph</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>15 dph</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>20 dph</td>
<td>6</td>
<td>2-4</td>
</tr>
<tr>
<td>30 dph</td>
<td>7</td>
<td>3-5</td>
</tr>
<tr>
<td>41 dph</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>48 dph</td>
<td>10-15</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 4. Semiquantitative assessment of thyroid hormones content by using immunohistochemical approaches. Asterisks indicate reaction colour intensities: */- weak; * moderate; ** intense; *** very intense.

<table>
<thead>
<tr>
<th>Days post-hatching</th>
<th>T₃ - immunoreactivity</th>
<th>T₄ - immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td>10 dph</td>
<td>*/-</td>
<td>*</td>
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<tr>
<td>15 dph</td>
<td>*/-</td>
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<tr>
<td>41 dph</td>
<td>*</td>
<td>*</td>
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<tr>
<td>48 dph</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>
Figure 7