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Title: Commercial products for Artemia enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalensis*) larvae.

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Corresponding Author: Mrs. Anaïs Boglino, Ph.D

Corresponding Author's Institution: IRTA

First Author: Anaïs Boglino, Ph.D

Order of Authors: Anaïs Boglino, Ph.D; Maria J Darias, PhD; Juan B Ortiz-Delgado, PhD; Filiz Özcan, MSc.; Alicia Estévez, PhD; Karl B Andree, PhD; Francisco Hontoria, PhD.; Carmen Sarasquete, PhD.; Enric Gisbert, PhD.

Abstract: Proper nutrition at first feeding in marine fish larvae is an important factor for successful larval rearing. However, live prey used to feed marine finfish larvae lack essential fatty acids requiring commercial hatcheries to enrich live prey to provide a balanced diet. Currently, the many commercially available enrichments for live prey present considerable differences in their formulas, and compositions of essential nutrients. Since nutritional requirements are species-specific it is necessary to define those enrichments most appropriate for each species in terms of larval development and quality. This study aimed to evaluate the effect of six enrichments on Senegalese sole larval performance including growth, digestive system development and skeletogenesis, to determine whether any of these products are more suitable for Senegalese sole larval rearing. From 8 to 37 days post hatching (dph), larvae were fed Artemia nauplii previously enriched with six different formulae: Easy Selco® (INVE, ES), Easy Selco® (INVE) half diluted with olive oil (ES/2), Multigain® (BioMar, MG), Red Pepper® (Bernaqua, RP), Aquagrow Gold® (ABN, AGG) and Aquagrow DHA® (ABN, AGD), with the last two being diluted by a third with olive oil. The dietary treatments significantly affected larval growth and performance; larvae fed Artemia enriched with AGG presented significantly higher final dry weight (2.0 ± 0.4 mg), growth and intestinal maturation than larvae fed ES-enriched Artemia (final dry weight: 1.1 ± 0.3 mg). Larvae fed the AGG treatment also were those most advanced in metamorphosis and ossification processes. Larvae fed ES/2, RP, AGD and MG treatments presented intermediary values for all these parameters. No significant effect of the dietary treatments was detected in final survival and incidence of skeletal deformities. We conclude that, among the enrichments tested, AGG is the most appropriate for larvae of Senegalese sole.

Commercial products for *Artemia* enrichment affect growth performance, digestive system maturation, ossification and incidence of skeletal deformities in Senegalese sole (*Solea senegalensis*) larvae.

Anaïs Boglino^{1*}, Maria José Darias¹, Juan Bosco Ortiz-Delgado³, Filiz Özcan¹, Alicia Estévez¹, Karl B. Andree¹, Francisco Hontoria², Carmen Sarasquete³, Enric Gisbert¹

¹IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aquícoles, Ctra. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain

²Instituto de Acuicultura de Torre de la Sal (CSIC), 12595 Torre de la Sal, Castellón, Spain

³Instituto de Ciencias Marinas de Andalucía (CSIC), Campus Universitario Río San Pedro, Apdo Oficial, 11510 Puerto Real, Cádiz, Spain

*Author for correspondence: Tel.: +34 977745427; Fax: +34 977443138; e-mail: anaïs.boglino@irta.cat

Abstract

Proper nutrition at first feeding in marine fish larvae is an important factor for successful larval rearing. However, live prey used to feed marine finfish larvae lack essential fatty acids requiring commercial hatcheries to enrich live prey to provide a balanced diet. Currently, the many commercially available enrichments for live prey present considerable differences in their formulas, and compositions of essential nutrients. Since nutritional requirements are species-specific it is necessary to define those enrichments most appropriate for each species in terms of larval development and quality. This study aimed to evaluate the effect of six enrichments on Senegalese sole larval performance including growth, digestive system development and skeletogenesis, to determine whether any of these products are more suitable for Senegalese sole larval rearing. From 8 to 37 days post hatching (dph), larvae were fed *Artemia* nauplii previously enriched with six different formulae: Easy Selco® (INVE, *ES*), Easy Selco® (INVE) half diluted with olive oil (*ES/2*), Multigain® (BioMar, *MG*), Red Pepper® (Bernaqua, *RP*), Aquagrow Gold® (ABN, *AGG*) and Aquagrow DHA® (ABN, *AGD*), with the last two being diluted by a third with olive oil. The dietary treatments significantly affected larval growth and performance; larvae fed *Artemia* enriched with *AGG* presented significantly higher final dry weight (2.0 ± 0.4 mg), growth and intestinal maturation than larvae fed *ES*-enriched *Artemia* (final dry weight: 1.1 ± 0.3 mg). Larvae fed the *AGG* treatment also were those most advanced in metamorphosis and ossification processes. Larvae fed *ES/2*, *RP*, *AGD* and *MG* treatments presented intermediary values for all these parameters. No significant effect of the dietary treatments was detected in final survival and incidence of skeletal deformities. We conclude that, among the enrichments tested, *AGG* is the most appropriate for larvae of Senegalese sole.

Keywords: Senegalese sole; larvae; enrichment; *Artemia* nauplii; lipid; fatty acid; morphogenesis; skeletogenesis; nutrition.

Abbreviations: AGD - Aquagrow DHA© treatment; AGG - Aquagrow gold© treatment; AP - alkaline phosphatase; ARA - arachidonic acid, 20:4n-6; DHA - docosahexaenoic acid, 22:6n-3; DPA - docosapentaenoic acid, 22:5n-3; dph – days after hatching; DW - dry weight; EA - eicosanoic acid, 20:1n-9; EFA - essential fatty acids; EPA - eicosapentaenoic acid 20:5n-3; ES - Easy Selco© treatment; ES/2 Easy Selco©- diluted by a half with olive oil treatment; FA - fatty acids; HUFA - highly unsaturated fatty acids; I_{EM} - eye migration index; LA - linoleic acid, 18:2n-6; LAP - leucine-alanine peptidase; LNA - linolenic acid, 18:3n-3; MG - Multigain© treatment; MUFA - monounsaturated fatty acids; OA - oleic acid, 18:1n-9; PCNA - proliferating cell nuclear antigen; PUFA - polyunsaturated fatty acids; RP - Red Pepper© treatment; SD – standard deviation; S.E.M. – standard error of the mean; SFA - saturated fatty acids; SGR - specific growth rate; SL - standard length; TFA - total fatty acids; VA - vitamin A

1. Introduction

A current major bottleneck in fish farming is the natural deficiency in essential fatty acids (EFA) of live prey, such as rotifers and *Artemia* nauplii, commonly used in marine fish hatcheries to feed larvae (Conceição et al., 2010). Enrichments have to be performed to attempt to provide live prey with the adequate nutritional value for the larvae (McEvoy et al., 1998). Many commercial enrichment products are formulated to contain high amounts of essential nutrients. However, among all these products there are large differences in their physical forms (emulsions, pastes, and spray-dried powders), ingredients (fish oils, vegetal oils, single-cell algal and fungal heterotrophic or phototrophic organisms, e.g. *Cryptocodinium cohnii* or *Schizochytrium* sp.) and composition of their primary nutrients, such as lipids, fatty acids, amino acids, minerals and vitamins. Although these enriching products are currently used in most commercial hatcheries to improve the nutritional value of live prey for feeding marine fish larvae, no comparative study has been conducted up to now

to determine whether these different commercial enriching products significantly affect larval performance and quality.

Senegalese sole (*Solea senegalensis* Kaup 1858) is a high-value flatfish, commonly reared in intensive aquaculture production systems in Spain and Portugal. Although enriched *Artemia* is commonly used in Senegalese sole larval rearing, high incidence of skeletal deformities and pigmentary disorders during larval stages were noticed (Soares et al., 2001; Gavaia et al., 2002; Fernández et al., 2009). These alterations to their normal appearance are a cause of reduced commercial value and limit the development of the fish production sector for this species.

Among other factors, nutritional imbalances are known to play a key role in morphogenesis and skeletogenesis at early stages and several dietary components have been identified that affect correct larval development (Lall and Lewis-McCrea, 2007; Cahu et al., 2003). Among them, dietary lipids have been shown to be particularly important for early development of marine finfish larvae (Sargent et al., 2002), because they represent the main energy source for larvae and a source of highly unsaturated fatty acids (HUFA) and EFA needed for the new cellular structures and required for normal larval growth, morphogenesis and bone formation (Izquierdo et al., 2000; Cahu et al., 2003; Lall and Lewis-McCrea, 2007). Moreover, the ingestion of HUFA from the diet is the only way for marine fish to get EFA from the n-3 series, such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), as well as from the n-6 series, such as arachidonic acid (ARA, 20:4n-6), since they are unable to synthesize these fatty acids from their precursors 18:3n-3 and 18:2n-6 (Sargent et al., 2002). Previous studies have highlighted the negative effect on flatfish larval growth performance, metamorphosis and survival (Tzoumas, 1988; Izquierdo et al., 1992; Dickey-Collas and Geffen, 1992; Morais et al., 2004; Villalta et al., 2005a), skeletal disorders (Dâmaso-Rodrigues et al., 2010) and in the incidence of pigmentary disorders (Estevéz and Kanazawa, 1995; Reitan et al., 1994; Villalta et al., 2005b; Vizcaíno-Ochoa et al., 2010), when these EFA are not provided in sufficient amount or in adequate form in the diet. Besides, previous studies on lipid nutrition of Senegalese sole larvae have

shown that this species has very specific requirements regarding EFA compared to other marine fish species (Morais et al., 2004 and Villalta et al., 2005a). Among all enriching products commercially available, it is unknown which is the most appropriate for Senegalese sole larval rearing.

The objective of this study was to compare the effect of six different enriching products for live prey, commonly used in Senegalese sole hatcheries, with graded levels of n-3 polyunsaturated fatty acids (PUFA) and graded DHA/EPA ratios on larval performance and skeletogenesis. Particular focus was made on the differential dietary content of two types of nutrients important in morphogenesis: fatty acids, because they are the main source of energy during the larval period, and vitamin A (VA) which plays an important role at early stages of larval development (Cahu et al., 2003; Fernandez and Gisbert, 2011). Further, because of the strong interactions existing between EFA (e.g. the competitiveness for the biosynthesis and molecular signaling pathways) this study set the focus not only on the effect of each nutrient in particular on larval performance, but in a more global and integrative approach considering the whole diet and its entire fatty acid profile. The results of this work provide practical guidelines to the industrial sector to improve both the current knowledge on Senegalese sole larval feedings, and the production of high quality animals.

2. Material and methods

2. 1. Larval rearing and feeding protocol

Two-day-old Senegalese sole larvae were obtained from Stolt Sea Farm SA (Carnota, A Coruña, Spain) and acclimated at the IRTA-SCR facilities. Larvae were reared in 18 cylindrical tanks of 60 l (initial density: 80 larvae·l⁻¹) connected to a water recirculation unit IRTAmarTM. Water conditions were as follows: temperature 20.0 ± 2.8 °C, salinity 35‰, pH 8.0 ± 0.2, dissolved oxygen 7.5 ± 1.3 ppm and 50% daily water renewal in the recirculation system with gentle aeration in each tank. Photoperiod was 16L: 8D, and light intensity was

500 lx at the water surface. Larvae were fed twice a day, from 2 dph to 10 dph, with rotifers (*Brachionus plicatilis*) enriched with microalgae (*Tetraselmis suecica*), at a density of 10 rotifers ml⁻¹ from 2 to 7 dph and of 5 rotifers ml⁻¹ from 8 to 10 dph. Enriched *Artemia* metanauplii were supplied to larvae from 8 to 38 dph twice a day, at increasing density from 0.5 to 12 metanauplii ml⁻¹, adjusted based upon the increase of weight of the larvae and to the daily food ration calculated as described by Cañavate et al. (2006).

2.2. Experimental diets and live prey enrichment

Several commercially available enriching products were used to enrich *Artemia*: Easy Selco© (INVE, Belgium, fish oil based-emulsion), Red Pepper© (Bernaqua, Belgium, encapsulated fish oil-based emulsion), Aquagrow Gold© (Advanced BioNutrition, ABN, USA, *Schizochytrium* sp. based spray dried product), Aquagrow DHA© (ABN, USA, *Cryptocodinium cohnii*-based spray dried product) and Multigain© (BioMar, Denmark, single cell marine organism-based spray dried product). Currently, Aquagrow Gold© and Aquagrow DHA© products are not manufactured anymore by ABN, but the composition of *Schizochytrium* sp. based spray dried Algamac 3050© (Bio-marine Inc., Aquafauna, USA) is the closest to the one of Aquagrow Gold©. It is worth noting that the formulation and biochemical composition of each commercial emulsion did not change from one batch to another, as should be expected, in order to maintain quality and the consumer's trust. *Artemia* was enriched with these products in order to obtain five dietary treatments with graded levels of n-3 PUFA and graded DHA/EPA ratios: Easy Selco© (ES), Red Pepper© (RP), Multigain© (MG), Aquagrow Gold© (AGG) and Aquagrow DHA© (AGD). Aquagrow Gold© and Aquagrow DHA© were diluted by a third with olive oil in order to adjust the increasing gradient of n-3 PUFA in enriched *Artemia*. Olive oil contains around 80% of oleic acid, so it is an appropriate solvent to dilute PUFA in saturated fatty acids (SFA). Moreover, a sixth dietary treatment containing a level of n-3 PUFA and a DHA/EPA ratio significantly was designed by enriching *Artemia* with Easy Selco© diluted by a half with olive oil and used as a negative control. One-day-old *Artemia* nauplii (EG strain, INVE) were enriched in

100 l containers at 300 nauplii ml⁻¹ for 16h at 28 °C with 0.6 g l⁻¹ of ES, AGG or AGD, 1.5 g l⁻¹ of RP and 0.5 g l⁻¹ of MG, following manufacturer's instructions. The biochemical analysis of lipids and fatty acid composition of enriched *Artemia* is shown in Table 1. After enrichment, *Artemia* metanauplii were washed with UV-treated, filtered seawater and freshwater to reduce the bacterial load and remove residues of the enrichment emulsions, and kept at 4 °C in UV-treated, filtered seawater with aeration until administered to larvae. From 20 dph onwards, when larvae began to settle to the bottom of the tank, enriched *Artemia* were supplied frozen to the larvae. The effect of the different dietary treatments on Senegalese sole skeletogenesis was only evaluated during the *Artemia* feeding period to avoid introducing variability due to the different pattern of fatty acid accumulation between both types of live preys (rotifers and *Artemia*) (Giménez et al., 2006).

2. 3. Lipid, fatty acid and retinoid analysis

Enriched live prey and larvae were sampled at 2 dph (endogenous feeding period before mouth opening), 8 dph (end of the rotifer feeding period and start of the *Artemia* feeding phase) and 38 dph, then washed with distilled water to remove salt and bacteria, and frozen at -80 °C until analysis. Total lipids were extracted in chloroform: methanol (2:1, v:v) using the method of Folch et al. (1957) and quantified gravimetrically after evaporation of the solvent under a nitrogen flow followed by vacuum desiccation overnight. Total lipids were stored in chloroform: methanol (2:1, 20mg ml⁻¹) containing 0.01% butylated hydroxytoluene (BHT) at -20 °C prior to analysis.

Acid catalyzed transmethylation was carried out using the method of Christie (1982). Methyl esters were extracted twice using isohexane: diethyl ether (1:1, v:v), purified on TLC plates (Silica gel 60, VWR, Lutterworth, UK) and analyzed by gas-liquid chromatography on a Thermo ElectronTraceGC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 m x 0,25 mm id; SGE, UK), using a two-stage thermal gradient from 50 °C (injection temperature) to 150 °C after ramping at 40 °C min⁻¹ and holding at 250 °C after ramping at 2 °C min⁻¹, helium (1.2 ml min⁻¹ constant flow rate) as the carrier gas and on-column injection

and flame ionization detection at 250 °C. Peaks of each fatty acid were identified by comparison with known standards (Supelco Inc., Spain) and a well characterized fish oil, and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrom-card for Windows (TraceGC, Thermo Finnigan, Italy). Results of fatty acid content are expressed as a percentage of total fatty acids (TFA).

Retinoids in enriching emulsions and live prey were analyzed by HPLC using a modification of the method proposed by Takeuchi et al. (1998). Extracted lipids were evaporated and redissolved on methanol:acetone (1:1 v:v) prior to their HPLC analysis. The HPLC system (Thermo Separation Products, San Jose, CA, USA) was equipped with a Lichrospher C-18 reverse phase column (Merck, Darmstadt, Germany) and a UV-visible detector set at a wavelength of 325 nm. The concentration of each retinoid was calculated from the 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).

2.4. Larval performance

2.4.1. Growth and survival

Standard length (SL) and dry weight (DW) of Senegalese sole larvae were measured at 2, 4, 8, 15, 22, 31 and 38 dph. Thirty larvae from each tank were randomly sampled and euthanized with an overdose of tricaine methane sulphonate (MS-222, Sigma). SL was measured with a digital camera connected to a microscope (Nikon SMZ 800) and an image analysis system (AnalySIS, Soft Imaging Systems, GmbH). DW determination was performed by rinsing larvae with distilled water to remove salt and then drying them at 60 °C for 24 h. Samples were weighed with an analytic microbalance (Sartorius BP211D). Specific growth rate (SGR, in % d⁻¹) was calculated as $(\ln DW_f - \ln DW_i) / (t_f - t_i) * 100$; where DW_f, DW_i, t_f and t_i represented final and initial DW and time of the experiment, respectively. Final survival was evaluated by counting the animals surviving at the end of the experiment and

calculated according to Buckley et al. (1984), which considers the number of sampled individuals during the experiment.

2.4.2. Metamorphosis

Eye migration in Senegalese sole larvae, used as a measure of the progress of the metamorphosis process, was assessed for 30 individuals per tank at 10, 15, 22, 31 and 38 dph, according to Villalta et al. (2005b). Data are presented as the relative amount of larvae in each stage of development at the same age. Eye migration index ($I_{EM} = \sum (\% \text{fish in each stage} * \text{stage}) / 100$) was calculated according to Solbakken et al. (1999).

2.4.3. Organization and functionality of the digestive tract

2.4.3.1. Digestive enzyme activity

The specific enzyme activity of one intestinal cytosolic enzyme (leucine-alanine peptidase), two intestinal brush border enzymes (alkaline phosphatase and N-aminopeptidase) and three pancreatic enzymes (amylase, lipase and trypsin) were used to assess the degree of development and maturation in the digestive system of Senegalese sole larvae fed the six dietary treatments. Enzyme activity was measured at 38 dph on 30 individuals per tank and each sample was assayed in triplicate.

Sampled fish were washed with distilled water and stored at -80 °C until enzyme activity analysis. Fish heads at 38dph were removed to reduce the protein content. Samples were homogenized (Ultra-Turrax D25 basic, IKA®-Werke) in thirty volumes (v/w) of Tris-Mannitol (50 mM, HCl 2 mM, pH = 7.5) and CaCl₂ (0.1M) for 5 minutes and subjected to sonication (Vibra-Cell®, Sonics) for 1.5 minutes, maintaining them in ice to restrict enzymatic activity. An aliquot was taken for pancreatic enzyme quantification and stored at -80 °C. Intestinal brush border membranes for determination of intestinal enzymes were purified according to Crane et al. (1979). The remaining fraction was centrifuged at 9,000 x g (10 min) at 4 °C, and then the supernatant was collected and centrifuged at 14,000 x g (30

min) at 4 °C. The pellet containing the intestinal brush border was recovered, diluted in a KCl, DTT, Hepes buffer (pH = 7.5) and kept at -80 °C.

Trypsin (E.C. 3.4.21.4) activity was assayed at 25 °C and 407 nm, using BAPNA (N- α -benzoyl-DL-arginine *p*-nitroanilide, Sigma) as substrate (Holm et al., 1988). Amylase (E.C. 3.2.1.1) activity was measured at 580 nm, 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 °C and 407 nm, using PNPP (4-nitrophenyl phosphate, Sigma) as substrate (Bessey et al., 1946). N-aminopeptidase (E.C.3.4.11.2) activity was determined at 25 °C and 410 nm, according to Maroux et al. (1973) using sodium phosphate buffer 80 mM (pH = 7.0) and L-leucine *p*-nitroanilide as substrate (Sigma, in 0.1 mM DMSO). Leucine-Alanine Peptidase (E.C.3.4.11) activity quantified at 37 °C and 530 nm, using leucine-alanine (Sigma) as substrate (Maroux et al., 1973). Lipase (E.C.3.1.1) activity was measured at 30 °C and 405 nm, using *p*-nitrophenyl myristate (Sigma) as substrate (Iijima et al., 1998). Enzymatic activities were read using a spectrophotometer (Synergy HT, Bio-Tech) and expressed as specific enzyme activity, in units per milligram of protein (U mg⁻¹ protein). Soluble protein of crude enzyme extracts was quantified by Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

4.3.2. Enterocyte height and cell proliferation capacity in the intestinal mucosa

For morphometric and cell proliferation capacity studies, ten larvae from each experimental tank at 38 dph were randomly sampled, euthanized with an overdose of MS-222 and fixed in 4 % formalin buffered to pH = 7.0 with 0.1 M phosphate buffer during 24 h. Samples were routinely dehydrated, embedded in paraffin wax, sectioned using a rotary microtome and placed on microscope slides precoated with poly-L-Lysine (Sigma-Aldrich).

The height of the mucosal epithelial cells, defined as the distance between the basal lamina and the extremity of the brush border was measured for 10 enterocytes located in the mucosal folds of the intestine in three larvae per treatment.

To detect the proliferation capacity of the intestinal mucosa, sections were stained immunohistochemically using a monoclonal antibody (PC10, Santa Cruz Biotechnology Inc., Heidelberg, Germany) against proliferating cell nuclear antigen (anti-PCNA), following the method of Piñuela et al. (2004). Mounted tissues were dewaxed, hydrated in ddH₂O and then placed in 3 % triton in phosphate buffer saline (PBS-T). Endogenous peroxidase activity was blocked by incubation in a solution of 3 % H₂O₂ in methanol. Background was reduced by incubation in a solution of 3 % BSA in triton/PBS with 1 % dimethyl sulfoxide (DMSO) for 30 min at RT. Sections were incubated with anti-PCNA IgG_{2a} mouse monoclonal antibody (PC10, Santa Cruz Biotechnology) 1:500 overnight in a humid chamber at RT. Samples were then incubated with a secondary antibody (biotinylated anti-mouse IgG from horse, Vector antibodies) for 1 h at RT. Before rinsing in PBS-T during 10 minutes, the slides were incubated with horseradish peroxidase-conjugated avidin-biotin complex (ABC complex; Vectastain, Vector Laboratories Inc., USA) for 1 h at RT. The colorimetric reaction was developed by exposing the slides to 0.04 % (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.015 % H₂O₂ in Trizma base (TB, 100 mM, pH = 7.6) for about 10 min. Sections were then dehydrated, mounted and counterstained with eosin.

Intestinal cell proliferation was calculated following the quantification criteria described by Sanden et al. (2005). For that purpose, five intestinal folds were examined randomly in two sections per fish, in three larvae from each dietary group. For each intestinal fold measured, the thickness of 10 cells was selected above the basal area and positive PCNA and negative PCNA cells were counted. Cell proliferation was expressed as the percentage of PCNA positive cells/non-PCNA cells.

Measurement of enterocyte height and proliferation capacity was realized by using a digital camera connected to a microscope (Leica DM 2000) and an image analysis system (ImageJ, National Institute of Health, Bethesda, USA).

2.5. Ossification degree and skeletal deformities

To evaluate the impact of different dietary treatments on the degree of ossification of the larval skeleton, identify different typologies of skeletal deformities, and quantify their incidence in larvae, 40 larvae per tank were randomly sampled at the end of the experiment, fixed in 4% formalin buffered to pH = 7.0 with 0.1 M phosphate buffer and stored until double staining. Animals were stained with alcian blue and alizarin red to detect cartilaginous and bony tissues, respectively (Darias et al., 2010a). Quantification of the degree of ossification was performed in 38 dph Senegalese sole larvae according to Darias et al. (2010a). Briefly, stained fish were placed on their blind (left) side and scanned using a digital scanner (HP Scanjet G3010) to create 1200 dpi images for subsequent analysis. A computerized image analysis package (IMAQ Vision Builder®, National Instruments) was used to determine the number of red pixels of each stained larvae which corresponded to the ossified structures.

Skeletal structures were identified and named according to Wagemans and Vandewalle (2001) and Gavaia et al. (2002). The incidence of skeletal abnormalities was determined in the cranium, vertebral column and caudal fin complex. Special attention was given to vertebral deformities, which were divided in two categories: severe (fusion, compression, deformed vertebral centrums, torsion and scoliosis) and light (haemal spines and neural spines) deformities and caudal fin complex deformities (modified haemal and neural spines). Fusion of the hypurals, parahypural and/or epural was not considered as a skeletal deformity, but rather the normal development of the caudal complex occurring in juveniles (Barrington, 1937).

2.6. Statistics

Results were expressed as means $\pm$ SD ($n = 3$). All data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test). A nested-ANOVA was performed with "treatment" as the fixed-effects factor nested within the random-effects factor "tank" (triplicates per treatment for final SL, final DW and ossification variables measured on 30 - 40 larvae per replica) or "larvae" (triplicates per treatment for enterocyte height or PCNA variables, measured on 10 cells or 10 intestinal folds per larvae) (Zar, 1999). Post-hoc Bonferroni's test was performed when significant differences were found at $P < 0.05$. However, for variables such as FA composition, SGR, survival,

lem, enzymatic activities and incidence of skeletal deformities, measurements were performed on a pool of individuals per replicate but with just one value of these variables per replicate, a nested ANOVA cannot be carried out. For these variables, a one-way ANOVA was performed among treatments and the post-hoc Tukey's test was performed when significant differences were found ($P < 0.05$). A correlation test of Pearson was performed between alkaline phosphatase specific activity and cell proliferation data. All the statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA).

3. Results

3.1. Lipid, fatty acid composition and retinoid content of live prey and fish larvae

3.1.1. Live prey

No significant differences were detected in the total lipid and total fatty acid contents among the *Artemia* nauplii enriched with the different commercial enrichments (Table 1, $P < 0.05$). *Artemia* enriched with ES/2 exhibited significantly higher total monounsaturated fatty acids (MUFA) levels than *Artemia* groups enriched with AGD, RP or MG. Significant differences were found in the proportions of 18:1n-9 (oleic acid, OA) and 20:1n-9 (eicosanoic acid, EA) between dietary treatments. *Artemia* enriched with ES/2 presented significantly higher amounts of OA than those enriched with RP or MG. *Artemia* enriched with ES showed higher levels of EA than groups enriched with AGG, RP or MG. In relation to n-6 PUFA, significantly different concentrations among dietary treatments were noticed for 18:2n-6 (linoleic acid, LA). Incorporation of LA was higher in the ES group and lower in the AGG group. For 20:4n-6 (ARA), the higher incorporation was observed in the RP group and the lower in the ES, ES/2 and AGD groups; with AGG having intermediate values. Concerning the n-3 PUFA, only EPA and DHA contents were significantly different between enrichment treatments. In the case of EPA, *Artemia* enriched with ES showed the highest content, those

enriched with RP, MG, AGD and AGG intermediate values and ES/2 the lowest. Regarding DHA, *Artemia* enriched with MG exhibited the highest content and ES/2 enrichment the lowest. The rest of the treatment groups presented intermediate DHA values. In terms of total PUFA content, *Artemia* enriched with ES showed an intermediate value between the lowest (the ES/2 group) and the highest (the MG, RP, AGD, and AGG groups). *Artemia* enriched with MG, AGD, RP and AGG presented significantly higher amounts of total n-3 PUFA than ES/2 but total n-6 PUFA were similar in all dietary groups, and the (n-3)/(n-6) ratio was not significantly different among the nauplii. *Artemia* enriched with MG, AGG and AGD showed the highest DHA/EPA ratios followed by the RP group, while the lowest DHA/EPA ratios were observed in *Artemia* enriched with ES and ES/2. *Artemia* enriched with ES/2 presented a significantly higher ARA/DHA ratio as compared with those enriched with RP, ES, AGG, MG and AGD. *Artemia* enriched with RP, AGG and MG showed significantly higher ARA/EPA ratios than those enriched with ES/2, ES and AGD. *Artemia* enriched with ES showed the highest retinoid content followed by ES/2 and the rest of the dietary treatments.

3.1.2. Fish larvae

In order of relative abundance, 2 dph-old larvae contained particularly high proportions of DHA, 16:0, OA, 18:0, EPA and 22:5n-3 (docosapentaenoic acid, DPA) (Table 2), resulting in high (n-3)/(n-6) and DHA/EPA ratios. At the end of the rotifer feeding period, 8dph-old larvae contained equivalent levels of total lipids, total FA, total SFA and PUFA and equivalent EFA ratios as 2dph-old larvae, but lower levels of 14:0, 16:0, 16:1n-7 and higher amounts of OA, EA, LA, LNA and 20:4n-3. At 38 dph, the fatty acid composition of the larvae reflected the composition of the diets (Tables 1 and 2). Significantly higher levels of MUFA, particularly OA and EA were identified in larvae fed *Artemia* enriched with ES/2. Larvae from the RP and MG dietary treatments contained significantly more n-6 PUFA than the rest of the groups, principally LA, 18:3n-6 (Gamma Linoleic Acid, GLA) and ARA for larvae from the RP group, and GLA, ARA and 22:5n-6 for larvae of the MG group. Larvae from the ES/2 and ES

groups also contained high LA levels. Larvae from the AGD, ES/2 and ES groups exhibited particularly significant low levels of ARA. Larvae from the MG group contained significantly higher amounts of total n-3 PUFA and DHA than the other groups; whereas EPA and DPA were significantly more abundant in larvae fed *Artemia* enriched with ES. Reflecting the *Artemia* profile, total PUFA amounts were higher in larvae from the MG group; intermediate in larvae from RP and AGG groups; and lower in the AGD, ES and ES/2 groups. The higher (n-3)/(n-6) PUFA ratio was noted in larvae fed *Artemia* enriched with AGD, followed by ES, AGG, MG, ES/2 and RP. The higher DHA/EPA ratio was obtained from larvae of the MG group (6.2 ± 0.5), and those from the ES and ES/2 groups the lowest, with the AGD, AGG and RP larvae showing intermediate values. Values of ARA/DHA and ARA/EPA ratios followed the same decreasing trend among larvae from the RP, AGG, MG, ES/2, ES and AGD groups. Larvae fed *Artemia* enriched with MG and RP contained significantly higher amounts of retinoid, while the ES group had lower values, and ES/2, AGD and AGG intermediate values in retinoid content.

3.2. Larval performance

3.2.1 Larval growth and survival

Senegalese sole larvae from the six dietary treatments did not show significant differences in SL and DW until 22 dph (metamorphosis period) (Table 3), the age at which larvae fed AGG-enriched *Artemia* appeared to be significantly longer in SL (except from larvae fed ES/2-enriched *Artemia*) and heavier in DW than those of the other groups (data not shown, $P = 0.007$ and $P = 0.004$, respectively). At the end of the experiment, larvae fed AGG-enriched *Artemia* presented significantly higher SL ($F_{10, 578} = 6.90$; $P \leq 0.001$), DW ($F_{10, 134} = 6.79$; $P \leq 0.001$) and SGR values ($P = 0.022$) during the whole larval rearing period than larvae fed ES-enriched *Artemia*, with the other groups showing intermediate values (Table 3). The SGR of larvae fed AGG-enriched *Artemia* was significantly higher than the others particularly during pre-metamorphic and metamorphic stages (2 - 22dph, $P = 0.006$).

Senegalese sole larvae final survival was not affected by any diet (Table 3, $P = 0.328$), reaching a mean of $95.7 \pm 1.0 \%$.

3.2.2. Metamorphosis

The process of eye migration in Senegalese sole larvae was not significantly affected by any dietary treatment (Table 4). During the metamorphosis process, no differences in I_{EM} were detected between the six experimental groups ($P > 0.05$). However, only in the AGG group was the eye migration process completed (stage 5) at the end of the experiment. No malpigmented fish were recorded at the end of the experiment in any of the six groups.

3.2.3. Organization and functionality of the digestive tract

3.2.3.1. Digestive enzyme activity

The specific activity of selected intestinal and pancreatic enzymes from fish fed the six different diets at 38 dph are represented in Fig. 1. Intestinal maturation in Senegalese sole larvae was measured as the ratio of specific activities of brush border membrane enzymes/cytosolic enzymes: alkaline phosphatase/leucine-alanine peptidase and N-aminopeptidase/leucine-alanine peptidase. These ratios were both significantly higher ($P = 0.001$ and $P = 0.006$) in larvae fed AGG-enriched *Artemia* (0.96 ± 0.04 and 17.01 ± 1.90 respectively), intermediate in larvae fed *Artemia* enriched with AGD (0.50 ± 0.12 and 10.83 ± 0.96) and lower in larvae fed MG (0.49 ± 0.15 and 8.51 ± 3.32), RP (0.34 ± 0.11 and 8.52 ± 3.46), ES (0.25 ± 0.01 and 8.09 ± 1.66) and ES/2-enriched *Artemia* (0.09 ± 0.01 and 6.04 ± 3.78). At 38dph, amylase specific activity was significantly ($P = 0.005$) higher in larvae from the ES group (2.11 ± 0.03 U mg protein⁻¹) than in those from RP, ES/2 and AGG groups (1.42 ± 0.15 , 1.34 ± 0.36 and 1.04 ± 0.01 U mg protein⁻¹, respectively) ($P < 0.001$), being intermediate in larvae from the AGD and MG groups (1.62 ± 0.32 and 1.50 ± 0.36 U mg protein⁻¹, respectively). Trypsin specific activity in 38 dph-larvae fed *Artemia* enriched with ES, AGG or AGD (13.42 ± 1.56 , 12.92 ± 3.94 and 11.94 ± 3.47 U mg protein⁻¹, respectively) was significantly ($P < 0.001$) higher than that of larvae from the RP, MG and ES/2 groups

(4.64 ± 0.63 , 4.26 ± 1.04 and 3.87 ± 0.89 U mg protein⁻¹, respectively). Lipase specific activity at 38 dph was not affected by the dietary treatments ($P = 0.816$).

3.2.3.2. Enterocyte height and cell proliferation capacity in the intestinal mucosa

The mean heights of enterocytes from fish fed the six different diets are represented in Fig. 2a. There were significant differences in enterocyte heights between dietary treatments ($F_{10, 162} = 6.24$; $P \leq 0.001$) being higher in larvae fed *Artemia* enriched with ES, lower in larvae fed *Artemia* enriched with RP, AGG and MG and intermediate in larvae fed ES/2- and AGD-enriched *Artemia*. The rate of cell proliferation was significantly different among the dietary groups, being higher in those exposed to AGG, AGD and MG with respect to those fed with ES-, ES/2- and RP-enriched *Artemia* (Fig. 2b, $F_{10, 162} = 3.98$; $P \leq 0.001$). Alkaline phosphatase specific activity was significantly and positively correlated with enterocyte proliferation ($R^2 = 0.83$, $P = 0.04$).

3.3. Ossification degree and skeletal deformities

At 38 dph, the ossification degree in Senegalese sole larvae was significantly affected by dietary treatments (Fig. 3, $F_{10, 213} = 4.61$; $P \leq 0.001$). Larvae fed *Artemia* enriched with ES/2, AGG or AGD exhibited a significantly higher ossification degree than those from the ES group, with the larvae from the RP and MG groups showing intermediate ossification.

Dietary treatments did not affect the incidence of total skeletal deformities in Senegalese sole (Table 5). The frequency of deformed fish was similar between the six groups ($P = 0.668$), ranging from 70 to 80 %. No cranial deformities were observed in any larvae of the different dietary treatments. In all experimental groups, skeletal abnormalities affected mainly vertebral and caudal fin complex regions, particularly the prehaemal vertebral region (Fig. 4b), haemal vertebral region in the middle of the column (Fig. 4c, d) and the last two vertebrae before the urostile (Fig. 4e, f). The main vertebral column in Senegalese sole is composed of 45 vertebrae, divided in 8 prehaemal and 37 haemal vertebrae (including the urostile). No significant differences were detected in the mean

percentage of fish with 43 (13.6%), 44 (39.0%) or 45 (47.3%) vertebrae among the six experimental groups ($P > 0.05$). The frequency of skeletal abnormalities in the vertebral column (prehaemal and haemal regions) was similar among the six dietary treatments ($P = 0.751$). However, when examining each kind of vertebral deformity, the incidence of vertebral fusion was affected by the dietary treatment ($P = 0.042$), with larvae from the AGD group presenting double the number of individuals ($30.6 \pm 7.1\%$) with total or partial vertebral fusion as compared to those from the MG group ($15.7 \pm 2.4\%$). No significant differences were detected in the remaining severe deformities (compression, torsion, scoliosis, vertebral centrums) nor in the minor ones (haemal or neural spines) among the dietary groups ($P > 0.05$). Skeletal structures composing the caudal fin complex were also affected by dietary treatments, associated with a total or partial fusion of the last two vertebrae, modified neural and haemal spines presenting deformities or defects in ossification. The incidence of deformities in these structures significantly decreased ($P = 0.017$) from larvae fed *Artemia* enriched with AGG (55.9%) and ES/2 (54.2%) to larvae fed *Artemia* enriched with ES (40.8%), whereas those fed *Artemia* enriched with MG, RP and AGD showed intermediate values relating to the incidence of abnormal modified neural and haemal spines (49.3, 47.4 and 44.7%, respectively).

4. Discussion

4.1. Effect of the dietary treatments on the fatty acid profiles in live prey and on fish larvae

Enriched *Artemia* nauplii from the six dietary treatments were isolipidic and contained similar content of total fatty acids. The fatty acid profile, particularly the profile of total (n-3) and total (n-6) HUFA, EPA, DHA, ARA, DHA/EPA ratio and the retinoid content of enriched *Artemia* nauplii varied among dietary treatments, in accordance to the composition of commercial emulsions (data not shown). However, *Artemia* are not passive carriers of fatty acids, but have specific physiological needs that can alter metabolically the original

composition of the diet by retroconverting DHA into EPA, and by redistributing the incorporated fatty acids among lipid classes with high unpredictability (Navarro et al., 1999). The metabolism inherent to *Artemia* nauplii cause them to accumulate EPA at higher levels than DHA, and consequently decreased the DHA/EPA ratio from the enriching product, as it had been previously described by Izquierdo (1988) and McEvoy et al. (1995).

In addition, the diverse forms in which enriching products are commercialized could be influencing the enrichment efficiency of *Artemia*. Indeed, during the enriching period, the digestive tract of *Artemia* nauplii were differentiating (Navarro et al., 1999) and the differences in the type of enriching compounds could likely be influencing their ontogenesis and, hence, their absorption efficiency and the nutritional value of this type of live prey. All the above suggests that a combination of the initial lipid composition of the commercial enrichment, its physical form of presentation, and the lipid metabolic pathways of *Artemia* could be responsible for the final lipid profile of the *Artemia*.

The fatty acid profile of Senegalese sole larvae at 38 dph closely reflected the composition of their diet, as observed in striped trumpeter *Latris lineata* (Brandsen et al., 2004), turbot *Scophthalmus maximus* (Estevez et al., 1999), seabream *Sparus aurata* (Koven et al., 2001), white bass *Morone chrysops* (Harel et al., 2000) or Senegalese sole larvae (Villalta et al., 2005a, b), among others. In agreement to Villalta et al. (2005b), diets with medium and high amounts of DHA (MG, AGD, RP and AGG) contained low levels of OA and MUFA, which were normally used as energy for larval growth and development, and this trend was reflected in fish larvae fed these diets. The DHA content in larvae reflected the DHA amounts of the diet. This level of DHA was maintained in larvae fed the MG treatment compared to 8 dph-old larvae, whereas DHA content decreased from 1.3 to 3.5-fold in larvae from the others. Among all dietary treatments, ES-enriched *Artemia* presented a significantly higher EPA content than the other enriched *Artemia* which might have induced the particular fatty acid profile observed in larvae from this treatment, with high retention of EPA and DPA in larval tissues, whereas larvae from the other treatments seemed to have used these fatty acids during their development. The provision of dietary

ARA in RP- and MG-enriched *Artemia* allowed the larvae to maintain the initial ARA content at 38 dph, but larvae fed ES-, ES/2-, AGG and AGD diets significantly depleted their ARA stock during development, with larvae from the AGD group presenting the lowest ARA content at the end of the experiment. Curiously, the retinoid content in larvae did not follow the profile found in the enriched *Artemia*. Amounts of VA in *Artemia* enriched with ES was between 50 to 205-times higher than in *Artemia* enriched with RP, AGD, AGG or MG, and twice as high as the ES/2-enriched *Artemia*, reflecting the composition of commercial emulsions. However, this trend was completely normalized in larvae, probably revealing a capacity of elimination and detoxification of the dietary VA surplus (Fernández and Gisbert, 2010).

4.2. Effect of the dietary treatments on the larval developmental performance

At 38 dph, Senegalese sole larvae fed AGG-enriched *Artemia* presented a significantly improved larval performance and development than fish fed ES-enriched *Artemia*, whereas larvae from the ES/2, RP, AGD and MG groups showed an intermediate profile. In this sense, larvae from the AGG group grew faster than the others, particularly during the pre-metamorphic phase, showing higher final DW and SL values than larvae fed ES-enriched *Artemia*. Larvae fed AGD, MG, ES/2 and RP showed intermediate growth values, but larvae from the AGD and MG groups tended to grow slightly faster during the post-metamorphic phase than larvae from ES/2 and RP groups. However, in this study, 38 dph-Senegalese sole larvae were smaller than those of Villalta et al. (2005a, b) and Morais et al. (2004) at the same developmental stage, far from the suitable window of 5 – 10 mg required to start weaning (Conceição et al., 2007). Such variations might be related to differences in the initial larval size among trials and rearing conditions.

Senegalese sole larvae were able to complete metamorphosis in all dietary treatments, but no significant effect of the different dietary treatments was observed in the process of eye migration. However, larvae from AGG, AGD and MG groups showed a trend toward a faster metamorphosis compared to larvae from the ES, ES/2 and RP groups.

Larvae that metamorphose faster seemed to grow faster too, as the relative profiles for the metamorphosis process among dietary treatments were in agreement with the trends for larval growth, as observed in Atlantic halibut *Hippoglossus hippoglossus* (Naess and Lie, 1998) and other flatfishes (Geffen et al., 2007).

Dietary treatments did not affect significantly the survival rate of Senegalese sole larvae. Survival at 38dph was higher than that reported in other studies where it ranged between 15 - 90 % (Dinis, 1992; Dinis et al., 1999; Cañavate and Fernández-Díaz, 1999; Morais et al., 2004; Villalta et al., 2005a, b; Fernández et al., 2008; Dâmaso-Rodríguez et al., 2010), but equivalent to the survival rate in Senegalese sole larvae fed *Artemia* HUFA-deficient during their post-metamorphic stage (> 90 %, Morais et al., 2005a, b).

The activity of the pancreatic and intestinal enzymes provides a reliable marker for assessing the development of the digestive function in fish larvae (Zambonino-Infante et al., 2008). During development, the specific activity of the brush border enzymes (alkaline phosphatase and N-aminopeptidase) increased with a parallel decrease in the activity of leucine-alanine peptidase (Ribeiro et al., 1999), increasing the ratios between the brush border and the cytosolic enzymes, which provide an indication of the degree of maturation of the digestive system. Significantly higher values for both ratios observed in larvae fed AGG-enriched *Artemia* than in larvae fed other diets indicated a more physiologically advanced digestive system of larvae fed the AGG diet than those from the other dietary treatments, which coincided with the results of growth and metamorphosis for this treatment. Larvae from the AGD group showed intermediate values of the N-aminopeptidase/leu-ala peptidase ratio in concordance with their intermediate growth profiles. Amylase and trypsin activities are markers of the maturation of the digestive tract (Zambonino Infante and Cahu, 2007; Ribeiro et al. 1999). These pancreatic enzymes play an extremely important role during early stages and their specific activities normally decrease during larval development with the maturation of the stomach and the intestine. At the end of the experiment, larvae fed AGG-enriched *Artemia* presented the lowest amylase specific activity values, whereas larvae fed ES-enriched *Artemia* showed the highest, suggesting a more advanced

maturation of the digestive system in larvae from the AGG group than those from the ES group. Larvae fed ES/2, RP, AGD or MG diets showed low or intermediate amylase specific activity values. Lipase specific activity was not significantly different among larvae fed *Artemia* enriched with the different products. Lipase secretion is regulated by lipid concentration in the diet (Martinez et al., 1999) and this observation probably reflected the fact that the treatments were isolipidic (Morais et al., 2006).

Parallel to these physiological changes in enzyme specific activities in Senegalese sole larvae during development, the intestinal mucosa matured as evidenced by structural changes. Enterocyte proliferation was significantly higher in 38 dph-larvae fed AGG-, AGD-, MG-enriched *Artemia* than in those fed ES-, ES/2- and RP-enriched *Artemia*. Apart from that, enterocyte height was lower in larvae fed AGG-, RP- and MG-enriched *Artemia* as compared to larvae from the ES fed group, and intermediate in larvae from the ES/2 and AGD fed groups. These results are inversely correlated to results of cell proliferation, since cells allocating energy in intensive mitotic divisions would spend less energy in cell growth (Lodish et al., 2000). Moreover, the significant and positive correlation between the specific activity of alkaline phosphatase and enterocyte proliferation indicated that both parameters are useful biomarkers providing complementary biochemical and histochemical information for the evaluation of gut maturation. These results supported the hypothesis of a more advanced degree of maturation of the digestive system for larvae from the AGG fed group, followed by those from the AGD and MG fed groups, afterwhich by those from the RP and ES/2 fed groups, and finally by larvae fed ES-enriched *Artemia*, which is in agreement with the results of growth and eye migration.

Dietary treatments also affected the degree of ossification of post-metamorphic Senegalese sole larvae. Larvae from the AGG group showed most of their skeleton ossified at 38 dph, whereas larvae from the ES dietary treatments were significantly less ossified, and larvae from the other groups showed intermediate ossification. Although there was no clear correlation with the trend observed in growth and intestinal maturation, it can be noticed that larvae fed ES-enriched *Artemia* showed the lowest growth, intestinal maturation

and ossification values; whereas larvae fed AGG-enriched *Artemia* showed the opposite pattern. A correlation between a delay in growth and ossification had been previously highlighted in Atlantic cod (Kjørsvik et al., 2009), gilthead sea bream (Fernández et al., 2008) and European sea bass (Darias et al., 2010b) larvae fed different diets. The former studies reported that animals with lower growth values were also delayed in digestive system maturation and skeletogenesis, resulting in the incidence of skeletal disorders.

However, in the current study, no correlation between the degree of ossification and the incidence of skeletal deformities could be established; although all experimental groups showed a large incidence of skeletal deformities (76 % in average), as previously observed in Senegalese sole larvae, particularly during early stages of development (Gavaia et al., 2002; Fernández et al., 2009; Engrola et al., 2009). No significant effect from the dietary treatment on the incidence of total abnormalities, nor malformations of the vertebral column were detected. Neither were there observed significant differences among treatments in types of skeletal deformities in the vertebral region, apart from the larvae fed AGD-enriched *Artemia* which were more affected by fusion of vertebral centrums than larvae fed MG-enriched *Artemia*. In the caudal fin complex, significant differences were detected, with larvae fed AGG- and ES/2-enriched *Artemia* presenting higher frequency of individuals with malformations of modified neural and haemal spines than larvae fed ES-enriched *Artemia*. However, these variations among treatments are too minor and therefore fail to indicate which dietary treatment is responsible for a higher frequency of skeletal deformities.

4.3. Effect of the dietary fatty acid profile on the larval performance in Senegalese sole larvae

The nutritional causes affecting larval developmental performance cannot be evaluated by taking into account the individual effect of each fatty acid alone due to the complexity of the interactions between the fatty acids and the nutrients composing the diet. Therefore it is necessary to examine the relative proportions of dietary fatty acids, especially the ratios between n-3 and n-6 HUFA and between EPA, DHA and ARA. Results of growth, eye

migration, intestinal maturation, and dietary amounts of the main essential nutrients were gathered in Fig. 5 in order to integrate and better visualize the differences in dietary fatty acid composition and larval performance between dietary treatments.

In this study, the AGG diet was associated with the best overall larval performance and quality among all dietary treatments tested. The fatty acid profile of the AGG diet presented intermediate values of total MUFA, ARA, EPA, DHA, total PUFA, DHA/EPA and (n-3)/(n-6) PUFA ratios (3.0:1 and 5.2:1, respectively). This subtle balance between all the components in the AGG diet certainly helps to explain the improved results observed in Senegalese sole larvae in the present study, since all the other diets that induced less efficient larval development had one or several compounds in a different proportion as compared to the composition of the AGG formulae.

The ES diet contained significantly higher EPA levels than AGG. This excessive dietary EPA accumulated in larval tissues in the form of EPA or DPA, probably because Senegalese sole larvae, as with many marine fish larvae, are unable to elongate and desaturate them into DHA at a significant rate (Morais et al., 2004). A surplus of EPA in larval tissues could have a detrimental effect on larval development. Previous studies (Leger et al., 1986; Izquierdo et al., 2000) estimated between 3 and 4 % TFA as the range of EPA levels, suitable for larval growth and survival of various marine organisms. Moreover, Villalta et al. (2008) proved that Senegalese sole larvae were able to grow and survive on *Artemia* nauplii with negligible EPA content (0.4 % of TFA) up to 40 dph, even showing a superior growth than when fed *Artemia* with 10.7 and 20.3 % EPA of TFA. Senegalese sole larvae have low requirements for EPA when DHA is present in the diet and increasing EPA amounts in the diet rather than enhance growth and development had a detrimental effect on morphogenesis, as demonstrated for the RP, AGD and MG diets.

The ES/2 diet was significantly poorer in DHA in comparison to the AGG diet, but both contained the same amounts of EPA; in spite of this, the ES/2 diet resulted in less developed larvae. Although previous studies have demonstrated that Senegalese sole larvae have low or negligible requirements for DHA, being able to survive, grow and

metamorphose on *Artemia* practically devoid of DHA, but containing other n-3 PUFA (Morais et al., 2004; Villalta et al., 2005a; Dâmaso-Rodriguez et al., 2010), the dietary DHA minimum requirement is an important nutritional parameter that should not be neglected even if it is “just a drop in the ocean” relative to the other nutritional factors affecting larval physiology. In this sense, an increase from 9.5 to 11.5-11.8 % TFA in the RP and AGD diets did not significantly improve larval development; and in the case of MG, DHA content was too excessive to be efficiently used by sole larvae and accumulated in larval tissue (Bogolino et al., 2011) which may have been detrimental for proper development (Roo et al., 2009). Such an increase in DHA was compensated by a diminution in MUFA, and particularly in OA; fatty acids that are more easily catabolized by fish larvae to produce energy for growth and development. The same trend was observed in the RP diet where higher (n-6) PUFA levels than in the AGG diet were offset by a lower MUFA content. As shown by Villalta et al. (2008), disrupting the balance between energy (saturated FA and MUFA) and essentiality (HUFA) had a detrimental effect on Senegalese sole larvae.

An optimal dietary DHA/EPA ratio of 2:1 for newly hatched larvae has been previously determined by Sargent et al. (1997) from the lipid composition of the yolk sac in marine fish eggs. According to these authors, a DHA/EPA ratio inferior or equal to 1 corresponds to a sub-optimal diet by providing to larvae insufficient amounts of DHA or an excess of EPA, and could be deleterious in larval fish feeds, as in the ES and ES/2 diets. The DHA/EPA ratio was largely reduced in ES, ES/2 and RP diets compared to the AGG diet. Morais et al. (2004) evaluated that Senegalese sole have a high dietary DHA requirement relative to EPA, with eggs of these species containing a DHA/EPA ratio (4.3:1) higher than that found in most marine species eggs (2:1 - 3:1); although no correlation was found between the prey's DHA/EPA ratio and Senegalese sole larval growth and survival. Brinkmeyer and Holt (1998) found that the optimal DHA/EPA ratio for red drum larvae was greater than 2.5:1, and further, a ratio of 3.78:1 in the diet produced larvae more resistant to stress. Other authors (Watanabe et al., 1989; Rodriguez et al., 1998; Harel et al., 2002) have demonstrated the wide superiority of DHA to EPA to improve growth and survival in other marine fish larvae,

such as red sea bream (DHA/EPA = 1.1:1), gilthead sea bream (DHA/EPA = 1.3:1), striped bass, European sea bass, Atlantic halibut (DHA/EPA = 2:1 - 2.5:1); and to ameliorate survival and vitality in blackspot sea bream *Pagellus bogaraveo* (DHA/EPA = 3.8:1). In this study, a DHA/EPA ratio of 3.0:1 provided the best results in terms of larval performance, whereas diets with diverging ratios were less efficient.

The decrease in n-3 PUFA content in the ES and ES/2 diets and the increase of n-6 PUFA content in the RP diet compared to the AGG diet also disrupted the balance between n-3 and n-6 PUFA, since a ratio of 5.2:1 seemed to produce better growth and development than a ratio of 3.9:1, 3.7:1 or 3.6:1 (ES, ES/2 and RP diets, respectively). The optimal balance between these two series of PUFA has been the subject of several reviews (Sargent et al., 1999; Izquierdo et al., 1996). A higher dietary (n-3)/(n-6) PUFA ratio is associated with beneficial effects on bone health (Maggio et al., 2009) and some evidence exists for higher requirements in n-3 EFA than in n-6 EFA in marine fish larvae. Sargent et al. (1999) showed that an excess of ARA to EPA impaired pigmentation and metamorphosis in turbot larvae. The dietary ARA/EPA ratio plays an important role, because ARA is the major eicosanoid precursor in fish and an excess of EPA can be harmful by reducing the desirable ARA/EPA ratio in live feeds (Sargent et al., 1997). Although ARA is included in enrichments in low but significant amounts (usually about 1 % TFA), there is no evidence if this amount is sufficient, nor if the relative proportion of ARA to DHA and EPA is optimal for marine fish larval feeds. In common sole *Solea solea*, neither standard growth nor larval survival was significantly affected by different dietary absolute concentrations of ARA, EPA and DHA, or their ratios (Lund et al., 2007). In this study, a 2-fold increase in the amount of ARA in the RP diet (1.4 % TFA) or a deficiency in ARA for the AGD, ES and ES/2 diets (0.2, 0.5 and 0.3 % TFA, respectively) in comparison to the AGG diet (0.7 % TFA), together with an excess of EPA in the ES diet, contributed to a disruption in the balance between EFAs, that might have had consequences on the overall developmental performance of Senegalese sole.

The effect of the origin of the dietary HUFA should not be neglected, since their efficiency of assimilation and their effect on larval performance depends on the dietary lipid class [neutral lipids (NL) or phospholipids (PL)]. EFA are generally provided in the diet by fish oils, which are mainly composed of NL (Izquierdo et al., 2000; Cahu et al., 2003). However, fish larvae have high requirements for dietary PL (Cahu et al., 2009). Adding PL to live-prey enrichment emulsions results in beneficial effects on the larval growth, survival, maturation of the digestive function, stress tolerance and larval quality (Sargent et al., 2002; Bell and Sargent, 2003; Izquierdo et al., 2000; Cahu et al., 2003; Coutteau et al., 1997; Gisbert et al., 2005) as marine fish larvae use dietary n-3 PUFA contained in the PL fraction more efficiently than those from the NL fraction (Salhi et al., 1999; Gisbert et al., 2005). Although the different origin of the ingredients composing the enriching products, under the present experimental conditions, enriched *Artemia* from the different dietary treatments showed similar total lipid content, as well as similar values of polar and neutral lipids (Boglino, unpublished data). As was found by Harel et al. (2002), this study demonstrated the potential of single cell heterotrophs (AGG, AGD, MG) as replacements for fish-based emulsions (ES, ES/2, RP) in larval diets, producing at least as good results in larval growth and development. However, further work is needed to examine possible effects of the different products on lipid absorption, transport and metabolism. Optimizing larval diets requires a careful balance of all the component nutrients and there is a need to consider the interactions between all of these components to create the most benefit for growth and development.

5. Conclusions

Under current experimental conditions, none of the tested enriching products were inappropriate for proper larval performance and quality, although they differentially affected Senegalese sole larval growth and development. Each dietary treatment presented its own

specific accumulation pattern for each fatty acid, none of which were optimal for proper larval performance and survival. Larvae fed the AGG diet had the best larval performance so that their FA profile can be considered as the most balanced for Senegalese sole larvae among all tested diets. Given the fatty acid profile of the AGG diet and our results from this study we recommend absolute levels of DHA, EPA and ARA of 9.5, 3.1 and 0.7 % TFA, respectively, and (n-3)/(n-6) PUFA, DHA/EPA, ARA/DHA and OA/PUFA ratios of 5.2, 3.0, 0.1, 0.5, respectively, as more suitable for Senegalese sole larval development.

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Figure captions

Figure 1. Intestinal maturation measured as the ratio of specific activity of brush border membrane enzymes/cytosolic enzymes: alkaline phosphatase/leucine-alanine peptidase (a), N-aminopeptidase/leucine-alanine peptidase (b), and specific activity of pancreatic enzymes: amylase (c), trypsin (d) and lipase (e) in 38dph-Senegalese sole larvae fed the six different dietary treatments. Data are expressed as the mean $\pm$ D.E. (n = 3). Different letters show significant differences between the six groups (ANOVA, $P < 0.05$).

Figure 2. Effect of the six different dietary treatments on the intestinal maturation at 38dph in Senegalese sole larvae fed the six different dietary treatments, measured as the enterocyte height (a) and cell proliferation capacity in mid intestine (b). Data are expressed as the

mean $\pm$ S.E.M ($n = 30$). Letters denote significant differences between groups (ANOVA, $P < 0.05$).

Figure 3: Degree of osification of Senegalese sole larvae fed *Artemia* enriched with the six different enrichments at 38dph. Data are expressed as the mean $\pm$ S.E.M ($n = 3$). Indexed letters indicate significant differences among treatments (ANOVA, $P < 0.05$).

Figure 4: Examples of different typologies of skeletal deformities found in 38-day-old Senegalese sole. (a) View of the vertebral region of a larva presenting scoliosis. (b) Strong compression of the first five prehaemal vertebrae resulting in abnormal neural spines. (c) Compression in the haemal region, resulting in a thickened area, raising the height of the vertebrae compared to the vertebral axis, and a defect in the haemal and neural spines parallelism. (d) Torsion in the haemal region, resulting in a deviation of the vertebral axis and of the apparent shape of the vertebral centrum (which seem inclined). Note the visible space of the neural and haemal arches as an indicator of the vertebral torsion and the absence of fusion of two haemal spines (arrow). (e) Partial fusion of the last two vertebrae before the urostile (the intervertebral space is still visible) and fusion of the modified haemal spines. (f) Total fusion of the last two vertebrae before the urostile and fusion of the modified neural spines.

Figure 5: Final DW (bars, in mg), I_{EM} at 22 dph (squares and solid line) and Alkaline phosphatase/leucine-alanine peptidase ratio at 22 dph (AP/LAP), diamonds and dotted line) in Senegalese sole larvae, DHA (red squares), EPA (green circles), ARA (yellow circles) contents and (n-3)/(n-6) PUFA (light blue triangles), DHA/EPA (dark blue triangles), ARA/EPA (white circles), OA/PUFA (black inversed triangles) ratios in *Artemia* enriched the six different dietary treatments. Data are expressed as mean $\pm$ D.E ($n = 3$). The highest final DW and the most advanced metamorphosis and maturation of the digestive system were obtained for larvae fed AGG-enriched *Artemia*, which diet presented intermediate values of

- 1052 DHA, EPA and ARA amounts (9.5, 3.1, 0.7 % TFA) and intermediate values of (n-3)/(n-6)
- 1053 PUFA, DHA/EPA, ARA/DHA and OA/PUFA ratios (5.2, 3.0, 0.1 and 0.5, respectively).

Table 1

Table 1. Total lipid and total fatty acids contents, fatty acid composition (in % TFA) and retinoid content in *Artemia* nauplii enriched with the six enriching products (mean $\pm$ SD; n = 3). Totals include some minor components not shown. Superscripts letters denote significant differences among diets ($P < 0.05$).

	Dietary treatments					
	ES	ES/2	RP	AGG	AGD	MG
Total lipids (mg·g ⁻¹ DW)	219.8 $\pm$ 17.4	175.3 $\pm$ 44.6	174.7 $\pm$ 17.3	184.4 $\pm$ 17.0	202.9 $\pm$ 1.3	159.6 $\pm$ 8.3
Total FA (mg·g ⁻¹ DW)	155.9 $\pm$ 54.8	112.2 $\pm$ 20.0	104.9 $\pm$ 15.6	124.0 $\pm$ 43.0	106.3 $\pm$ 6.0	116.1 $\pm$ 24.1
14:0	0.7 $\pm$ 0.1	0.3 $\pm$ 0.1	0.7 $\pm$ 0.5	0.8 $\pm$ 0.4	0.6 $\pm$ 0.4	0.6 $\pm$ 0.3
16:0	9.9 $\pm$ 0.4	8.3 $\pm$ 0.9	12.2 $\pm$ 1.2	10.3 $\pm$ 1.7	9.5 $\pm$ 3.6	10.7 $\pm$ 0.4
18:0	4.2 $\pm$ 0.2	4.3 $\pm$ 0.4	4.7 $\pm$ 0.8	4.5 $\pm$ 0.2	4.2 $\pm$ 1.5	3.8 $\pm$ 0.7
Total saturated	14.9 $\pm$ 0.3	13.0 $\pm$ 1.2	17.8 $\pm$ 1.2	15.9 $\pm$ 2.6	14.6 $\pm$ 5.4	15.1 $\pm$ 0.7
16:1n-7	2.1 $\pm$ 0.6	1.1 $\pm$ 0.1	1.0 $\pm$ 0.9	1.1 $\pm$ 0.3	1.6 $\pm$ 0.6	1.1 $\pm$ 0.2
18:1n-9	33.3 $\pm$ 2.6 ^{ab}	50.8 $\pm$ 2.0 ^a	20.2 $\pm$ 7.0 ^b	26.2 $\pm$ 10.1 ^{ab}	27.7 $\pm$ 19.0 ^{ab}	17.8 $\pm$ 6.1 ^b
18:1n-7	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	3.6 $\pm$ 6.2	4.1 $\pm$ 7.0	0.0 $\pm$ 0.0	3.1 $\pm$ 5.4
20:1n-9	2.5 $\pm$ 0.2 ^a	1.7 $\pm$ 0.2 ^{ab}	1.2 $\pm$ 0.1 ^b	1.1 $\pm$ 0.2 ^b	1.5 $\pm$ 0.7 ^{ab}	1.2 $\pm$ 0.2 ^b
Total monounsaturated	38.8 $\pm$ 3.7 ^{ab}	54.1 $\pm$ 1.6 ^a	26.5 $\pm$ 1.9 ^b	32.7 $\pm$ 3.2 ^{ab}	30.9 $\pm$ 17.7 ^b	23.5 $\pm$ 2.4 ^b
18:2n-6	8.28 $\pm$ 0.04 ^a	6.3 $\pm$ 0.5 ^{ab}	7.6 $\pm$ 0.4 ^{ab}	5.2 $\pm$ 1.0 ^b	6.9 $\pm$ 1.9 ^{ab}	5.5 $\pm$ 0.5 ^{ab}
18:3n-6	0.56 $\pm$ 0.03	0.3 $\pm$ 0.2	0.4 $\pm$ 0.2	0.5 $\pm$ 0.2	0.6 $\pm$ 0.5	0.4 $\pm$ 0.1
20:4n-6	0.5 $\pm$ 0.1 ^{cd}	0.3 $\pm$ 0.1 ^{cd}	1.4 $\pm$ 0.3 ^a	0.7 $\pm$ 0.1 ^{bc}	0.2 $\pm$ 0.2 ^d	1.1 $\pm$ 0.2 ^{ab}
22:5n-6	0.1 $\pm$ 0.07	0.0 $\pm$ 0.0	2.8 $\pm$ 2.5	2.2 $\pm$ 1.9	0.04 $\pm$ 0.04	3.7 $\pm$ 3.2
Total n-6 PUFA	9.5 $\pm$ 0.2	7.0 $\pm$ 0.4	12.3 $\pm$ 2.3	8.6 $\pm$ 2.3	7.8 $\pm$ 2.6	10.8 $\pm$ 3.7
18:3n-3	18.9 $\pm$ 1.8	17.4 $\pm$ 2.3	20.5 $\pm$ 3.4	24.1 $\pm$ 6.1	25.1 $\pm$ 5.5	21.4 $\pm$ 2.1
18:4n-3	2.3 $\pm$ 0.3	1.92 $\pm$ 0.02	2.4 $\pm$ 0.3	2.9 $\pm$ 0.6	3.4 $\pm$ 1.0	2.9 $\pm$ 0.4
20:4n-3	0.4 $\pm$ 0.6	0.3 $\pm$ 0.3	0.5 $\pm$ 0.4	0.6 $\pm$ 0.5	0.4 $\pm$ 0.4	0.8 $\pm$ 0.1
20:5n-3	7.6 $\pm$ 0.6 ^a	3.1 $\pm$ 0.9 ^c	5.1 $\pm$ 0.6 ^b	3.1 $\pm$ 0.4 ^{cb}	3.7 $\pm$ 1.2 ^{cb}	4.9 $\pm$ 0.4 ^{cb}
22:5n-3	1.4 $\pm$ 0.3	1.6 $\pm$ 1.8	1.8 $\pm$ 2.6	1.2 $\pm$ 2.0	1.9 $\pm$ 2.7	1.8 $\pm$ 2.4
22:6n-3	5.5 $\pm$ 1.5 ^{cd}	1.3 $\pm$ 0.5 ^d	11.5 $\pm$ 1.0 ^b	9.5 $\pm$ 0.9 ^{bc}	11.8 $\pm$ 2.7 ^b	16.9 $\pm$ 2.0 ^a
Total n-3 PUFA	36.5 $\pm$ 3.5 ^{ab}	25.7 $\pm$ 3.2 ^b	42.9 $\pm$ 1.6 ^a	42.5 $\pm$ 4.6 ^a	46.4 $\pm$ 9.5 ^a	50.4 $\pm$ 2.3 ^a
Total PUFA	46.0 $\pm$ 3.7 ^{ab}	32.7 $\pm$ 2.9 ^b	55.3 $\pm$ 2.8 ^a	51.1 $\pm$ 5.9 ^a	54.1 $\pm$ 12.0 ^a	61.1 $\pm$ 2.3 ^a
(n-3)/(n-6)	3.9 $\pm$ 0.3	3.7 $\pm$ 0.6	3.6 $\pm$ 0.7	5.2 $\pm$ 1.5	6.2 $\pm$ 0.9	5.2 $\pm$ 2.4

DHA/EPA	0.7±0.1 ^c	0.4±0.1 ^c	2.3±0.3 ^b	3.0±0.1 ^a	3.3±0.3 ^a	3.5±0.3 ^a
ARA/DHA	0.1±0.0 ^{bc}	0.22±0.04 ^a	0.12±0.01 ^b	0.08±0.01 ^{bc}	0.01±0.01 ^d	0.07±0.01 ^{cd}
ARA/EPA	0.07±0.01 ^b	0.09±0.02 ^b	0.3±0.1 ^a	0.23±0.02 ^a	0.04±0.03 ^b	0.23±0.03 ^a
OA/PUFA	0.73±0.11 ^b	1.57±0.20 ^a	0.37±0.14 ^b	0.53±0.24 ^b	0.58±0.43 ^b	0.29±0.10 ^b
Retinoids (µg·g DW ⁻¹)	24.8±9.6 ^a	13.2±7.4 ^{ab}	0.5±0.3 ^b	0.2±0.2 ^b	0.4±0.6 ^b	0.1±0.2 ^b

Table 2

Table 2. Total lipid and total fatty acids contents. fatty acid composition (in % TFA) and retinoid content in newly hatched Senegalese sole larvae at 2 dph and 38 dph fed *Artemia* enriched with the six commercial enrichments (mean $\pm$ SD. n = 3). Totals include some minor components not shown. Superscripts denote significant differences among diets ($P < 0.05$).

	Dietary treatments (38 dph)							
	(2dph)	(8dph)	ES	ES/2	RP	AGG	AGD	MG
Total lipids (mg·g ⁻¹ DW)	43.4 $\pm$ 8.4	49.9 $\pm$ 10.1	67.3 $\pm$ 17.0	88.3 $\pm$ 11.3	79.0 $\pm$ 8.6	97.9 $\pm$ 5.8	93.4 $\pm$ 11.1	86.6 $\pm$ 7.5
Total FA (mg·g ⁻¹ DW)	26.2 $\pm$ 7.8	25.7 $\pm$ 7.3	34.7 $\pm$ 10.0	53.5 $\pm$ 10.3	36.9 $\pm$ 9.6	41.6 $\pm$ 3.0	49.0 $\pm$ 9.7	41.9 $\pm$ 2.3
14:0	1.0 $\pm$ 0.5	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1 ^{bc}	0.3 $\pm$ 0.0 ^c	0.6 $\pm$ 0.1 ^{ab}	0.6 $\pm$ 0.1 ^a	0.5 $\pm$ 0.0 ^{ab}	0.6 $\pm$ 0.0 ^a
16:0	20.0 $\pm$ 2.6	12.8 $\pm$ 1.5	10.4 $\pm$ 1.1 ^{bc}	8.6 $\pm$ 0.1 ^c	12.8 $\pm$ 1.1 ^a	11.4 $\pm$ 0.4 ^{ab}	10.0 $\pm$ 1.1 ^{bc}	11.5 $\pm$ 0.6 ^{ab}
18:0	7.3 $\pm$ 0.4	10.2 $\pm$ 2.0	5.8 $\pm$ 0.5 ^{bc}	4.7 $\pm$ 0.4 ^d	7.3 $\pm$ 0.4 ^a	6.4 $\pm$ 0.2 ^{ab}	5.1 $\pm$ 0.4 ^{cd}	6.2 $\pm$ 0.1 ^b
Total saturated	29.1 $\pm$ 3.4	23.6 $\pm$ 3.5	16.9 $\pm$ 1.7 ^{bc}	13.6 $\pm$ 0.3 ^c	20.7 $\pm$ 1.6 ^a	18.5 $\pm$ 0.6 ^{ab}	16.1 $\pm$ 2.0 ^{bc}	18.4 $\pm$ 0.5 ^{ab}
16:1n-7	3.7 $\pm$ 0.9	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1	1.3 $\pm$ 0.8	0.7 $\pm$ 0.0	0.7 $\pm$ 0.2	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1
18:1n-9	12.9 $\pm$ 5.8	23.9 $\pm$ 3.9	32.1 $\pm$ 4.9 ^{bc}	42.4 $\pm$ 4.9 ^a	24.5 $\pm$ 0.7 ^{cd}	30.3 $\pm$ 1.2 ^{bc}	34.8 $\pm$ 1.2 ^{ab}	21.5 $\pm$ 1.6 ^d
20:1n-9	1.1 $\pm$ 1.1	2.6 $\pm$ 0.3	2.8 $\pm$ 0.3 ^a	2.7 $\pm$ 0.3 ^a	1.8 $\pm$ 0.4 ^{ab}	2.0 $\pm$ 0.1 ^{ab}	2.1 $\pm$ 0.3 ^{ab}	1.6 $\pm$ 0.7 ^b
Total monounsaturated	21.3 $\pm$ 0.8	27.5 $\pm$ 4.0	35.9 $\pm$ 5.1 ^b	46.4 $\pm$ 4.3 ^a	27.1 $\pm$ 0.6 ^{cd}	33.0 $\pm$ 1.2 ^{bc}	37.6 $\pm$ 1.5 ^b	23.7 $\pm$ 1.5 ^d
18:2n-6	1.9 $\pm$ 1.4	5.2 $\pm$ 0.5	7.9 $\pm$ 1.0 ^{ab}	9.2 $\pm$ 0.1 ^a	7.9 $\pm$ 0.4 ^{ab}	5.8 $\pm$ 0.8 ^c	6.8 $\pm$ 0.4 ^{bc}	5.4 $\pm$ 0.4 ^c
18:3n-6	0.17 $\pm$ 0.03	0.3 $\pm$ 0.1	0.6 $\pm$ 0.1 ^a	0.4 $\pm$ 0.0 ^b	0.5 $\pm$ 0.1 ^a	0.6 $\pm$ 0.0 ^a	0.6 $\pm$ 0.1 ^a	0.5 $\pm$ 0.0 ^{ab}
20:4n-6	2.9 $\pm$ 0.1	2.3 $\pm$ 1.3	1.1 $\pm$ 0.2 ^c	0.9 $\pm$ 0.2 ^{cd}	3.3 $\pm$ 0.1 ^a	2.1 $\pm$ 0.1 ^b	0.5 $\pm$ 0.1 ^d	3.0 $\pm$ 0.1 ^a
22:5n-6	0.66 $\pm$ 0.03	0.9 $\pm$ 0.5	0.3 $\pm$ 0.2 ^c	0.1 $\pm$ 0.1 ^c	4.6 $\pm$ 0.6 ^b	3.8 $\pm$ 0.1 ^b	0.1 $\pm$ 0.2 ^c	5.6 $\pm$ 0.4 ^a
Total n-6 PUFA	6.2 $\pm$ 1.4	9.2 $\pm$ 2.0	10.2 $\pm$ 1.3 ^{cd}	10.8 $\pm$ 0.3 ^{bc}	16.6 $\pm$ 1.0 ^a	12.5 $\pm$ 0.6 ^b	8.1 $\pm$ 0.7 ^d	14.9 $\pm$ 0.6 ^a
18:3n-3	0.4 $\pm$ 0.1	9.7 $\pm$ 5.0	14.3 $\pm$ 1.6	15.5 $\pm$ 1.1	12.6 $\pm$ 1.2	15.5 $\pm$ 0.8	15.4 $\pm$ 1.1	14.5 $\pm$ 0.6
18:4n-3	0.15 $\pm$ 0.02	1.1 $\pm$ 0.7	1.6 $\pm$ 0.3 ^{ab}	1.3 $\pm$ 0.2 ^{ab}	1.1 $\pm$ 0.2 ^b	1.7 $\pm$ 0.2 ^{ab}	1.8 $\pm$ 0.1 ^a	1.6 $\pm$ 0.2 ^{ab}
20:4n-3	0.23 $\pm$ 0.05	1.0 $\pm$ 0.1	0.8 $\pm$ 0.1 ^a	0.7 $\pm$ 0.1 ^{ab}	0.5 $\pm$ 0.1 ^b	0.8 $\pm$ 0.1 ^a	0.7 $\pm$ 0.0 ^{ab}	0.7 $\pm$ 0.0 ^{ab}
20:5n-3	4.4 $\pm$ 0.7	3.9 $\pm$ 0.7	5.1 $\pm$ 0.7 ^a	3.4 $\pm$ 0.5 ^b	3.0 $\pm$ 0.2 ^b	2.3 $\pm$ 0.2 ^b	2.6 $\pm$ 0.4 ^b	3.2 $\pm$ 0.3 ^b
22:5n-3	4.1 $\pm$ 0.1	2.0 $\pm$ 0.3	4.0 $\pm$ 0.7 ^a	2.1 $\pm$ 0.2 ^b	2.0 $\pm$ 0.4 ^b	1.7 $\pm$ 0.3 ^b	1.5 $\pm$ 0.1 ^b	1.9 $\pm$ 0.1 ^b
22:6n-3	28.6 $\pm$ 6.3	19.8 $\pm$ 4.1	10.4 $\pm$ 1.9 ^c	5.6 $\pm$ 1.4 ^d	15.3 $\pm$ 0.7 ^b	12.9 $\pm$ 1.5 ^{bc}	15.1 $\pm$ 1.7 ^b	20.0 $\pm$ 1.6 ^a
Total n-3 PUFA	38.1 $\pm$ 7.2	37.6 $\pm$ 4.3	36.2 $\pm$ 4.8 ^{ab}	28.6 $\pm$ 3.5 ^b	34.6 $\pm$ 2.1 ^{ab}	34.9 $\pm$ 2.2 ^{ab}	37.2 $\pm$ 3.1 ^{ab}	42.0 $\pm$ 2.1 ^a
Total PUFA	44.2 $\pm$ 8.6	46.8 $\pm$ 5.3	46.4 $\pm$ 6.1 ^b	39.4 $\pm$ 3.8 ^b	51.3 $\pm$ 2.3 ^{ab}	47.5 $\pm$ 1.6 ^{ab}	45.2 $\pm$ 3.7 ^b	56.9 $\pm$ 2.0 ^a
(n-3)/(n-6)	6.2 $\pm$ 0.3	4.3 $\pm$ 1.1	3.6 $\pm$ 0.0 ^b	2.7 $\pm$ 0.3 ^{cd}	2.1 $\pm$ 0.2 ^d	2.8 $\pm$ 0.3 ^c	4.6 $\pm$ 0.3 ^a	2.8 $\pm$ 0.2 ^c
DHA/EPA	6.5 $\pm$ 0.4	5.2 $\pm$ 1.6	2.0 $\pm$ 0.1 ^c	1.7 $\pm$ 0.2 ^c	5.1 $\pm$ 0.4 ^b	5.6 $\pm$ 0.2 ^{ab}	5.8 $\pm$ 0.6 ^{ab}	6.2 $\pm$ 0.5 ^a
ARA/DHA	0.1 $\pm$ 0.0	0.1 $\pm$ 0.1	0.1 $\pm$ 0.0 ^c	0.2 $\pm$ 0.0 ^b	0.2 $\pm$ 0.0 ^a	0.2 $\pm$ 0.0 ^b	0.03 $\pm$ 0.0 ^d	0.2 $\pm$ 0.0 ^b
ARA/EPA	0.7 $\pm$ 0.1	0.6 $\pm$ 0.4	0.2 $\pm$ 0.0 ^c	0.3 $\pm$ 0.0 ^c	1.1 $\pm$ 0.1 ^a	0.9 $\pm$ 0.1 ^b	0.2 $\pm$ 0.0 ^c	0.9 $\pm$ 0.1 ^b

OA/PUFA	0.3±0.1	0.5±0.1	0.7±0.2 ^b	1.1±0.2 ^a	0.5±0.0 ^b	0.6±0.1 ^b	0.8±0.1 ^{ab}	0.4±0.0 ^b
Retinoids (µg·g DW ⁻¹)			3.2±0.5 ^b	4.6±0.4 ^{ab}	6.3±1.1 ^a	4.1±1.1 ^{ab}	4.4±1.6 ^{ab}	6.5±0.9 ^a

Table 3. Final larval size in standard length (SL, in mm) and dry weight (DW, in mg), specific growth rate (SGR, in % d⁻¹) during the whole period of rearing (2-38), the pre-metamorphic and metamorphic periods (2-22) and the post-metamorphic period (22-38), and survival rate (in %) of Senegalese sole larvae fed *Artemia* nauplii enriched the different commercial products. Initial standard length and dry weight of larvae were 2.90 ± 0.28 mm and 16.5 ± 0.0 µg, respectively. Values are expressed as mean ± SD (n = 3). Different letters within the same column show significant differences ($P < 0.05$).

	Final SL	Final DW	SGR (2-38)	SGR (2-22)	SGR (22-38)	Survival rate
ES	7,21±1,14 ^e	1,07±0,25 ^c	0,115±0,007 ^b	0,191±0,004 ^b	0,044±0,010 ^b	94,1±2,3
ES/2	7,76±1,17 ^{cd}	1,33±0,26 ^c	0,122±0,002 ^{ab}	0,196±0,004 ^{ab}	0,054±0,001 ^{ab}	96,3±0,2
RP	7,59±1,39 ^{de}	1,25±0,41 ^c	0,119±0,008 ^{ab}	0,191±0,004 ^b	0,054±0,015 ^{ab}	96,4±0,4
AGG	8,93±1,29 ^a	2,01±0,42 ^a	0,133±0,004 ^a	0,203±0,004 ^a	0,072±0,013 ^{ab}	94,8±2,6
AGD	8,42±1,47 ^{ab}	1,68±0,45 ^b	0,129±0,004 ^{ab}	0,189±0,004 ^b	0,077±0,005 ^a	96,2±0,4
MG	8,20±1,41 ^{bc}	1,63±0,47 ^b	0,127±0,006 ^{ab}	0,189±0,002 ^b	0,073±0,013 ^{ab}	96,2±0,8

Table 4. Eye migration index (I_{EM}) of Senegalese sole larvae fed *Artemia* nauplii enriched the different commercial products at 10, 15, 22, 31 and 38 dph. Values are expressed as mean $\pm$ SD ($n = 3$). No significant differences ($P > 0.05$) were detected among dietary treatments for any sampled day.

	Age (days post hatching)				
	10	15	22	31	38
ES	0,96 $\pm$ 0,10	1,25 $\pm$ 0,23	2,82 $\pm$ 0,23	4,17 $\pm$ 0,03	4,77 $\pm$ 0,29
ES/2	0,97 $\pm$ 0,08	1,25 $\pm$ 0,14	2,93 $\pm$ 0,07	4,21 $\pm$ 0,54	4,84 $\pm$ 0,14
RP	0,93 $\pm$ 0,09	1,50 $\pm$ 0,17	2,99 $\pm$ 0,45	4,17 $\pm$ 0,62	4,78 $\pm$ 0,16
AGG	0,96 $\pm$ 0,07	1,29 $\pm$ 0,13	3,13 $\pm$ 0,10	4,26 $\pm$ 0,49	5,00 $\pm$ 0,00
AGD	0,93 $\pm$ 0,03	1,12 $\pm$ 0,16	2,99 $\pm$ 0,23	4,50 $\pm$ 0,30	4,95 $\pm$ 0,07
MG	0,98 $\pm$ 0,04	1,36 $\pm$ 0,08	2,77 $\pm$ 0,06	4,04 $\pm$ 0,57	4,90 $\pm$ 0,07

Table 5

Table 5. Incidence of skeletal deformities (in %) in Senegalese sole fed with the six different dietary treatments, considering the number of abnormal skeletal elements per fish (mean $\pm$ SD). Indexed letters show significant differences among treatments (ANOVA. $P < 0.05$). Mns, modified neural spine; Mhs, modified haemal spines; HYP, hypurals; PHY, parahypural; EP, epural.

	Dietary treatments					
	ES	ES/2	RP	AGG	AGD	MG
Total deformities	71.9 $\pm$ 9.9	74.2 $\pm$ 10.2	74.5 $\pm$ 2.0	79.2 $\pm$ 3.4	77.9 $\pm$ 5.3	79.3 $\pm$ 3.9
Vertebral abnormalities	68.0 $\pm$ 13.6	60.4 $\pm$ 11.4	65.2 $\pm$ 5.1	69.7 $\pm$ 4.6	66.3 $\pm$ 9.4	67.3 $\pm$ 7.1
<i>Severe</i>	46.7 $\pm$ 15.0	40.0 $\pm$ 5.7	37.7 $\pm$ 7.0	39.6 $\pm$ 1.6	50.8 $\pm$ 10.2	36.7 $\pm$ 4.3
Fusion	18.7 $\pm$ 6.9 ^{ab}	20.0 $\pm$ 0.6 ^{ab}	17.2 $\pm$ 5.7 ^{ab}	22.5 $\pm$ 4.4 ^{ab}	30.6 $\pm$ 7.1 ^a	15.7 $\pm$ 2.4 ^b
Compression	13.1 $\pm$ 12.6	10.4 $\pm$ 6.4	13.4 $\pm$ 3.6	18.9 $\pm$ 3.6	16.8 $\pm$ 7.1	15.0 $\pm$ 4.9
Vertebral centrums	16.4 $\pm$ 4.0	9.4 $\pm$ 4.0	14.8 $\pm$ 3.2	7.0 $\pm$ 4.2	15.2 $\pm$ 6.7	8.0 $\pm$ 3.1
Torsion	8.5 $\pm$ 7.2	6.8 $\pm$ 5.4	10.7 $\pm$ 7.6	3.4 $\pm$ 3.8	10.9 $\pm$ 10.5	6.9 $\pm$ 3.9
Scoliosis	7.5 $\pm$ 7.9	2.8 $\pm$ 0.4	4.4 $\pm$ 1.4	6.8 $\pm$ 3.8	6.5 $\pm$ 1.4	2.5 $\pm$ 2.4
<i>Light</i>	56.5 $\pm$ 9.3	57.6 $\pm$ 8.9	62.6 $\pm$ 4.9	61.3 $\pm$ 6.9	47.1 $\pm$ 2.6	62.8 $\pm$ 5.8
Haemal spines	40.3 $\pm$ 11.3	33.2 $\pm$ 7.2	35.5 $\pm$ 8.2	31.8 $\pm$ 2.8	30.0 $\pm$ 7.2	36.1 $\pm$ 12.2
Neural spines	50.6 $\pm$ 11.2	56.8 $\pm$ 10.3	59.0 $\pm$ 4.6	58.7 $\pm$ 6.9	42.3 $\pm$ 2.0	57.6 $\pm$ 8.4
Caudal abnormalities	40.8 $\pm$ 1.9 ^b	54.2 $\pm$ 6.5 ^a	47.4 $\pm$ 2.8 ^{ab}	55.9 $\pm$ 4.4 ^a	44.7 $\pm$ 4.4 ^{ab}	49.3 $\pm$ 6.7 ^{ab}

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