Effects of dietary eicosapentaenoic acid on growth, survival, pigmentation and fatty acid composition in Senegal sole (*Solea senegalensis*)

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Keywords: Eicosapentaenoic acid; Fatty acid; Larvae; *Solea senegalensis*; Enrichment; Live food

Abstract

We examined the effect of dietary eicosapentaenoic acid (20:5n-3, EPA) on growth, survival, pigmentation and fatty acid composition of Senegal sole larvae using a dose-response design. From 3 to 40 days post hatch (dph), larvae were fed live food that had been enriched using one of four experimental emulsions containing graduated concentrations of EPA and constant docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, ARA). Proportions of EPA in the enriched *Artemia* nauplii were described as "nil" (EPA-N, 0.5% total fatty acids, TFA), "low" (EPA-L, 10.7% TFA), "medium" (EPA-M, 20.3% TFA) or "high" (EPA-H, 29.5% TFA). Significant differences among dietary treatments in larval length were observed at 25, 30 and 40 dph, and in dry weight at 30 and 40 dph, although no significant correlation could be found between dietary EPA content and growth. The stage of eye migration at 17 and 25 dph was significantly affected by dietary levels of EPA. Significantly lower survival was observed in fish fed EPA-H enriched nauplii. A significantly lower percentage of fish fed EPA-N (82.7%) and EPA-L (82.9%) diets were normally pigmented compared to the fish fed EPA-M (98.1%) and EPA-H (99.4%) enriched nauplii. Tissue fatty acid concentrations reflected the corresponding dietary composition. Arachidonic and docosahexaenoic acid levels in all the tissues examined were inversely related to dietary EPA. There was an increase in the proportion of docosapentaenoic acid (22:5n-3, DPA) in the tissues relative to the diet, which is indicative of chain elongation of EPA. This work concluded that Senegal sole larvae have a very low EPA requirement during the live feeding period.

Introduction

Senegal sole (*Solea senegalensis*) has been identified as a candidate species for commercial culture in the Mediterranean and South Atlantic coasts (Dinis et al., 1999). However, anomalous pigmentation of cultured flatfish (Naess and Lie, 1998) and high mortalities during the weaning period (Padrós et al., 2003; Zarza et al., 2003), are still major obstacles to the successful culture of this species. Both problems have been associated with the highly unsaturated fatty acid (HUFA) content of the live food used in first feeding in most flatfish species studied to date (Bell et al., 1985; Izquierdo, et al., 1992; Bell et al., 1995, 2003; Rodriguez, et al., 1997; McEvoy et al., 1998). Previous research has demonstrated associations between dietary HUFA with larval pigmentation and performance (Villalta et al., 2005a,b).

During the larval stage of teleosts, lipids play an important role as sources of metabolic energy, components of membrane phospholipids and as precursors of bioactive molecules (Sargent et al., 1999b; Tocher, 2003). In particular, the role of HUFA on larval development has been extensively studied (Rainuzzo et al., 1991; Izquierdo, 1996; Takeuchi et al., 1996; Estévez et al., 1999; Sargent et al., 1999a, 1999b; Izquierdo et al., 2000). Determining the dietary requirements for normal growth and development is an important step in the successful culture of candidate species (Izquierdo, 1996; Takeuchi et al., 1996). In particular, many marine larvae require n-3 HUFA, like DHA or EPA, for normal larval development and survival (Castell et al., 1994; Takeuchi et al., 1996; Furuita et al., 1998; McEvoy et al., 1998; Copeman et al., 2002). Conversely, we have previously established the non-essentiality of dietary DHA for Senegal sole to 35 days post hatch (dph). Further, we found that larvae fed DHA

deficient *Artemia* nauplii show normal development and skin pigmentation as well as high survival rates (Villalta et al., 2005b). The requirement of Senegal sole larvae for ARA has also been determined (Villalta et al., 2005a), but the requirement for EPA still remains unclear. Thus, this study was designed to investigate the role of dietary EPA on growth, survival and pigmentation of Senegal sole larvae from 1 to 40 dph. A dose-response design, which are commonly used in juvenile and adult fish to determine nutrient requirements, was also utilised in this study and had been used successfully in our previous studies (Villalta et al., 2005a,b).

2. Material and methods

2.1. Experimental emulsions

Commercially available DHA, ARA and EPA rich oils were obtained, respectively, from the heterotrophically grown algae *Cryphecodinium cohnii* (Neuromins®, Martek Bioscience, USA), fungus *Mortierella alpina* (Vevodar®, DSM, Netherlands) and fish oil triglycerides (Croda®, Incromega EPA500TG, UK). Different combinations of these oils were formulated in order to produce emulsions with "nil" (EPA-N), "low" (EPA-L), "medium" (EPA-M) or "high" (EPA-H) EPA contents. The components used in the formulation of each emulsion and the major fatty acid compositions in the enriched *Artemia* nauplii are shown in Table 1. The emulsions were made as described by Dunstan el al. (2003).

2.2. Live food enrichment

Rotifers were enriched in 10 l containers at a density of 500 rotifers ml^{-1} for 6 h at 20ºC using 0.1 g l^{-1} of each emulsions. After 6 h, the rotifers were gently washed with

UV filtered seawater, rinsed for a further 1 min with freshwater to reduce bacterial load, and subsequently fed to the larvae.

An Argentinean strain of *Artemia* (*Artemia persimilis*) with low initial levels of EPA was selected; the fatty acid compositions of non enriched *Artemia* are shown in Table 1. Hatching of the cysts and enrichment of the nauplii were carried out at 18‰ salinity and a temperature of 25ºC, following the recommendations of the supplier. Six hour-old *Artemia* nauplii were enriched in 10 l containers at 100 nauplii ml^{-1} for 18 h with 0.6 g l^{-1} of the emulsions. Enriched metanauplii were thoroughly washed with UV filtered seawater and freshwater for 15 min before feeding to the larvae.

Artemia was sampled at three time points during the experimental period, for lipid analysis.

2.3. Sole larviculture

Senegal sole (*Solea senegalensis*) eggs were obtained from CIFPA "El Toruño" (Cádiz, Spain) from a broodstock held under natural photo and thermo periods. Newly hatched larvae were transported by road to Centre d'Aqüicultura-Institut de Recerca i Tecnologia Agroalimentàries (CA-IRTA). Once at CA-IRTA, larvae were randomly distributed (50 larvae l^{-1}) into twelve 35 l, 150 μm mesh baskets distributed amongst four 1500 l holding tanks. Three baskets were distributed in each holding tank. The three baskets within one tank received the same dietary treatment. All 1500 l tanks were connected to a recirculation unit assuring the same conditions of light, flow rate and water quality, described in previous experiments (Villalta et al., 2005a,b). Larvae were fed on rotifers from 3 to 5 dph at a density of 10 rotifers ml^{-1} and *Artemia* nauplii from 5 until 40 dph. Triplicate baskets of larvae were fed on each of the experimentally enriched live feeds. The *Artemia* ration was adjusted as in Villalta et al., (2005a,b) to

avoid unenriched leftovers (70% body weight, BW, day^{-1} from 5 to 15 dph, 20% BW from 16 to 25 dph, 15% BW from 26 to 30 dph and 7% BW from 31 to 40 dph). Live preys were given twice per day (9.00 and 16.00 h). Water changes (200%) in the holding tanks were performed daily.

Standard length and dry weight were measured at 1, 4, 6, 10, 12, 15, 17, 20, 25, 30 and 40 dph. Twenty larvae were sampled, placed in beakers and euthanased using a lethal concentration of 3-amino benzoate methane sulphonate (1000 mg l^{-1}, MS 222). Length was measured using a dissecting microscope and image analyser (AnalySIS, SIS Gmbh, Germany). Dry weight (DW) determination was carried out by rising larvae with distilled water to remove salt and then oven-dried at 60ºC for 24 h. Eye migration during sole metamorphosis was assessed according to the description of Fernandez-Diaz et al. (2001). Data are presented as the relative amount of larvae at each stage of development at the same age. Eye migration index (I_{EM}) was calculated according to Solbakken et al. (1999) considering stage 3b and 4 of Cañavate and Fernández-Díaz (1999) as 4 and 5 in the calculation of the index.

$$I_{EM} = \Sigma(\%\text{fish in each stage}*\text{stage}) / 100$$

Triplicate samples were taken at 1 dph (N = 2076) for initial biochemical analysis, additional larvae were sampled at 15 dph (100 larvae $basket^{-1}$). For an assessment of final biochemical composition, 85 larvae $basket^{-1}$ were sampled at 40 dph, with the heads, guts and carcasses dissected on ice and kept separately for lipid analysis. The samples were placed directly into chloroform:methanol (2:1, v:v) and stored under nitrogen at -20ºC until analysis.

Survival and pigmentation success were determined at the end of the experiment (40 dph) by counting and assessing all the remaining larvae. Abnormally pigmented individuals (totally or partially malpigmented) were considered as a whole group

without giving any category or pigmentation index due to the difficulties of such classifications (Bolker and Hill, 2000).

2.4. Lipid analysis

Total lipids from enriched live food and larval tissues 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 stream of nitrogen followed by vacuum desiccation overnight. Total lipids were stored in chloroform:methanol (2:1, 10 mg ml^{-1}) at -20ºC until final analysis.

Acid catalysed transmethylation was carried out using the method of Christie (1982). Methyl esters were extracted twice as described by Ghioni et al. (1996). Fatty acid methyl esters were analysed by gas-liquid chromatography on a Thermo Electron TraceGC (Runcorn, UK) instrument fitted with a ZB-Wax capillary column (30m x 0.25 mm id; Phenomenex, Macclesfield, UK), using a two stage thermal gradient from 50ºC (injection temperature) to 150ºC at 40ºC min^{-1} and then to 225ºC at 2ºC min^{-1} and finally holding for 5 min at 225ºC. Hydrogen was used (2.0 ml min^{-1} constant flow rate) as the carrier gas, injection was on-column and detection was by flame ionisation at 250ºC. Individual fatty acids were identified by comparison with well characterised fish oil, and quantified by means of the response factor to the internal standard, 17:0 fatty acid, added prior to transmethylation.

2.6. Statistics

The variance of the data is given as standard deviation (SD) of the mean of three replicates with differences tested for statistical significance ($P \leq 0.05$) by one-way ANOVA followed by a pair-wise multiple comparisons of means using Tukey's test,

following testing for normaility and homogeneity of variance. Percentage data were square root transformed for normality. A Statgraphics package (Microsoft) was used for all statistical analysis. Due to a significant mortality event in one of the feeds (EPA-H), this treatment was not included in the statistical analysis, but the data remain for comparison.

3. Results

3.1. Lipid composition of live prey

Lipid and fatty acid composition of *Artemia* nauplii before and after enrichment is presented in Table 1. The composition of the rotifers is not shown due to the short time of rotifer feeding (only 3 days). No significant differences were found in absolute amounts of total lipid and total fatty acids among the nauplii. Significant differences were found in total saturated fatty acids (SFA) due to the differences in the relative content of 14:0 and 16:0, and in total monounsaturated fatty acids (MUFA) due to the differences in 18:1n-9 and 18:1n-7. No significant differences were found in n-6 PUFA. The significant differences observed in n-3 PUFA are due to the differences in EPA, reflecting the composition of the emulsions. The relative ratios of DHA/EPA, ARA/DHA and ARA/EPA in the nauplii varied significantly between EPA-N (being the highest) and the rest of the groups.

3.2. Larval performance

Results obtained for growth, survival and pigmentation success are shown in Table 2. Survival was poorest in larvae fed EPA-H. When this was analysed it was found to be significantly different ($P < 0.01$) to all other treatments. As a reduced

number of larvae may have resulted in larger fish through reduced competition for food items and interactions between larvae, this treatment was excluded from the statistical analysis to eliminate the possible confounding factors. Significant differences in growth in length were observed between EPA-N and EPA-M and -L larvae at days 17, 25 and 40 dph (Fig. 1a), although at the end of the experiment the largest larvae were those of groups –N and –H. Growth in weight for EPA-H group of larvae was higher from day 30 onwards, whereas EPA-L and EPA-M fed larvae showed the lowest growth in weight along the study period (Fig. 1b).

Significant differences ($P<0.05$) in the stage of metamorphosis were found at 17 and 25 dph, with EPA-H fed larvae being in a more advanced stage of eye migration (I_{EM} 2.22 and 4.25 at 17 and 25 dph, respectively) than the larvae fed lower levels of EPA (Fig. 2). The highest dietary EPA accelerated the degree of eye migration at 17 and 25 dph. At 17 dph the most delayed eye migration stage occurred in the EPA-M treatment (I_{EM} = 1.79), followed by EPA-L and EPA-N (I_{EM} = 1.83 and 1.93, respectively) while larvae fed EPA-H had the most advanced (I_{EM} = 2.22) eye migration stage. Similarly, at 25 dph the most delayed eye migration stage was in the EPA-L (I_{EM} = 3.52), followed by EPA-N and EPA-M (I_{EM} = 3.78 and 3.98, respectively) while larvae fed EPA-H had the most advanced (I_{EM} = 4.25) eye migration. Eye migration was complete and normal in all the groups at the end of the experiment.

3.3 Effects of diet on pigmentation rate

Significant differences (Table 2) were found in pigmentation rate with significantly higher number of malpigmented larvae found in groups fed EPA-N and EPA-L enriched nauplii. A significant positive regression was found between

pigmentation rate and EPA relative dietary content (Pigmentation = 80.45+0.77EPA, $P<0.05$, $r^2=0.75$).

3.4. Dietary effects on lipid and fatty acid composition of larval tissues

After 40 days of feeding, larval whole body lipid composition reflected dietary changes (Table 3). No significant differences were found in total lipid or total fatty acid content among the groups. The fatty acid composition (% total fatty acid, %TFA) of the whole fish reflected the composition of the diets. Significant differences were found in total monounsaturated fatty acids primarily due to 18:1n-9 content. Significant differences were also found in total n-6 PUFA content between EPA-N and the other groups due to the higher linoleic (18:2n-6) and ARA contents. A significant negative regression was found between *Artemia* nauplii relative EPA content and whole body relative ARA content (Fig. 3a). Total n-3 PUFA composition was significantly different among the groups, whereas linolenic (18:3n-3) and EPA contents were significantly higher as EPA dietary content increased. DHA was found to be higher in EPA-N fed larval body followed by EPA-L and EPA-M fed fish. Fish fed EPA-H nauplii had the lowest DHA body content. A significant positive regression was found between *Artemia* nauplii relative EPA content and whole body relative EPA content (Fig. 3b), and a significant negative regression between *Artemia* nauplii relative EPA content and whole body relative DHA content (Fig. 3c).

4. Discussion

No dose-response relationship was recorded between dietary EPA and growth in Senegal sole larvae to 40 dph. This suggests Senegal sole larvae are able to grow and

survive on *Artemia* nauplii with negligible EPA content up to 40 dph. Significant mortality occurred in larvae fed EPA-H, the exact cause of which remains unknown. As a consequence of this mortality it is possible EPA-H larvae had access to larger quantities of food than the others and less interactions between each other. This may have resulted in the greater growth recorded in these larvae (7.2mg dry weight compared with 5mg as the next best growth). The timing of the mortality is unclear, as daily assessment of dead and moribund larvae is not possible in the experimental system used. However at 17 dph (mid metamorphosis) the EPA-H group of fish showed a more advanced stage of development than the other groups of fish, possibly indicating the mortality event had already occurred. Considering these results and in order to elucidate the effects of dietary EPA levels on growth, pigmentation and fatty acid composition of the larvae, the EPA-H group were excluded from the statistical analysis.

With removal of EPA-H, significant differences between growth of EPA-N and –L and –M larvae were recorded, with EPA-N fed larvae being approximately 60% larger than those fed EPA-L and EPA-M. A possible explanation for these differences is the availability of dietary energy. Fish obtain the energy for growth and metabolic activity mainly from the saturated and monounsaturated fatty acids (16:0, 18:1n-9, 20:1n-9) that are deposited in tissue storage lipids (triglycerides) and are easily catabolized (Sargent et al., 2002). EPA-N enriched *Artemia* were particularly rich in 18:1n-9, making this diet more energy dense than the others. Conversely, fish fed EPA enriched *Artemia* nauplii were less abundant in monounsaturated fatty acids compared to EPA-N. This may have resulted in other fatty acids, including DHA and EPA, to be used for energy production (Villalta et al., 2005b), which may also explain the decrease in the concentration of tissue DHA (table 3). However, it may also be explained by competitive interactions between fatty acids.

In general, Senegal sole larvae fed the experimental diets had fatty acid compositions that reflected dietary composition, which is consistent with other studies carried out with other marine fish larvae (Mourente et al., 1993; Rodríguez et al., 1994; Harel et al., 2000; Koven et al., 2001; Bransden et al., 2004, 2005a,b,c; Villalta et al., 2005a,b). The emulsions in the present study were formulated to ensure DHA and ARA remained constant among the four groups, as was reflected in the composition of *Artemia* nauplii (see Table 1). However, larval tissue composition showed a different trend such that the content of DHA and ARA actually decreased concomitant with increasing dietary EPA (see Figs. 3a, 3c). While catabolism for energy is one possible explanation, more likely these reductions in DHA and ARA can be explained by the interactions of some HUFA. Displacement of tissue EPA by ARA has been described by several authors (Tocher and Sargent, 1986; Bell et al., 1995; Bessonart et al., 1999; Willey et al., 2003), and is explained by the competitive interaction between these two fatty acids for the *sn*-2 position on membrane phospholipids. In the present study EPA content in the tissues increased in parallel with a clear and significant reduction in ARA concentration, a fact already observed by Bell et al. (1989) in salmon. There was also an apparent competition between EPA and DHA such that larval DHA concentrations were reduced, even though dietary concentrations were maintained or increased. This suggests that at high dietary EPA concentrations DHA was out-competed by EPA for acylation to phospholipids. The molecular speciation of fish phospholipids suggest that the *sn*-2 position is favoured by ARA, EPA and DHA so that if one is present in excess then it will dominate over the other two HUFA for acylation in membrane phospholipids (Bell and Dick, 1991a,b).

Increasing dietary EPA resulted in higher accumulation of tissue docosapentaenoic acid (22:5n-3, DPA). Accumulation of DPA is due to the elongation

of EPA and the very low rate of conversion of DPA to DHA, as has been observed in several marine fish species (Mourente and Tocher, 1994; Takeuchi et al., 1996; Bell et al., 1995; Bransden et al., 2004). However some fish, especially freshwater fish, are better able to convert EPA into DHA than marine species (Sargent et al., 1989, 1995). In this case, the first step in the elongation of EPA to DPA is by the action of elongase enzymes, followed by DPA conversion to DHA in a complex chain of reactions including elongation, Δ6 desaturation and chain shortening, making this a rate limiting process. The conversion of EPA to DHA may have been further compromised by the relatively high levels of dietary 18:3n-3 in the present study. Mammalian studies have shown that high levels of dietary 18:3n-3 can inhibit synthesis and incorporation of DHA due to the competition of 18:3n-3 and 24:5n-3 for the Δ6 desaturase enzyme (Cleland et al., 2005). As a consequence, the larvae are not able to elongate and desaturate EPA to DHA at a significant rate, and, thus, the intermediate product, DPA, accumulates, as has been observed in Senegal sole larvae in the present study. Our previous work (Villalta et al., 2005b), however, indicated the non-essentiality of dietary DHA for Senegal sole during the early larval rearing stages, so it is unlikely that this accumulation of DPA is indicative of a DHA deficiency, with a subsequent physiological attempt to rectify this through EPA to DHA conversion. More probably the increasing tissue DPA only reflects the increased availability of substrate (i.e. EPA) as more EPA is provided in the diet.

There is a positive correlation between the dietary content of EPA, the accumulation of EPA in larval tissues and the final pigmentation rate of the fish. Previous studies have indicated that pigmentation success in flatfish is related negatively to dietary ARA, as well as the ratios ARA/EPA (Bell et al., 2003; Villalta et al., 2005) and DHA/EPA (Reitan et al., 1994). An intrinsic role of eicosanoids, of which

ARA and EPA are precursors, has been postulated to be involved in the pigmentation process (Sargent et al., 1999b), and recent studies have provided further evidence for this (Bransden et al., 2005b). In the present study we have found that the malpigmentation observed in the groups EPA-N and EPA-L was a consequence of dietary EPA content and the sub-optimal ratios of ARA/EPA and DHA/EPA. Malpigmentation in flatfish aquaculture is a serious economic problem and improvements to our understanding of how it is nutritionally induced can lead to better management in the future.

In the present study Senegal sole larvae grew as well on live foods with negligible EPA compared with those larvae fed diets with higher concentrations of this essential fatty acid. While the best growth was recorded in larvae fed the highest dietary EPA concentrations (29.5% TFA) these larvae also had the highest rate of mortality, which probably resulted in a greater availability of food items per larva, and subsequently greater growth. Tissue fatty acid profiles generally reflected the dietary proportions, with the exception of DHA and ARA, which was explained by the competitive interactions of the EFA. Along with our previous work on DHA and ARA requirements (Villalta et al., 2005a,b), these data provide further evidence that Senegal sole larvae are unusual compared to many other marine fish species in that they have a very low requirement for n-3 HUFA during the live feeding period. Abnormal pigmentation can be a serious economic problem in commercial aquaculture of flatfish species and this study provides further evidence for how these problems can be avoided through careful nutritional management during the larval stages.

Acknowledgements

Senegal sole eggs were generously donated by CIFPA "El Toruño" (Cádiz, Spain). Neuromins® oil was provided by Martek Biosciencies, USA. Vevodar® oil was provided by DSM, Netherlands. Olive oil was generously provided by Enrique García Tenorio (D.O. Montes de Toledo, Toledo, Spain). MV would like to thank the financial support provided by Generalitat de Catalunya (DURSI scholarship). Funding was partially provided to AE by the Spanish Ministries of Science and Education (INIA project ACU-02-006) and Agriculture, Fisheries and Food (Jacumar), and by the Generalitat de Catalunya (CRA). MPB was financially supported by the Australian Aquafin CRC. We owe thanks to O. Bellot, J.Canoura, G. Macià and M. Monllaó for their help during larval rearing, and to J. Dick, F. Strachan and R.J. Henderson for analytical assistance. This investigation was supported through an award to AE from the European Union Access to Research Infrastructures (ARI) Action of the Improving Human Potential (IHP) Programme (contract HPRI-CT-2001-00180).

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Table 1. Formulation (mg g^{-1}) of the experimental emulsion, the resulting enriched *Artemia* nauplii and non enriched *Artemia* profile of key fatty acids (% weight of total fatty acids (TFA), mean±SD, n=3).

	EPA-N	**EPA-L**	**EPA-M**	**EPA-H**	**Non enriched *Artemia***
Formulation (mg g^{-1})					
Vevodar® oil[a]	47	31.3	15.1	0.0	
Neuromins® oil[b]	114.8	76.6	38.3	0.0	
Croda® oil[c]	0.0	175.7	351.5	527.8	
Olive oil[d]	366	244.2	123	0.0	
Supplements[e]	52.2	52.2	52.2	52.2	
Total lipids (mg g^{-1} DW)	187.4±11.7	200.3±22.2	208.4±18.0	208.1±56.7	148.2±14.1
Total FA (mg g^{-1} DW)	119.9±16.4	121.6±9.2	155.4±69.2	115.0±44.1	77.9±0.3
14:0	1.9±0.3^{c}	1.2±0.3^{b}	0.7±0.2^{a,b}	0.4±0.1^{a}	0.6±0.1
16:0	12.3±0.8^{b}	10.2±0.2^{a,b}	9.1±0.2^{a,b}	8.3±2.3^{a}	14.4±0.3
18:0	3.9±1.5	4.1±0.1	3.8±0.2	3.7±1.0	5.3±0.1
Total saturated	19.4±1.8^{b}	16.2±0.6^{a,b}	14.2±0.5^{a,b}	12.9±3.5^{a}	21.3±0.4
16:1n-7	2.2±0.3	2.2±0.2	2.3±0.1	2.4±0.6	4.5±0.0
18:1n-9	42.6±5.2^{d}	31.3±1.6^{c}	21.6±0.4^{b}	11.5±2.8^{a}	18.2±0.1
18:1n-7	5.0±0.7^{a}	5.3±0.5^{a}	5.5±0.1^{c}	5.9±1.5^{b}	9.1±0.1
20:1n-9	0.5±0.1	0.3±0.3	0.4±0.3	0.4±0.3	0.3±0.5
20:1n-7	0.1±0.1	0.1±0.1	0.1±0.0	0.1±0.0	0.2±0.0
Total monounsaturated	50.7±4.3^{d}	39.6±1.0^{c}	30.4±0.3^{b}	21.0±5.2^{a}	33.1±0.4
18:2n-6	5.8±0.5	5.7±0.2	5.1±0.2	5.2±0.8	6.3±0.1
20:2n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.0
20:3n-6	0.3±0.1	0.3±0.0	0.3±0.0	0.2±0.0	0.2±0.0
20:4n-6	2.1±0.3	2.2±0.1	2.1±0.1	2.0±0.6	0.0±0.0
Total n-6 PUFA	8.9±0.9	9.0±0.4	8.3±0.3	8.3±0.1	7.5±0.1
18:3n-3	12.8±3.9	14.7±2.1	15.7±1.2	16.0±3.7	30.6±0.4
20:5n-3	0.5±0.2^{a}	10.7±0.3^{a,b}	20.3±0.7^{b,c}	29.5±9.7^{c}	0.3±0.5
22:5n-3	0.2±0.4	0.3±0.0	0.5±0.0	0.7±0.3	0.0±0.0
22:6n-3	4.3±0.8	4.7±0.3	4.8±0.2	4.8±1.8	0.1±0.1
Total n-3 PUFA	20.9±5.3^{a}	35.2±1.6^{b}	47.1±1.0^{b,c}	57.8±8.7^{c}	38.1±0.1
Total PUFA	29.8±6.1^{a}	44.2±1.6^{b}	55.4±0.8^{b,c}	66.1±8.7^{c}	45.7±0.0
DHA / EPA	8.9±2.6^{b}	0.4±0.0^{a}	0.2±0.0^{a}	0.2±0.0^{a}	0.1±0.1
ARA / DHA	0.5±0.0^{b}	0.5±0.0^{a,b}	0.4±0.0^{a}	0.4±0.0^{a}	0.0±0.0
ARA / EPA	4.6±1.6^{b}	0.2±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.0±0.0

Totals include some minor components not shown.
[a] Vevodar® oil, DSM, Delft, Netherlands.
[b] Neuromins® oil, Martek Biociences, Columbia, MD, USA.
[c] Croda® oil, Incromega EPA500TG, UK.
[d] Olive oil, variety Cornicabra, D.O. Montes de Toledo.
[e] Supplements: soy lecithin, 40.6 mg; vitamin E, 11.6 mg.
Values in the same row assigned a different superscript letter are significantly different ($P<0.05$, $F_{3,11}$).

Table 2. Growth, survival and pigmentation success of Senegal sole larvae after 40 days of being fed *Artemia* enriched on experimental emulsions containing "nil", "low", "medium" or "high" EPA concentrations (EPA-N, -L, -M, -H). Mean ± SD (n=3).

	EPA-N	**EPA-L**	**EPA-M**	**EPA-H**
Final length (mm)	12.1±2.0[b]	10.0±1.6[a]	9.8±2.0[a]	13.0±2.2
Final dry weight (mg)	5.0±1.3[b]	3.0±0.8[a]	3.3±0.8[a]	7.2±1.8
Survival (%)	50.6±7.1	50.3±0.1	57.7±6.8	34.1±1.8
Pigmentation (%)	82.7±3.3[a]	82.9±0.3[a]	98.1±0.9[b]	99.4±0.1

Values in the same row assigned a different superscript letter are significantly different ($P<0.05$).

Table 3. Fatty acid composition (%TFA) of the whole body of newly hatched (1 day post-hatch, dph) and 40 dph larvae of Senegal sole larvae after being fed *Artemia* enriched on experimental emulsions containing "nil", "low", "medium" or "high" EPA concentrations (EPA-N, -L, -M, -H) (mean±SD, n=3).

	Initial	**EPA-N**	**EPA-L**	**EPA-M**	**EPA-H**
Total lipids (mg g^{-1} DW)	176.7±23.1	180.5±17.1	198.8±46.1	219.6±16.3	219.0±26.8
Total FA (mg g^{-1} DW)	116.1±16.6	90.5±15.7	89.8±17.2	92.2±5.7	91.0±29.1
14:0	1.5±0.6	1.2±0.1^{b}	0.8±0.1^{a}	0.7±0.1^{a}	0.5±0.1
16:0	20.6±2.0	12.5±0.8	12.2±0.9	12.0±1.3	11.7±3.0
18:0	7.7±1.4	5.5±0.3	5.8±0.4	5.7±0.7	6.1±1.5
Total saturated	30.8±2.5	20.1±1.3	19.7±1.5	19.3±2.1	19.3±4.8
16:1n-7	4.5±0.7	2.1±0.1^{a}	2.4±0.1^{a,b}	2.6±0.2^{b}	2.8±0.7
18:1n-9	10.6±1.9	32.7±0.5^{c}	26.6±1.6^{b}	23.1±1.5^{a}	12.3±1.7
18:1n-7	3.0±0.1	6.3±0.1^{a}	7.1±0.3^{a,b}	7.2±0.5^{b}	4.9±4.1
20:1n-9	1.1±0.4	0.9±0.1^{b}	0.8±0.0^{a,b}	0.7±0.1^{a}	0.4±0.4
20:1n-7	0.6±0.2	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
Total monounsaturated	21.4±3.2	43.7±0.6^{b}	38.0±2.2^{a}	34.6±2.3^{a}	21.6±5.4
18:2n-6	1.1±0.4	6.7±0.1^{c}	5.3±0.3^{b}	4.7±0.2^{a}	5.2±0.4
20:2n-6	0.2±0.1	0.3±0.0^{c}	0.3±0.0^{b}	0.2±0.0^{a}	0.3±0.0
20:3n-6	0.3±0.1	0.4±0.2	0.4±0.0	0.2±0.1	0.3±0.0
20:4n-6	3.0±0.1	4.0±0.2^{b}	2.7±0.4^{a}	2.1±0.3^{a}	2.0±0.1
Total n-6 PUFA	5.6±0.5	12.2±0.5^{c}	9.2±0.8^{b}	7.8±0.5^{a}	8.5±0.2
18:3n-3	0.4±0.1	11.7±0.5^{a}	13.6±0.3^{b}	13.4±0.6^{b}	15.5±1.5
20:5n-3	5.8±2.1	1.2±0.3^{a}	7.0±0.9^{b}	12.1±1.5^{c}	19.3±1.1
22:5n-3	4.1±1.1	1.0±0.1^{a}	3.5±0.5^{b}	3.8±0.6^{b}	5.0±0.2
22:6n-3	28.1±2.5	6.5±0.4^{b}	5.3±0.4^{a}	4.9±0.8^{a}	4.8±0.8
Total n-3 PUFA	41.1±1.7	24.0±1.4^{a}	33.1±2.9^{b}	38.3±3.9^{b}	50.6±1.0
Total PUFA	47.9±0.8	36.2±1.8^{a}	42.3±3.7^{a,b}	46.0±4.4^{b}	59.1±1.1
DHA / EPA	5.2±1.3	5.7±1.5^{b}	0.7±0.0^{a}	0.4±0.0^{a}	0.3±0.0
ARA / DHA	0.1±0.0	0.6±0.0^{c}	0.6±0.0^{b}	0.4±0.0^{a}	0.4±0.0
ARA / EPA	0.6±0.2	3.5±1.0^{b}	0.4±0.0^{a}	0.2±0.0^{a}	0.1±0.0

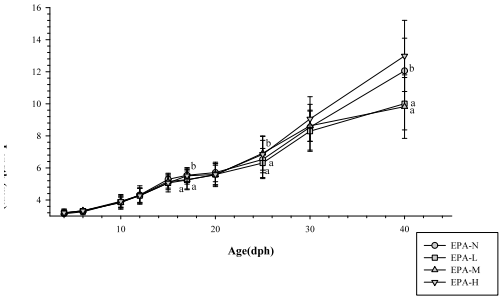
Totals include some minor components not shown.
Values in the same row assigned a different superscript letter are significantly different ($P<0.05$, $F_{3,11}$).

Figure captions.

Fig 1. Temporal changes in legth and weight of Senegal sole larvae fed *Artemia* enriched on experimental (EPA-N, -L, -M, -H) emulsions (mean±SD, n=3). Points assigned different letters denote a significant difference ($P<0.05$) among treatment at that age.

Fig. 2. Changes in eye migration of Senegal sole larvae (stages as in Cañavate and Fernández-Díaz, 1999) and in the values of the eye migration index (I_{EM}) after being fed *Artemia* enriched with experimental emulsions containing "nil", "low", "medium" or "high" EPA concentrations (EPA-N, -L, -M, -H). Vertical bars show the percentage±SD of fish at each stage of development. Columns assigned different letters denote significant differences ($P<0.05$) among dietary treatments at the same age.

Fig. 3. a. The relationship between *Artemia* nauplii relative EPA content (%TFA) and larval whole body relative ARA content (%TFA). b. The relationship between *Artemia* nauplii relative EPA content (%TFA) and larval whole body relative EPA content (%TFA). c. The relationship between *Artemia* nauplii relative EPA content (%TFA) and larval whole body relative DHA content (%TFA). Each value (O) represents a replicate from a treatment. Polynomial regressions were fitted to the data.



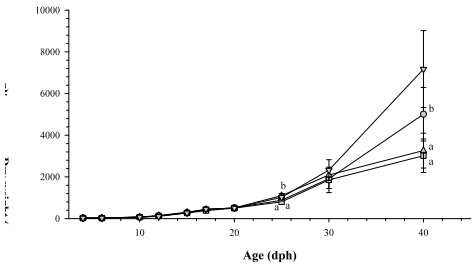


Fig. 1. Villalta et al.

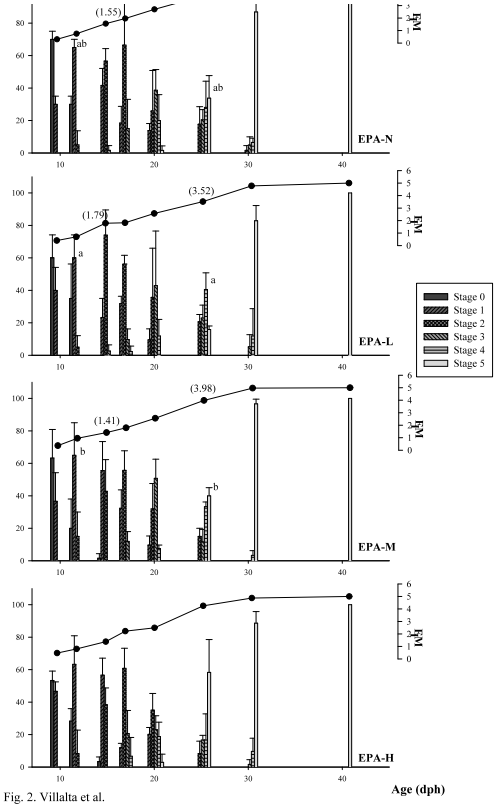
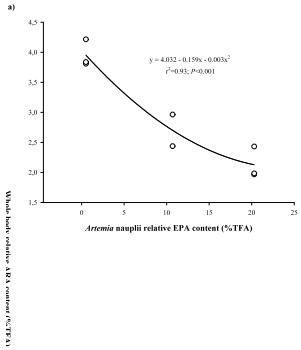
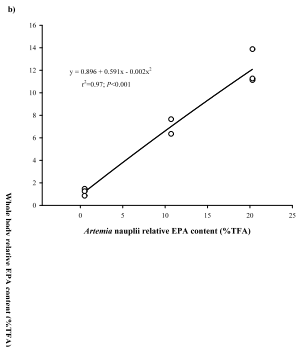


Fig. 2. Villalta et al.





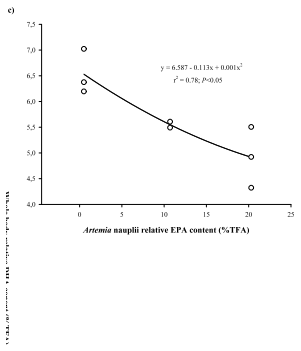


Fig. 3. Villalta et al.