Lack of essential fatty acids in live feed during larval and post-larval rearing: effect on the performance of juvenile *Solea senegalensis*

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Abstract Despite the large progress obtained in recent years, Senegalese sole (*Solea senegalensis*) production of high quality juveniles is still a bottleneck. This paper examines the effect of larval and post-larval lipid nutrition on juvenile performance and quality. Four dietary treatments were tested: A—enriched *Artemia* spp. (EA); B—non-enriched *Artemia* spp. (NEA); C—EA during the pelagic larval period and NEA after larval settlement; D—50% EA and 50% NEA. Juvenile fatty acid profile at 60 days after hatching (DAH) clearly reflected the larval and post-larval diet composition. Feeding sole larvae on NEA (poor in lipids and essential fatty acids-EFA) had a negative effect, reducing growth (total length and dry weight) after 30 DAH and decreasing digestive enzyme activity at the end of the rearing period (60 DAH). However, relatively good performance compared to the EFA-richest treatment (A) was obtained when larvae were fed 50% EA and 50% NEA (D) or even EA only during the pelagic larval period followed by NEA after larval settlement (C). Malpigmentation was not affected by the dietary regimes and its incidence was very low. However, skeletal deformities were prevalent, particularly in the caudal complex, independently of diet. The results confirm that Senegalese sole appear to have lower larval EFA requirements than most cultured marine species and potentially even

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lower requirements during the post-larval stage. The importance of studying the impact of early nutrition on later juvenile stages was clearly highlighted in this study.

Keywords Digestive enzymes · Larval rearing · Lipid nutrition · Pigmentation · Skeletal deformities · *Solea senegalensis*

Abbreviations

ARA	Arachidonic acid (20:4n-6)
DAH	Days after hatching
DHA	Docosahexaenoic acid (22:6n-3)
DW	Dry weight
EA	Enriched *Artemia*
EPA	Eicosapentaenoic acid (20:5n-3)
FA	Fatty acid
FAME	FA methyl esters
HUFA	Highly unsaturated fatty acids
LA	Linoleic acid (18:2n-6)
LNA	Linolenic acid (18:3n-3)
NEA	Non-enriched *Artemia*
OA	Oleic acid (18:1n-9)
PUFA	Polyunsaturated fatty acid
TL	Total length

Introduction

The aquaculture production in southern Europe has long been characterized by an intensification and massive increase in the production of European sea bass (*Dicentrarchus labrax* Linnaeus, 1758) and gilthead seabream (*Sparus aurata* Linnaeus, 1758), which has led to a decline in market prices for these species. Therefore, diversification of product supply is critical, and an intense effort is being undertaken to develop rearing methods for several potential new species for aquaculture with high price and demand in European markets. One of the most promising of these species is Senegalese sole (*Solea senegalensis* Kaup, 1858). It is commonly raised in extensive or semi-intensive polyculture in the south of Portugal and Spain (Drake et al. 1984; Dinis 1992), where it attains a higher growth rate than sea bass, being second to seabream (Drake et al. 1984; Dinis and Reis 1995). Its high price and market demand have stimulated producers to on grow this species more intensively since the early nineties. Hatchery production of Senegalese sole for intensive or semi-intensive culture has been economically and biologically viable for at least 2 decades (Dinis 1992) and larvae can be produced with high survival and growth rates, compared to other marine species, even if there is still incomplete knowledge regarding larval and juvenile nutritional requirements. Compared to other flatfishes, malpigmentation is a less severe problem in Senegalese sole rearing (Dinis et al. 1999). However, skeletal deformities are more prevalent (Gavaia et al. 2002; Engrola et al. 2005), reflecting problems related to rearing and/or nutrition.

A major difference between sole and other marine species is that nutritional deficiencies in essential highly unsaturated fatty acids (HUFA), particularly docosahexaenoic acid

(DHA; 22:6n-3), during the larval stage do not result in mass mortalities at the larval stage itself. In fact, good larval and post-larval rearing results have been obtained even when feeding Senegalese sole larvae and early post-larvae on non-enriched *Artemia* (NEA) metanauplii, devoid of DHA, and with low lipid and very low (n-3) HUFA levels (Morais et al. 2004, 2005a, b). In addition, Villalta et al. (2005) have also not found a significant relationship, up to 36 DAH, between dietary DHA supply and growth and survival of Senegalese sole larvae. It appears therefore that Senegalese sole larvae are less demanding in terms of prey lipid content and (n-3) HUFA dietary requirements. This has been claimed to be a major advantage of Senegalese sole for commercial rearing, as it might lead to some economical savings and less problems with live prey enrichment. However, Heath and Moore (1997) found that the larval diet of Dover sole had a significant impact in juvenile growth. In addition, studies performed on turbot larvae (Støttrup and Attramadal 1992) revealed that the polyunsaturated fatty acid (PUFA) content of rotifers did not have a significant immediate effect on growth and survival but became later evident in larval survival, during the *Artemia* stage. Having this in mind, the present study was conducted in order to evaluate the impact of lipid and (n-3) HUFA-deficient dietary regimes during the larval and post-larval stages on juvenile (until 10 days after complete weaning) performance. Effects on survival, growth, fatty acid (FA) profile, development of the digestive system (activity of trypsin, alkaline phosphatase, amylase and lipase), as well as on the incidence of malpigmentation and malformations (juvenile quality), were analyzed.

Materials and methods

Larval rearing and experimental diets

Senegalese sole larvae hatched from eggs obtained from natural spawning of a broodstock from wild origin, maintained at the IPIMAR Aquaculture Research Centre (Olhão, Portugal). The experiment was conducted until 60 days after hatching (DAH) in a semi-closed system composed of twelve 300-L fiber glass U-shaped tanks (triplicate tanks per treatment) with 20-μm-filtered and UV-treated seawater, temperature between 16 and 20°C, a salinity of 36–38, a photoperiod of 14 h light:10 h dark and a starting density of 15 larvae L^{-1}. At the start of the experiment, water recirculation was very low but it was increased over the rearing period to the maximal flow rate of 60 Lh^{-1}, corresponding to a renewal rate of 20% tank volume h^{-1}. From start feeding (2 DAH) to 8 DAH, all treatments were fed newly hatched *Artemia* AF nauplii (INVE Aquaculture NV, Dendermonde, Belgium) plus rotifers *Brachionus* spp. enriched with microalgae (*Isochrysis galbana* clone T. Iso and *Nannochloropsis gaditana*), until 5 DAH. Green water was maintained only during the rotifer feeding period. Larvae started feeding on the experimental treatments at 6 DAH, with *Artemia* AF nauplii co-feeding until 8 DAH, and exclusively afterward.

The dietary treatments were as follows: A—*Artemia* metanauplii (Salt Lake Aquafeed™ Premium Artemia Cysts, Catvis BV, 's-Hertogenbosch, The Netherlands) enriched in Rich® (Catvis BV) (EA) during the whole larval and post-larval rearing period; B—non-enriched *Artemia* metanauplii (NEA) during the whole larval and post-larval rearing period; C—EA during the pelagic larval stage (up to 19 DAH), followed by NEA after post-larval settlement; and D—50% EA and 50% NEA during the whole larval and post-larval rearing period. At 40 DAH, post-larvae from all treatments started to be weaned into formulated feed (AgloNorse® 0, 1 and 2, Ewos, Bergen, Norway), in co-feeding with the experimental *Artemia* treatments until 50 DAH, after which the treatments were all fed

similarly and exclusively with formulated feed, but were still kept in separate tanks, until 60 DAH. During co-feeding, the amount of formulated feed given to each tank gradually increased from 40 to 50 DAH, while live feed meals decreased until being completely replaced by formulated feed.

The enrichments were conducted over 22–30 h, at 25°C, with strong aeration and at 100 metanauplii mL^{-1}, with two doses of 0.08 g Rich$^{®}$ L^{-1} being added at the start and at 6 h of enrichment. A third dose was given at 24 h of enrichment, but only for the *Artemia* that was fed to the larvae in the afternoon. The NEA was kept in clean seawater during the same period of time. *Artemia* was fed to the experimental tanks in four meals during the day (1.5 metanauplii mL^{-1} day^{-1} in average).

The FA composition of the two *Artemia* diets used at different stages of larval rearing, as well as of the formulated feed on which all the post-larvae were weaned is shown in Table 1. EA presented a higher total FA methyl esters (FAME) level (171.5 vs. 107.0 µg FAME/mg dry weight-DW) and higher absolute levels of all analyzed FA (not shown) than NEA. The only exceptions were the levels of arachidonic acid (ARA; 20:4n-6) and lino-lenic acid (LNA; 18:3n-3), which were very similar in absolute levels in both *Artemia* diets (not shown), leading to a higher percentage of these FA in NEA. NEA was also charac-terized by a higher percentage of oleic acid (OA; 18:1n-9). As for the DHA content, it was not detected in NEA and was 3.6% (corresponding to 6.3 µg/mg DW) in EA, while eicosapentaenoic acid (EPA; 20:5n-3) relative level was 2.9 and 7.3% in NEA and EA, respectively (corresponding to 3.2 and 12.8 µg/mg DW). The AgloNorse$^{®}$ diet (analysis

Table 1 Fatty acid composition, including the main identified FA, total FA groups (% total FAME) and total FAME level (µg mg^{-1} DW) of the non-enriched *Artemia* (NEA) and enriched *Artemia* (EA) used in the experimental treatments and of the formulated feed onto which all treatments were weaned (AgloNorse$^{®}$ 0–2)

Fatty acids	*Artemia*		Formulated feed
(% FAME)	NEA	EA	
14:0	0.7 ± 0.0	1.2 ± 0.1	5.7 ± 0.2
16:0	11.2 ± 0.2	10.9 ± 0.2	16.6 ± 1.1
18:0	6.1 ± 0.1	4.1 ± 0.1	2.2 ± 0.2
Total SFA	19.6 ± 0.3	17.4 ± 0.3	26.3 ± 1.0
16:1	3.6 ± 0.1	4.2 ± 0.1	3.8 ± 0.2
18:1	27.6 ± 0.3	23.6 ± 0.2	14.2 ± 0.3
20:1	0.5 ± 0.1	2.8 ± 0.2	8.8 ± 0.9
Total MUFA	32.4 ± 0.3	33.8 ± 0.5	39.1 ± 2.4
18:2n-6	6.0 ± 0.0	8.0 ± 0.2	14.5 ± 1.5
18:3n-3	25.2 ± 0.2	18.8 ± 0.6	1.8 ± 0.1
20:4n-6	1.1 ± 0.0	0.7 ± 0.0	0.4 ± 0.0
20:5n-3	2.9 ± 0.1	7.3 ± 0.3	4.9 ± 0.1
22:6n-3	0.0 ± 0.0	3.6 ± 0.2	8.8 ± 0.5
Total PUFA	43.8 ± 0.3	45.7 ± 0.4	33.4 ± 1.8
DHA/EPA	0.0 ± 0.0	0.5 ± 0.0	1.8 ± 0.1
FAME			
(µg mg^{-1} DW)	107.0 ± 12.8	171.5 ± 8.2	107.0 ± 25.7

Values are means ± SD

performed on a pool of all the granulometric sizes used) presented a higher relative level of linoleic acid (LA; 18:2n-6), a much lower level of LNA (at least tenfold), lower relative and absolute levels of ARA, higher relative and absolute levels of DHA and lower level of EPA (only in relation to EA), and thus, a higher DHA/EPA ratio. The total FAME content in the AgloNorse® diet was equivalent to that in NEA.

Sampling and biochemical analysis

Samples of 20 larvae or juveniles were collected from each triplicate tank (before adding the first meal of the day) at specific key points in development: at the end of the pelagic larval stage (19 DAH), at the end of the post-larval stage and just before weaning (40 DAH) and at the end of the experimental period (60 DAH). At 60 DAH, however, only two tanks from treatment C could be sampled, due to loss of one replicate tank (for zootechnical reasons) in this treatment. Additionally, samples of 20 individuals per triplicate tank were also collected at 6, 10 and 30 DAH, to determine length and weight. The total length (TL) of the specimens was measured under a binocular (Zeiss Stemi SV 8, Carl Zeiss AG, Oberkochen, Germany) until 30 DAH, or using a graduated scale afterward. After length measurement, specimens were rinsed in 3% ammonium formate solution and in distilled water, pooled in groups of five (until 10 DAH) or kept individually, frozen at −20°C and later freeze-dried (Savant RVT 400, Farmingdale, NY 11735, USA). Dry weight (DW) was assessed using a Sartorius Supermicro S4 (Sartorius AG, Goettingen, Germany) precision balance. The FA composition was also determined in 19, 40 and 60 DAH sole, when a pool of 30, 25 and 20 individuals per tank was sampled, respectively. For the enzyme assays, a pool of 50, 25 and 20 individuals was sampled per tank at 19, 40 and 60 DAH, respectively. The samples for FA and enzymatic determinations were washed in distilled water, frozen in liquid nitrogen and, for FA analysis, were later freeze-dried. In order to assess the incidence of malpigmentations and skeletal malformations at the end of the experiment, 15 animals were randomly sampled per tank and were measured and photographed under a binocular microscope equipped with a digital camera (digital Canon Power Shot G5, Canon Inc., Tokyo, Japan) and individually stored in 4% buffered formaldehyde (pH 7.4). These specimens were later processed using a whole-mount double staining methodology described in Gavaia et al. (2000) that uses alcian blue 8GX and alizarin red S for cartilage and bone staining, respectively, in combination with KOH treatment to render soft tissues transparent and permit observation of skeletal structures. Skeletal malformations were classified according to the affected structures as follows: pleural vertebra, parapophysis, caudal vertebra, neural arches, hemal arches, preural 1–3 and urostyle, modified neural arches, modified hemal arches, epural, hypurals, parahypural, caudal fin rays, dorsal fin pterigophores, dorsal fin rays, anal fin pterigophores, anal fin rays.

At 60 DAH, the survival rate was determined by counting the juveniles remaining in the tanks, after correcting for the individuals sampled. Survival values at 50 DAH are also shown and were obtained by adding the number of mortalities recorded daily to the number of fish counted at 60 DAH.

FA analysis of Senegalese sole and of the tested diets were performed in triplicate, using the transesterification method by basic catalysis (Park et al. 2001). FAME were separated and quantified using a Varian CP 3800 gas chromatograph (Varian Inc., Palo Alto, USA) equipped with a flame ionization detector (250°C) and a DB-WAX Polyetilene Glicol column (30 m × 0.32 mm ID; 0.25 μm). Injector temperature was maintained constant at 250°C over the 40 min of the analysis. The column was submitted to a temperature gradient, 5 min at 180°C, an increase of 4°C min^{-1} for 10 min, and kept at 220°C for

25 min. FA results were obtained in absolute levels of FAME (μg mg^{-1} DW) and of each individual assayed FA (not shown) and, in order to eliminate differences in total lipid composition and compare experimental treatments, they were transformed into relative amounts and expressed as percentages of total FAME.

The individuals used for enzymatic assays were defrosted on ice, and their abdominal portions were dissected on a glass maintained at 0°C (Ribeiro et al. 1999) and homogenized (Ultra Turrax T25, IKA Labortechnik, Staufen, Germany) in ice cold distilled water, at a dilution of 1:15 (wet weight of tissue:ml water). Trypsin activity was assayed using BAPNA (Na-benzoyl-DL-arginine-p-nitroanilide) (Sigma–Aldrich, St. Louis, MO, USA) as substrate (Holm et al. 1988). The activity of alkaline phosphatase was measured following the method of Bessey et al. (1946), using as substrate p-nitrophenylphosphate (pNPP) (Sigma–Aldrich). Amylase activity was measured using starch as the substrate (Métais and Bieth 1968) and lipase activity was assayed according to a spectrophotometric method from Iijima et al. (1998), using as substrate p-nitrophenyl myristate (Sigma–Aldrich) dissolved in DMSO (Dimethyl sulfoxide, Sigma–Aldrich). Protein level was determined by the Bradford procedure (Bradford 1976). Enzyme activities were calculated as μmoles of substrate hydrolyzed per min (U), at 25°C for trypsin, 37°C for alkaline phosphatase and amylase and 30°C for lipase. Enzyme activities were expressed as specific activities (U mg^{-1} of protein) and segmental activities, i.e., total activity per larval segment (digestive portion).

Statistical analysis

To assess differences induced by the dietary treatments in terms of survival, growth, FA composition and enzymatic activity, results were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey HSD multiple comparisons test when significant differences were found between treatments, at a significance level of 0.05. In the cases where an unequal sample number had to be used, the Spjotvoll/Stoline multiple comparisons test was employed instead. The homogeneity of variances was checked by the Levene test and whenever the assumption could not be met, the non-parametric Kruskal–Wallis test, followed by the Dunn's multiple comparisons non-parametric test, were used. All percentage results—survival and FA composition—were arcsin transformed (Underwood 1997). The statistical software package used was Statistica^{TM} 5.5 (StatSoft Inc., Tulsa, USA).

Results

Survival and growth performance

Survival was highly variable between replicate tanks of the same treatment, and no significant differences were found between dietary treatments. Nonetheless, a trend for lower survival of larvae fed NEA during the whole larval and post-larval period (treatment B) could be noticed at 50 DAH (Fig. 1). This trend was no longer evident at 60 DAH, possibly as a result of massive mortality from 54 to 60 DAH in all treatments. One of the triplicates of treatment C had to be disregarded at 60 DAH and one of the triplicates of treatment D had to be disregarded at 50 and 60 DAH (but only for the determination of survival), as a large number of the larvae of those replicates were suddenly lost due to problems unrelated to feeding: a technical defect of a tank during the early pelagic larval rearing stage, for treatment D, and almost complete overnight mortality, possibly due to ammonia
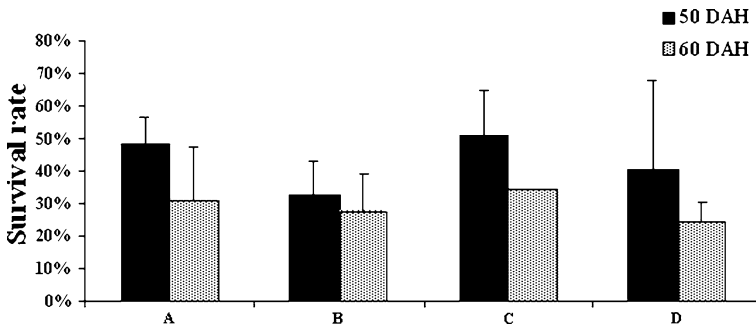
Fig. 1 Survival rate of Senegalese sole submitted to the different dietary treatments, at 50 and 60 DAH. No significant differences were observed ($P < 0.05$). Values are means ± SD. At 60 DAH in treatments C and D and at 50 DAH in treatment C only two replicate tanks were considered for determination of survival rate, given that a large reduction in juvenile numbers occurred due to reasons unrelated to dietary treatment (as explained in the "Results" section). In treatment C, at 60 DAH, both replicate tanks presented the same survival rate, hence SD is zero

intoxication, in one replicate of treatment C, at 53 DAH. Globally, mortality was high after complete weaning (from 54 DAH onwards) but it was also relatively important during the pelagic larval stage independently of dietary treatment, with a peak being noted at the end of the yolk-sac stage, when larvae started to feed exogenously (detected by counting the number of dead larvae, when cleaning the tanks daily; not shown). However, once the metamorphosis was completed and the animals acquired a benthic life (at 19 DAH), almost no mortality was noticeable for weeks, until after weaning.

No significant differences were found in total length and dry weight at 19 DAH (Fig. 2a and b). However, at 30 DAH, post-larvae fed exclusively on NEA (treatment B) presented a significantly lower growth than those fed exclusively EA (treatment A) and, at 40 and 60 DAH, post-larvae from treatment B were significantly smaller and lighter than those from the other treatments. DW allowed a better discrimination between treatments A, C and D given that a slightly lower weight was noted for treatments C and D at the end of the experiment (Fig. 2b), even though no significant differences were found between them.

Fatty acid composition

The FA composition of the larvae, post-larvae and juveniles was analyzed at three times of the experimental period, corresponding to the end of the pelagic larval stage (19 DAH), just before weaning (40 DAH) and at the end of the experiment (60 DAH) (Table 2). The results show only a very slight effect of dietary treatment at 19 DAH. At this time, significant differences were only found in the percentage of LA that was significantly lower in treatment B (although not significantly different from treatment D). A similar pattern was found for the percentage of DHA but it was only a non-significant trend at this point.

At 40 DAH, dietary effects became more noticeable. The total FAME level was higher in larvae fed EA during the whole larval and post-larval rearing stage (treatment A), followed by treatment D (50% EA and 50% NEA) and was lower in larvae fed NEA during the same period (treatment B) or only after larval settlement (treatment C), although
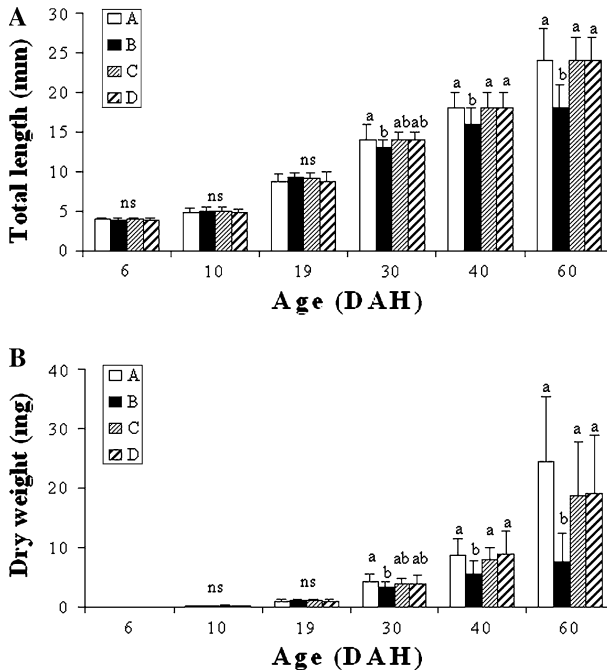
Fig. 2 Growth, in terms of total length (mm) and dry weight (mg), of Senegalese sole larvae, post-larvae and juveniles submitted to different dietary treatments. Values are means ± SD. *Different letters* indicate significant differences ($P < 0.05$) between treatments at each age; *ns* non-significantly different

differences were only significant between treatments A and C. Significant differences were found in the relative levels of LA, LNA, ARA, EPA and DHA, reflecting the diet composition. Post-larvae from treatment A presented significantly higher levels of LA, EPA and DHA, and significantly lower levels of LNA and ARA, compared to treatments B and C, for which no significant differences were found between each other. As for treatment D, it presented intermediate levels of these FA. Post-larvae from treatment B showed a significantly lower DHA/EPA ratio compared to treatments A and D, followed by treatment C, which was not significantly different from any of the other treatments.

At 60 DAH, there were more important differences in terms of total FAME levels, with larvae from treatment B presenting the significantly lowest absolute amounts of total FAME, treatments A and D the highest total FAME levels and treatment C showed intermediate and non-significantly different total FAME level. Significant differences were also found in total PUFA, and in LA, LNA, ARA, EPA, and DHA/EPA ratio. Larvae from treatment B presented significantly different FA percentages (higher LNA, ARA, EPA and total PUFA and lower LA and DHA/EPA ratio) than those from treatments A and D, while those from treatment C showed intermediate and non-significantly different levels. At this age there were no significant differences in the percentage of DHA between treatments, although in absolute amounts larvae from treatment B presented significantly lower values than those from treatments A and D (absolute amounts of DHA were 17.5, 6.5, 10.9 and 19.1 μg mg^{-1} DW respectively in treatments A, B, C and D), reflecting the differences in total FAME levels.

Table 2 Fatty acid composition, including the main identified FA, total FA groups (% total FAME) and total FAME level (μg mg^{-1} DW) of sole submitted to the different dietary treatments, at 19, 40 and 60 DAH

Fatty acids (% FAME)	19 DAH				40 DAH				60 DAH			
	A	B	C	D	A	B	C	D	A	B	C	D
14:0	0.8 ± 0.2	0.9 ± 0.5	0.9 ± 0.6	0.9 ± 0.3	0.6 ± 0.1[a]	0.5 ± 0.0[bc]	0.4 ± 0.0[b]	0.5 ± 0.1[c]	4.1 ± 0.4[a]	1.5 ± 0.4[b]	2.8 ± 0.7[ab]	3.5 ± 0.4[a]
16:0	13.3 ± 0.9	13.7 ± 1.0	13.5 ± 1.1	13.5 ± 0.7	12.9 ± 0.7	13.2 ± 0.7	13.1 ± 0.3	12.7 ± 0.5	15.6 ± 0.4[a]	13.8 ± 0.6[b]	14.8 ± 0.7[ab]	15.2 ± 0.4[a]
18:0	8.5 ± 0.9	8.4 ± 1.1	8.1 ± 0.8	8.5 ± 0.9	8.6 ± 0.9	9.2 ± 0.6	9.2 ± 0.2	8.8 ± 0.4	3.9 ± 0.4[a]	8.5 ± 1.0[b]	5.9 ± 1.0[ab]	4.8 ± 0.9[a]
Total SFA	24.8 ± 1.7	24.9 ± 1.0	24.7 ± 1.4	25.0 ± 1.1	24.1 ± 1.8	24.6 ± 0.9	24.7 ± 0.6	23.9 ± 0.8	25.2 ± 0.5	25.5 ± 0.7	25.3 ± 0.1	25.2 ± 0.3
18:1n-7	6.7 ± 0.3	7.0 ± 0.5	6.6 ± 0.3	6.9 ± 0.5	6.6 ± 0.2[a]	7.3 ± 0.3[b]	7.4 ± 0.8[b]	7.1 ± 0.5[ab]	2.4 ± 0.1[a]	3.7 ± 0.5[b]	2.8 ± 0.3[ab]	2.5 ± 0.3[a]
18:1n-9	16.5 ± 0.7	17.6 ± 0.6	16.6 ± 0.7	17.1 ± 0.7	17.1 ± 0.3	17.4 ± 0.3	17.2 ± 0.4	17.0 ± 0.7	13.5 ± 0.2	14.1 ± 0.4	13.7 ± 0.1	13.6 ± 0.4
20:1n-7	1.7 ± 0.3	1.4 ± 1.1	1.6 ± 0.3	1.6 ± 0.6	1.7 ± 0.1[a]	0.7 ± 0.1[b]	0.8 ± 0.1[b]	1.0 ± 0.4[ab]	7.1 ± 0.2[a]	2.9 ± 1.1[b]	5.0 ± 0.8[ab]	5.8 ± 0.5[a]
Total MUFA	29.3 ± 1.7	30.7 ± 2.7	29.6 ± 1.6	30.6 ± 1.6	30.0 ± 0.5[a]	28.9 ± 0.7[b]	28.8 ± 1.0[b]	29.2 ± 1.8[b]	34.6 ± 0.6[a]	27.2 ± 2.7[b]	31.6 ± 2.0[ab]	33.0 ± 1.3[a]
18:2n-6	8.8 ± 0.6[a]	7.6 ± 0.6[b]	8.7 ± 0.8[a]	8.0 ± 0.5[ab]	8.9 ± 0.2[a]	7.6 ± 0.2[b]	7.8 ± 0.2[b]	8.3 ± 0.3[c]	11.6 ± 0.4[a]	8.7 ± 0.9[b]	10.9 ± 1.3[ab]	11.0 ± 0.7[a]
18:3n-3	10.9 ± 0.9	12.7 ± 2.3	11.0 ± 1.4	11.8 ± 1.3	10.1 ± 0.4[a]	12.6 ± 0.7[b]	12.0 ± 0.5[bc]	11.0 ± 1.3[ac]	2.0 ± 0.3[a]	2.9 ± 0.3[b]	2.2 ± 0.3[ab]	1.8 ± 0.3[a]
20:4n-6	2.2 ± 0.3	2.5 ± 0.6	2.2 ± 0.5	2.4 ± 0.6	2.2 ± 0.2[a]	3.8 ± 0.3[b]	3.5 ± 0.2[b]	3.1 ± 0.5[ab]	1.0 ± 0.2[a]	4.2 ± 0.6[b]	2.4 ± 0.9[b]	1.6 ± 0.6[a]
20:5n-3	5.1 ± 0.7	4.3 ± 0.2	5.2 ± 1.1	4.6 ± 0.5	4.2 ± 0.4[a]	3.7 ± 0.2[b]	3.6 ± 0.1[b]	3.9 ± 0.1[ab]	2.7 ± 0.1[a]	3.6 ± 0.3[b]	3.2 ± 0.2[ab]	2.9 ± 0.1[a]
22:6n-3	7.3 ± 0.9	5.4 ± 1.7	7.0 ± 0.9	5.8 ± 1.4	8.6 ± 1.1[a]	4.9 ± 0.2[b]	5.9 ± 0.5[bc]	7.8 ± 1.5[ac]	14.3 ± 0.5	14.6 ± 1.3	14.5 ± 0.5	14.9 ± 0.8
Total PUFA	43.1 ± 1.9	40.9 ± 2.6	42.4 ± 1.8	40.9 ± 1.8	43.3 ± 1.8	43.4 ± 0.7	43.6 ± 0.8	43.9 ± 1.3	37.4 ± 0.4[a]	44.6 ± 1.8[b]	41.3 ± 1.2[ab]	39.2 ± 1.2[a]
DHA/EPA	1.4 ± 0.1	1.3 ± 0.41	1.4 ± 0.2	1.3 ± 0.2	2.0 ± 0.3[a]	1.3 ± 0.1[b]	1.6 ± 0.1[ab]	2.0 ± 0.4[a]	5.3 ± 0.1[a]	4.0 ± 0.2[b]	4.5 ± 0.4[ab]	5.2 ± 0.3[a]
Total FAME (μg mg^{-1} DW)	85.7 ± 21.8	86.6 ± 31.3	94.2 ± 23.9	93.4 ± 22.1	60.6 ± 12.7[a]	53.1 ± 5.5[ab]	49.9 ± 4.6[b]	56.8 ± 6.0[ab]	118.6 ± 13.6[a]	44.1 ± 10.4[b]	73.1 ± 19.9[ab]	124.2 ± 19.8[a]

Values are means ± SD. Columns with different superscript letters for a given FA and at a specific sampling time are significantly different ($P < 0.05$)

Enzymatic activities

At 19 DAH, the segmental activity of the assayed enzymes was generally much lower than in later stages and neither specific nor segmental activities were significantly affected by dietary treatment, except for the segmental activity of trypsin (Fig. 3b), which was significantly higher in treatment A, significantly lower in treatment D and intermediate in treatments B and C. At 40 DAH, post-larvae from treatment A presented a significantly
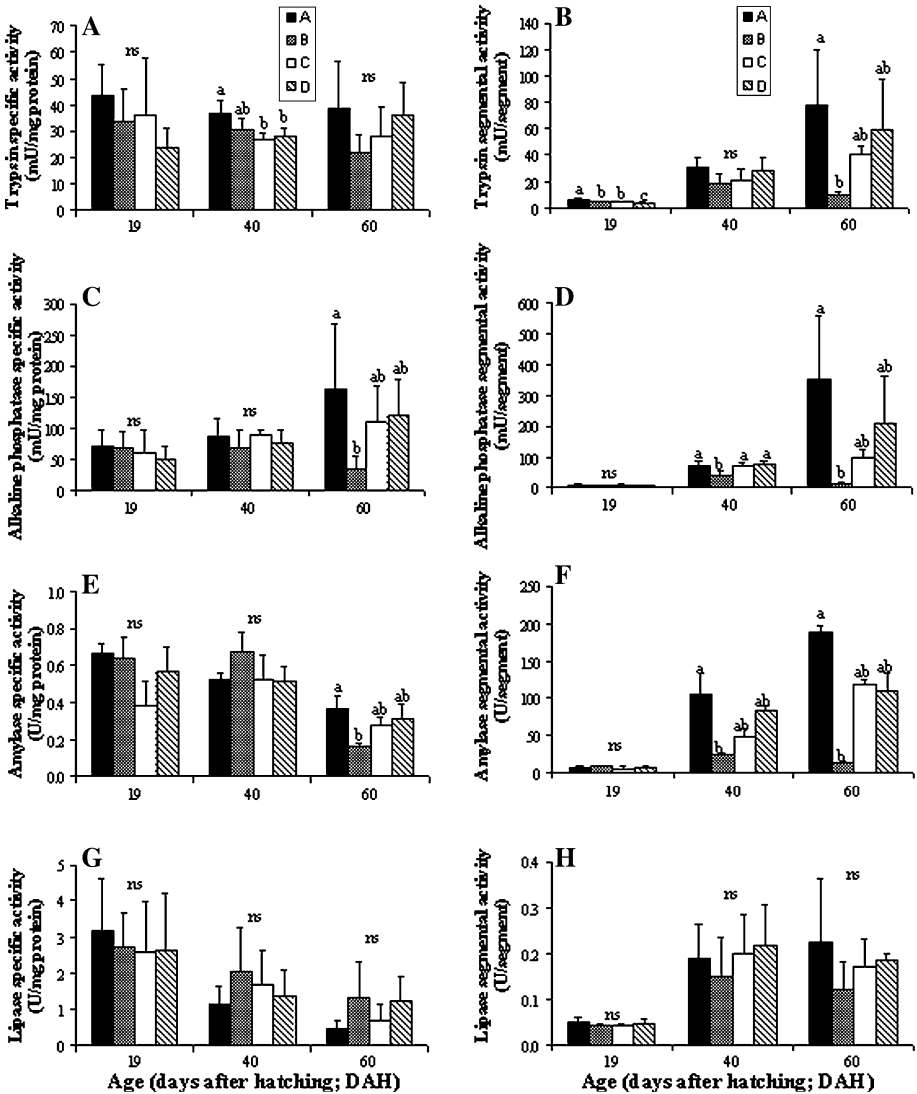
Fig. 3 Enzymatic specific (U or mU mg^{-1} protein) and segmental (U or mU segment^{-1}) activity of the enzymes trypsin (**a**, **b**), alkaline phosphatase (**c**, **d**), amylase (**e**, **f**) and lipase (**g**, **h**) in Senegalese sole at 19, 40 and 60 DAH. Values are means ± SD. *Different letters* indicate significant differences ($P < 0.05$) between treatments at each age; *ns* non-significantly different

higher trypsin-specific activity, and treatments C and D were responsible for significantly lower specific activities (Fig. 3a). However, no significant dietary effect could be noted on the specific activities of alkaline phosphatase, amylase or lipase at 40 DAH (Fig. 3c, e, g). As for the segmental activities at 40 DAH, treatment B was responsible for a significantly lower alkaline phosphatase and amylase activity (Fig. 3d, f), although in the case of amylase it was only statistically different from treatment A. No significant differences were observed in trypsin or lipase segmental activities at this time (Fig. 3b, h). At the end of the experimental period (60 DAH), a significant dietary effect was observed in the specific activities of alkaline phosphatase (Fig. 3c) and amylase (Fig. 3e) and in the segmental activities of trypsin (Fig. 3b), alkaline phosphatase (Fig. 3d) and amylase (Fig. 3f). In all these cases, significantly higher specific and segmental activities were measured in juveniles from treatment A and significantly lower activities in treatment B, while treatments C and D induced intermediate and non-significantly different activities.

Pigmentation and malformations

In order to assess juvenile quality and establish potential relationships with dietary regime during the larval and post-larval stages, the percentage of malpigmented juveniles and those presenting skeletal malformations was determined at the end of the experiment. In terms of pigmentation, almost no malpigmentations were observed and these were not significantly different between dietary treatments, with 97.8–100% presenting normal pigmentation at 60 DAH. In terms of skeletal malformations, the incidence was much more important, even though they could not be visually detected based on external body appearance and did not appear to have been related to dietary treatment. Globally, the most common types of skeletal malformations that were detected were the ones affecting the caudal fin complex. Treatments A, B, C and D presented $49 \pm 15\%$ (40, 40, 67% per triplicate tank, respectively), $69 \pm 10\%$ (60, 80, 67%), $57 \pm 14\%$ (47, 67%) and $61 \pm 13\%$ (70, 67, 47%) individuals with skeletal malformations, respectively, from which 88, 85, 81 and 73%, respectively, presented caudal fin malformations, more specifically corresponding to the fusion of the last two vertebrae before the urostyle (preural) along with fused and/or malformed hemal and neural arches and fusions affecting hypuralia and malformed caudal fin rays. Malformations affecting pleural and caudal vertebra (except preural) represented 27, 39, 49 and 37% from the total of deformations found in treatments A, B, C and D, respectively.

Discussion

Survival and growth performance

The relationship between dietary levels of (n-3) HUFA, particularly DHA and EPA, and larval survival in marine species remains unclear. A positive relationship has been observed in several species, such as seabream (Salhi et al. 1994) and red seabream, *Pagrus major* (Temminck and Schlegel, 1843) (Watanabe et al. 1989; Watanabe 1993), but other studies conducted also with seabream (Koven et al. 1990; Koven et al. 1992; Pousão-Ferreira et al. 1999) and turbot (Rainuzzo et al. 1994; Reitan et al. 1994; Estévez et al. 1999) failed to demonstrate such a relationship. The results of previous studies (Morais et al. 2004; Villalta et al. 2005) appeared to support a lack of correlation between the prey's HUFA, particularly DHA, concentration and larval survival in Senegalese sole.

Other studies that used low lipid and/or HUFA-deficient (devoid in DHA and low EPA) *Artemia* only during the benthic post-larval stage (Morais et al. 2005a, b) found very high survival rates (over 90%) from 16 to 36 DAH. This post-larval period appears to correspond to a phase of very low mortality, as was also noted in the present study. In this study, the high variability obtained within treatments did not enable finding significant differences in survival, but a trend for lower survival of larvae fed NEA during the whole larval and post-larval rearing (treatment B) was found at 50 DAH (end of the weaning period), which was no longer evident at 60 DAH.

Studies by Rainuzzo et al. (1994), Reitan et al. (1994) and Estévez et al. (1999) showed no correlation between dietary (n-3) HUFA content and the growth rate of turbot larvae. Additionally, Howell and Tzoumas (1991) found that a higher DHA inclusion level did not improve *Solea solea* (Linnaeus, 1758) growth rate, and high growth and survival rates could be achieved with diets deficient in DHA. These authors suggest that Dover sole and Senegalese sole are less demanding in terms of (n-3) HUFA dietary requirements, when compared with many other marine species, and that there is no need for enhancing the *Artemia* DHA concentration, as long as there is a sufficient provision of EPA. In the present study, there was no immediate effect of dietary treatment on larval TL and DW at the end of the pelagic stage (19 DAH) but at 30 DAH there was a significant difference in growth, both in terms of TL and DW, between larvae that had been fed only EA (treatment A) and NEA (treatment B), being the latter ones smaller. The differences between treatment B and the other three treatments were accentuated over time, with these larvae being significantly smaller and lighter at the start of weaning (40 DAH) and at the end of the experiment (60 DAH). However, in spite of their lower weight at 40 DAH (5.5 vs. 7.9–8.9 mg), post-larvae from treatment B were still within the 5–10 mg DW window considered suitable to start weaning sole (Conceição et al. 2007).

The results obtained in this experiment have thus shown that the idea that Senegalese sole larvae could be fed on NEA or on EA containing low HUFA, and negligible DHA levels without deleterious effects on performance was a misconception, derived from the fact that the performance of those larvae was evaluated for a short period of time after acquisition of benthic life (up to 36 DAH) (Morais et al. 2004, 2005a, b; Villalta et al. 2005). Moreover, our results have shown that as long as EA was fed during the pelagic stage, as in treatment C, no significant differences were found in either growth or survival, in comparison with larvae that were fed EA during the whole larval and post-larval period (treatment A), indicating that larvae might be more sensitive to (n-3) HUFA deficiencies during the pelagic stage.

Fatty acid composition

As observed in different species, the FA profile of Senegalese sole larvae closely reflected the composition of their diet (e.g., Rainuzzo et al. 1994; Reitan et al. 1994; Estévez et al. 1999; Pousão-Ferreira et al. 1999; Villalta et al. 2005). There appears, however, to be a certain delay in the dietary effect, which might depend on the age of the animals, i.e., the rate at which they are growing, and on the previous nutritional regime. In the present study, no significant differences were observed at 19 DAH in the FA composition of sole larvae despite differences in live prey composition. However, after this age it was possible to observe a dietary effect in sole body composition. In fact, from 19 to 40 DAH post-larvae from treatments A and D (fed 100 or 50% EA) showed the same pattern of change, while treatment B (100% NEA) and C (fed 100% NEA from 19 DAH onwards) behaved similarly in terms of the trends of variation of their FA composition. Moreover, the changes in

juvenile FA profile from 40 to 60 DAH reflected the introduction of the formulated feed in the feeding protocol. The higher percentage of LA in formulated diet and its much lower level of LNA were clearly reflected in the increase and decrease of these FA, respectively, from 40 to 60 DAH in all experimental treatments. Likewise, the higher relative and absolute level of DHA in the formulated feed resulted in an increase in the percentage of this essential FA in all treatments after weaning. As for the EPA level, it decreased from 40 to 60 DAH in the treatments that had been previously fed 100 or 50% EA (A and D), which had a higher relative level of EPA, while it remained constant in the treatments that had been fed NEA (treatments B and C), which was a poorer source of this FA. In spite of this, at the end of the experiment (60 DAH), the differences in juvenile FA profile were quite marked and reflected the previous nutritional history, both in absolute and relative FA levels. This was somewhat surprising since at this age fish from different experimental groups were under the same feeding regime for already 10 days. It seems therefore that juveniles that were reared on different dietary regimes during their larval and post-larval stages need a very long time before they can recover (if they ever do) from eventual lipid and (n-3) HUFA deficiencies, after they are weaned into formulated feed.

The results have also shown that even if Senegalese sole larvae are fed EA during the pelagic larval stage, up to 19 DAH, and NEA is only introduced in the benthic post-larval stage (treatment C), the effect of feeding this total lipid and (n-3) HUFA-deficient diet for about 13 days is still noticeable in the larval biochemical profile at 60 DAH, as they have still not fully recovered in terms of total FAME levels. It might be speculated whether the weaning at the same age rather than at the same size could have produced different results in the capacity of post-larvae to recover from previous nutritional deficiencies. Nonetheless, not only all treatments at 40 DAH had a weight that is considered suitable for weaning (Conceição et al. 2007), but also there was no significant difference in TL and DW between treatments A, C and D at this age. Still, for most FA analyzed, and in relative terms, the composition of treatment C was intermediate and not significantly different from the treatments fed 100 or 50% EA (A and D) during the whole larval and post-larval period, but was also not significantly different from treatment B, fed exclusively NEA until 40 DAH. Even though no significant effects were noticed in all the performance and quality parameters analyzed, it cannot be ascertained whether the lower body total FAME level of this treatment would not have a negative impact at an even later stage in juvenile development. On the other hand, the effect of feeding NEA during only the pelagic larval stage was not tested in the present experiment and would be interesting to investigate in future studies, in order to verify whether the larvae are capable of recovering their total lipid levels if the total lipid and (n-3) HUFA deficiency occurs at an earlier larval stage.

At the end of the experiment, no significant differences were found in the FA profile of sole fed 100% EA during the whole larval and post-larval rearing period (treatment A) and those fed 50% EA and 50% NEA (treatment D), both in terms of the absolute amounts (not shown) and relative levels of total FAME and individual FA analyzed. Furthermore, no significant differences were found in terms of survival, growth, activity of the assayed digestive enzymes and incidence of malpigmentations or malformations (see "Discussion" below). It has been shown that dietary FA composition may affect seabream larval food intake and absorption efficiency (Morais et al. 2006) but we have no knowledge of studies on larval feeding selectivity and prey attractability in relation to the HUFA content of the prey, when preys differing in their FA composition are presented simultaneously. However, if we assume that Senegalese sole larvae do not selectively ingest EA, in detriment of NEA, we might hypothesize that the dietary supply of (n-3) HUFA that, in the present study was 6.3 mg DHA g^{-1} DW and 12.8 mg EPA g^{-1} DW in EA, can be greatly reduced

(up to half, in the case of DHA), without deleterious effects on larval and juvenile performance and quality. Although this remains speculative, a previous study of Villalta et al. (2005) using prey enriched on graded levels of DHA indicated that a better growth (only significantly for TL) was obtained with 2.4 mg DHA g^{-1} DW than with 1.5 or 4.7 mg DHA g^{-1} DW. However, Villalta et al. (2005) observed no differences between the best performing treatment and that containing no DHA, up to 36 DAH.

Digestive enzymes

The analysis of digestive enzyme activities at different stages of the experimental period revealed, in general, more important differences in terms of segmental activity, directly reflecting the differences observed in Senegalese sole growth and development. Hence, for most enzymes analyzed, with the exception of lipase, a significantly lower segmental activity was found in the smallest animals (treatment B) at 60 DAH. Interestingly, significant differences were detected at 60 DAH, when all treatments had been submitted to a similar feeding regime during the previous 10 days, and less so when fish were being fed diets with different lipid and (n-3) HUFA contents. The digestive capacity of sole larvae was not significantly affected by the lipid and (n-3) HUFA content of the diet at 19 DAH. However, at 40 DAH, significant differences were observed for alkaline phosphatase and amylase segmental activity between the NEA treatment (B) and the remaining treatments, for alkaline phosphatase, or treatment A, for amylase. Moreover, although non-significantly, trypsin activity exhibited a similar tendency. This suggests that post-larvae from treatment B presented a delayed development and were not prepared to start weaning. This was clearly confirmed at 60 DAH by the globally significantly lower digestive enzyme activities (both specific and segmental) exhibited by sole from treatment B. However, based on the knowledge gathered so far, the lower size of larvae from treatment B at weaning is unlikely to have been responsible for these effects, as the introduction of formulated feed in co-feeding with *Artemia* has not been seen to affect the digestive enzymes profile as long as post-larvae have more than 2 mg of DW (Conceição et al. 2007; Engrola et al. 2007), which was the case in the present study (average of treatment B: 5.5 ± 2.26 mg; minimum weight 2.8 mg). It is therefore possible that the size of the larvae is not a sufficient indication of readiness for weaning. During normal development, sole larvae acquire an adult mode of intestinal digestion around 25 DAH (Ribeiro et al. 1999). In this study, although no immediate differences were observed on growth, the lower FAME and (n-3) HUFA content of NEA might have affected the biosynthesis of some structures, such as intestinal membranes. As a consequence, an adult mode of intestinal digestion might have been achieved later than 25 DAH in treatment B, thus affecting the activity of alkaline phosphatase. Alkaline phosphatase levels are related to the gut absorptive capacity, given that it is an enzyme involved in nutrient absorption and transport across the intestinal epithelia (Fraisse et al. 1981), and higher levels of alkaline phosphatase are considered a marker of better developmental and nutritional status (Ribeiro et al. 2002).

Juvenile quality

Juvenile quality was assessed at the end of the experiment using as parameters the percentage of malpigmented juveniles and incidence of skeletal malformations. In both cases, no significant differences were found between experimental treatments, indicating that these parameters were not affected by dietary levels of lipid and (n-3) HUFA. As is usually

the case in Senegalese sole culture (Dinis et al. 1999), malpigmentations were extremely rare and did not represent a problem in the present experiment. On the other hand, skeletal malformations, although not apparent externally, were prevalent, with 40–80% incidence. Of these, a large percentage of deformations were in the caudal complex, resulting from the fusion of the preural vertebrae. A predominance of malformations in the caudal region had been previously described by Gavaia et al. (2002). These levels of malformations are comparable to those observed in another flatfish, the Japanese flounder, that presented up to 60% of axial skeleton deformities (Hosoya and Kawamura 1995, 1998), but are much lower than the levels observed for other species commonly produced in Mediterranean aquaculture such as seabream and sea bass that can reach levels of 100 and 75% deformed fish, respectively (Boglione et al. 1993; Boglione et al. 2001; Marino et al. 1993). In addition, a high variability between replicate tanks was noticeable in this experiment, indicating more likely rearing problems due to zootechnical rather than feeding conditions.

Conclusion

The results obtained in this study highlight the importance of examining the effects of larval and post-larval nutrition on later stages of development. Globally, the results support the notion that Senegalese sole have lower requirements for lipid and (n-3) HUFA during their larval and post-larval rearing stage, compared to other marine species. In fact, a diet of 50% NEA and 50% EA did not produce significant differences in any of the parameters analyzed, compared to a standard dietary regime composed of 100% EA. Nonetheless, it has now been shown that deleterious effects on juvenile performance (growth, FA composition and development of the digestive system), but not in quality (malpigmentation and skeletal malformations), may arise if rearing Senegalese sole larvae on NEA during the whole larval and post-larval stage. Still, the pelagic stage seems more critical and sole appear to have even lower (n-3) HUFA requirements during the post-larval benthic stage, given that good juvenile performance (not significantly different from treatment A fed EA during the whole larval and post-larval period) was achieved when larvae were fed EA up to 19 DAH and post-larvae were fed NEA afterward. At present, and based solely on the present experiment, we cannot advocate the use of only 50% of EA in feeding protocols in Senegalese sole hatcheries. However, it seems clear that enrichment strategies and products leading to lower DHA levels than those currently employed in marine hatcheries can be implemented in Senegalese sole larval rearing without deleterious effects on larval, post-larval and juvenile survival, growth and quality. There is thus a need for more and even longer-term studies to estimate more accurately (n-3) HUFA, and particularly DHA, requirements during different stages of larval and post-larval rearing.

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