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DIETARY MODULATION OF ARACHIDONIC ACID METABOLISM IN SENEGALESE SOLE (*SOLEA SENEGALENSIS*) BROODSTOCK REARED IN CAPTIVITY

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Abstract

Previous studies have shown higher levels of arachidonic acid (20:4n-6, ARA) in testis, liver, and muscle of wild Senegalese sole (*Solea senegalensis*) compared to fish reared in captivity (first generation, G1). The present study was conducted to establish the optimal level of dietary ARA for G1 Senegalese sole broodstock, using as a reference the fatty acid profile of wild broodstock (gonads, liver and muscle). A total of 120 Senegalese sole broodstock were randomly distributed into 12 tanks and (1:1 male and female), fed in duplicate with six experimental diets containing increasing amounts of ARA (0.7%, 1.6%, 2.3%, 3.2%, 5.0%, and 6.0 % of total fatty acids) for nine months. The relative ARA levels in liver, muscle and male and female gonads at the end of the feeding perio increased in a dose dependent manner. Dietary ARA was mainly incorporated and stored in testis or ovary, followed by liver and muscle. Fish fed 2.3% and 3.2% ARA showed no differences in the ARA content of testis, ovary and liver when compared to

wild fish. In male fish, a significant increase in the levels of 22:4n-6 and 22:5n-6 fatty acids was also observed, which was consistent with the up-regulation of fatty acyl elongase (*elovl5*) and desaturase (*d4fad*) transcript levels in the liver of fish fed 0.7%, 2.3% and 6% ARA. These results suggest that dietary inclusion of 3.2% ARA during periods shorter than nine months, or of 2.3% ARA for prolonged periods, can maintain optimal levels of tissue ARA in captive Senegalese sole broodstock. In addition, the data indicate that male Senegalese sole is able to elongate and desaturate ARA to 22:4n-6 and 22:5n-6, suggesting that these fatty acids may be important for male reproduction.

Keywords: Arachidonic acid, broodstock fish, fish nutrition, fatty acids.

1. Introduction

One of the most important nutritional factors for successful fish reproduction, and among the most studied, is the fatty acid arachidonic acid (20:4n-6, ARA) [1-6]. Arachidonic acid is the main precursor for production of 2-series prostaglandins (PGs) [7,8], which stimulate ovarian and testicular steroidogenesis, triggering oocyte maturation in females and milt production in males, and are involved in female sexual behaviour [9-13]. Arachidonic acid itself and its metabolites regulate cholesterol (CHOL) transfer from the outer to inner mitochondrial membrane where the P450 enzyme resides to initiate steroid hormone synthesis [11,14]. Moreover, ARA had differential effects on steroid biosynthesis. Although it stimulates testosterone production by elevating cAMP levels in a dose-dependent manner, ARA at high doses can also inhibit steroidogenesis by affecting the availability of CHOL [11,15].

Senegalese sole (*Solea senegalensis*) is a promising species for aquaculture in Southern Europe. However, an important problem encountered in this species is the fact that first generation (G1) of reared fish often fail to spawn viable eggs, contrary to wild animals, which produce eggs of sufficient quality and quantity after variable times of acclimation in captivity [16]. This is hindering the expansion of Sole aquaculture and hence several studies have been recently performed in an attempt to understand the underlying causes. Studies on wild sole broodstock showed higher levels of ARA and ARA-derived fatty acids in different tissues compared to those observed in G1 fish [17], similarly to that has been reported in other fish species [18-27]. High accumulation of ARA in sperm has also been reported in rainbow trout (*Oncorhynchus mykiss*)

fed diets low in docosahexaenoic acid (22:6n-3, DHA) [28], and in wild European seabass (*Dicentrarchus labrax*) [29]. In addition, previous studies on Senegalese sole showed that differences in ARA tissue content resulted in differences in cyclooxygenase (COX-2) gene expression, which was significantly up-regulated in the sperm-duct, oviduct and gills of males from wild origin compared to G1 fish [30]. Thus, wild fish showed significantly higher levels of 2-series PGs compared to cultured fish, especially in testis, whereas G1 Senegalese sole, with a lower ARA tissue content, exhibited significantly higher levels of 3-series PGs and lower levels of CHOL [17], the precursor of steroid hormones in vertebrates [31]. On the other hand, Senegalese sole fed artificial diets formulated with graded ARA levels showed an increase in ARA levels in circulating blood, which in turn may induce an increase in CHOL and steroid production, especially in males [32]. Higher levels of ARA in the tissues of wild fish and increased levels in the blood of G1 fish previously fed ARA-enriched diets resulted in an increase in ARA-derived fatty acids, 22:4n-6 and 22:5n-6 [32]. A similar increase in these n-6 long-chain polyunsaturated fatty acids (LC-PUFA) was observed in the sperm of wild European seabass [29]. These fatty acids are present in the cells of reproductive (i.e., seminiferous tubules, sperm) and nervous tissues in larger quantities (human, bull, boar and rabbit) [33-36] than those reported in any other fish tissue and mammals [37,38]. On the other hand, although the physiological function of these LC-PUFAs in sperm is not well known, in mammals they are considered indicators of normal testicular development, spermatogenesis, germ cell populations and fertility [36,39-42] as well as in sperm formation and transportation in the rat testicle [36,38].

Biologically active essential fatty acids such as ARA, eicosapentaenoic acid (20:5n-3, EPA) and DHA can be synthesized to some extent by some mammals and freshwater fish through elongation and desaturation of dietary shorter chain precursors. Carnivores and marine fish have only negligible biosynthetic capacity and hence require preformed LC-PUFA in the diet [6,43]. However, it was recently demonstrated that desaturation of 22:4n-6 to 22:5n-6 may be carried out by a direct pathway involving a delta 4 desaturase in both a marine herbivorous fish [44], as well as in Senegalese sole larvae [45]. This indicates that there is more than one possible pathway for the synthesis of 22:5n-6 and DHA in vertebrates, i.e., not only the classical 'Sprecher pathway' [43].

Based on these latest observations, in the present study we conducted a nine-month feeding trial on broodstock G1 Senegalese sole using a standard commercial feed formulation with six graded levels of dietary ARA. The objectives were: (1) to determine the optimal dietary level of ARA for G1 Senegalese sole, using as a reference the fatty acid profile in gonads, liver and muscle of wild broodstock [17]; and (2) to investigate the regulation of fatty acyl desaturase (*d4fad*) and elongase (*elovl5*) gene expression in the liver of G1 sole fed different amounts of ARA.

2. Materials and Methods

Research involving animal experimentation conformed to the principles for the use and care of laboratory animals, in agreement with the Spanish and European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA).

2.1. Fish and Diets

One hundred and twenty Senegalese sole (four year old and 524 ± 11 g average weight), reared in captivity were PIT tagged (AVID, UK) and sexed using a heterologous vitellogenin ELISA for European seabass (*Dicentranchus labrax*) and validated for Senegaleses sole [46]. The fish were distributed among twelve experimental tanks (10 fish per tank, 5 males and 5 females) and fed in duplicate standard commercial (extruded) diet with six graded ARA contents (Tables 1 and 2) for nine months (from September 2009 until May 2010). The fish were held in a recirculation system with simulated natural photoperiod and temperature (40º 37' and 40º 48' N and between 0º 21' and 0º 40' E., Tarragona, Spain), with minimum temperature observed during two weeks in January - February (13ºC) and maximum temperature over twelve weeks during June - September (21°C). The fish were fed six days per week at a daily ration of 0.15- 0.3% body weight.

2.2. Fish Sampling

In May 2010, seventy two fish were sacrificed by pithing the spinal cord (12 fish per dietary treatment, 6 males and 6 females) after anaesthesia with 0.3 ml L^{-1} Aqui-S® (Scan Aqua A.S, Årnes, Norway) [47]. Gonads, liver and muscle were collected, and 2 g of liver frozen immediately in liquid nitrogen and subsequently stored at −70°C until RNA extraction, whereas the rest of the tissues for lipid and fatty acid profile were stored at -20 °C. All the fish used for

these analyses were in advanced stages of sexual maturation, females with vitellogenic oocytes and males containing spermatozoa in the seminiferous tubules.

2.3. Lipid and Fatty Acid Analyses

Samples of tissues and feeds were homogenized and total lipids extracted [48] and quantified gravimetrically. Tissue samples of six males and six females for each diet treatment were analyzed, and feeds were analyzed in triplicate every three months during the experiment. Fatty acid methyl esters were prepared by acid-catalyzed transmethylation [49], and extracted and purified following [50]. Methyl esters were separated and quantified by gas-liquid chromatography (Thermo Trace GC, Thermo Finningan, Milan, Italy) using a 30 m x 0.25 mm ID capillary column (BPX 70, SGE Europe Ltd., UK) with on-column injection and flame ionization detection using helium as carrier gas (1.2 mL min^{-1} constant flow rate). Individual methyl esters were identified by comparison with known standards (Supelco Inc., Madrid) and a well-characterized fish oil, and quantified in relation to the internal standard, 21:0. The results are presented as percentage of the total fatty acids (TFA) as mean ± standard error of the mean (SEM). Water content was calculated by drying samples at 105ºC until a constant weigh was obtained [51].

2.4. Tissue RNA Extraction and Quantitative Real-Time (qRT-PCR)

In order to study the expression of fatty acyl desaturase (*d4fad*) and elongase (*elovl5*) in liver, which is the main metabolic organ where LC-PUFA biosynthesis occurs, total RNA was extracted by organic solvent (Tri-reagent), according to the manufacturer's instructions (Ambion, Applied Biosystems). RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, USA), respectively. One microgram of total RNA per sample was reverse-transcribed into cDNA using a Verso™ cDNA kit (ABgene, Surrey, UK), following the manufacturer's instructions, using a mixture of random hexamers and anchored oligo-dT (3:1, v/v). The cDNA was then diluted 50-fold with water, after a similar amount of cDNA was pooled from all samples. The expression levels of *d4fad* and *elovl5* transcripts were determined by real-time quantitative (qRT-PCR) and normalized using ubiquitin (*UBQ*) and ribosomal protein S4 (*RPS4*) expression.using primers described previously by Morais et al. [45] and normalized using ubiquitin (*UBQ*) and ribosomal

protein S4 (*RPS4*) [52]. Details of the primers used can be found in Table 3. The amplification efficiency of the primer pairs was assessed using serial dilutions of cDNA pooled from the samples. All amplifications were carried out in duplicate using an Eppendorf qPCR Cycler (Stanford) in a final volume of 20 µl containing 2 µl (for reference genes) or 5 µl (for *d4fad* and *elovl5*) diluted cDNA (1/50), 0.5 µM of each primer and 10 µl of AbsoluteTM qPCR SYBR® Green mix (ABgene). Every amplification experiment also included non-template controls (NTC). The RT-qPCR profiles contained an initial activation step at 95°C for 15 min, followed by 35 cycles: 15 s at 95°C, 15 s at the specific primer pair annealing Tm (Table 3), and 30 s at 72°C. After the amplification phase, a melt curve of 0.5°C increments from 75°C to 90°C was performed, enabling confirmation of the amplification of a single product in each reaction. The RT-qPCR product sizes were checked by agarose gel electrophoresis and their identity was confirmed by sequencing. No primer–dimer formation occurred in the NTC.

2.5. Statistical Analysis

Statistical differences in lipid and fatty acid compositions among the six experimental groups and wild fish measured previously [17] were analysed separately in males and females by one-way ANOVA followed by the post-hoc multiple comparison test Tukey's HSD, at a significance level (P) of 0.05. Moreover, correlations among ARA and C22 fatty acids of the n-6 series were calculated at $P = 0.05$. The compliance of data with normality and homogeneity of variance were tested using the Kolmogorov–Smirnov and Bartlett (Chi-Sqr) tests and, when necessary, log-transformation was carried out. Fatty acid content was expressed as mean % TFA ± SEM. Statistical analysis was performed using the Statistica® package for windows (version 6.0; StatSoft Inc, Tulsa, USA).

The relative expression of *d4fad* and *elovl5* in fish from groups A (control), C and F was normalized by the expression of *UBQ* and *RPS4* using the normalization factor calculated by geNorm© Software version 3.5 [53] and then analyzed for statistical significance using the relative expression software tool (REST-MCS©, version 2, http://www.gene-quantification.info/), which employs a pairwise fixed reallocation randomization test (10,000 randomizations) with efficiency correction [54].

3. Results

After 9 months of feeding the experimental diets the weight of females increased 1.8-fold (from 533 ± 13g to 950 ± 25g, SGR = 0.20% day^{-1}) whereas weights of males increased 1.5-fold, (from 515 ± 16g to 755 ± 25g, SGR = 0.13% day^{-1}), with a feed conversion ratio of 1.3 ± 0.04. No significant differences were noted among the six experimental groups in either growth or feed conversion.

3.1. Fatty acid composition of tissues

The total fatty acid compositions of the different male and female tissues are shown in Tables 4-9. All the tissues analyzed showed a significant accumulation of ARA in a dose dependent manner. Thus, in testis the fish in groups C, D, E and F showed a significantly higher ARA content compared to the control group A, particularly group F which had 13% ARA, 2.7-fold higher than group A with 5% ARA. In the ovary, fish from group F showed a 3.2-fold higher ARA content than group A, whereas groups B, C, D and E did not show significant differences compared to A. The liver of the fish from groups fed diets C, D, E and F had higher ARA levels than control diet A both in males and females. Additionally, males from group B also had significantly higher liver ARA than those from group A, while in females there were no significant differences. The muscle of male fish from groups C, D, E and F showed significantly higher ARA levels than those from group A, whereas in females only those from groups E and F had significantly higher ARA levels than group A.

In the case of 22:4n-6 and 22:5n-6 contents, fish fed the six experimental diets showed a dose dependent and significant increase in testis (Table 4) and liver (Table 6) of male fish, whereas no differences could be found in females, although a similar trend was observed but with much smaller differences (Table 5 and 7). The relative level of 22:4n-6 in testis was significantly higher in groups B (2.2-fold), C (4.2-fold), D (4.5-fold), E (6.1-fold) and F (8.1-fold) compared with the lowest levels found in the control group A (0.2% TFA) (Table 4). The 22:5n-6 content in testis was also higher in groups C (1.5-fold), D (1.8-fold), E (2.2-fold) and F (2.4-fold) compared with the control group A (0.4% TFA). The 22:4n-6 levels in the liver of males were also significantly higher in groups E (7.1-fold) and F (9.5-fold) compared to control group A

201 (0.1% TFA), while 22:5n-6 was 5-fold higher in group F compared with group A (0.2% TFA)
202 (Table 6).

203 As a consequence of the increasing ARA levels, the EPA/ARA ratio was significantly reduced in
204 similar dose-dependent manner in gonads (Tables 4 and 5), liver (Tables 6 and 7) and muscle
205 (Tables 8 and 9) of both males and females. The increase of ARA in gonad, liver and muscle of
206 males resulted in a concomitant significant increase in total n-6 PUFA whereas a significant
207 reduction in total n-3 PUFA was only observed in testis.

208 **3.2. Comparison of tissue ARA levels with wild fish**

209 The tissue ARA levels in the present study were compared those of wild broodstock reported
210 previously [17], and different results were obtained depending on the tissue (Fig. 1). In gonads
211 ARA was significantly higher in males compared with females however in liver and muscle no
212 differences in ARA content between males and females were observed. The levels of ARA in
213 testis of fish from groups E and F showed significantly higher accumulation of ARA compared
214 with those from wild fish. However, ARA levels in testis from groups A, B, C, and D were
215 similar and not significantly different to those from wild fish. In ovary no differences in ARA
216 between the experimental groups and wild fish were observed. ARA content in liver (males and
217 females) from groups A and B were significantly lower than the wild group. However, groups C,
218 D, E and F showed no differences compared to the wild group. In muscle of males the ARA
219 levels in all the fish groups were significantly lower compared to wild fish, and the same as in
220 females from groups A, B, C and D. However, females from groups E and F showed similar
221 ARA levels to that found in the wild.

222 **3.3. Elongase and desaturase gene expression (RT-qPCR)**

223 The expression of *d4fad* and *elovl5* increased in a dose dependent manner in fish fed diets C and
224 F in comparison to the control (A) treatment, but only in males (Fig. 2). Levels of *d4fad*
225 transcripts were significantly higher in the liver of male fish from groups C (3.2-fold) and F (6.8-
226 fold). On the other hand, expression of *elovl5* increased significantly in group F, being 5.3-fold
227 higher than in group A, but not in group C (1.8-fold increase).
228
229

4. Discussion

One of the main bottlenecks to the culture of Senegalese sole, a species with high market value and interest for aquaculture diversification in Southern Europe, is the poor reproductive performance of G1 fish. Previous studies have indicated that the problem is more likely associated with male sperm production and quality than female performance [55-58]. When comparing the fatty acid profile of cultured and wild fish major differences were found in the contents of ARA which, due to its important multiple roles in reproduction, was deemed a potential factor associated with reproductive failure of fish produced in captivity. Hence, the objective of the present study was to test a gradient of dietary ARA to determine the levels that would raise ARA tissue contents in G1 fish to those similar to wild fish. In addition, given previous observations that increased ARA in the tissue and blood of fish raise the levels of its elongated and desaturated products, 22:4n-6 and 22:5n-6, [17,32], the pathway of LC-PUFA biosynthesis was also studied by assessing the expression levels of two genes coding for Elovl5 and d4Fad, which are two key enzymes in this pathway.

The results obtained show that the relative content of ARA in tissues of G1 Senegalese sole generally correlated with dietary ARA levels, increasing in a dose-dependent manner in both sexes. Feeding fish with diets containing 2.3% and 3.2% ARA levels (diets C and D, respectively) resulted in similar ARA contents in testis, ovary and liver compared to wild fish. Muscle, on the other hand, did not accumulate ARA at the same rate as liver and gonads and only the female fish fed diets containing 5% or higher ARA achieved similar ARA levels in muscle as the wild fish. A previous study on dietary ARA preference of Senegalese sole broodstock using a self-feeding system over 16 months revealed that fish regulate the ingestion of ARA to around 3.0% TFA in diet and the resulting ARA content in the tissues was 8.9% TFA in testis, 4% in ovary and 2.5% in liver [59]. These values were similar to those found previously in wild Senegalese sole [17] and also to those obtained in the present study in the testis, ovary and liver of G1 fish in groups C and D. Thus, the ARA accumulation in the muscle was lower than in the self-feeding experiment, except for females of groups E and F. Considering that the self-feeding experiment was conducted for 16 months and the present study lasted only 9 months, optimal dietary ARA level seems to be dependent on the duration of the feeding period. If fish are fed for a period shorter than nine months it might be appropriate to use at least 3.2%

ARA in the diet. For longer feeding periods, 2.3% ARA in the diet might be sufficient for Senegalese sole G1, such that no differences in ARA content could be found in the testis and ovary of the G1 fish compared to wild fish, or fish in the self-feeding experiment. Alorend [4], studied ARA requirements for G1 Atlantic halibut over 3 years and suggested 2.3% as the optimal dietary content, as this level led to the longest milt production period and highest fecundity. This result is likely due to ARA being rapidly incorporated into the reproductive tissues [4].

Previous studies on ARA requirements for broodstock fish showed that the optimal dietary level is species-specific, with higher or lower ARA levels producing detrimental effects in reproductive physiology [2-4,11,12,15,30,60]. A significant increase in the production of steroids was observed in Senegalese sole males fed 3.2% ARA [32], and the highest egg production was obtained using diets with 3.6% ARA for Japanese flounder (*Paralichthys olivaceus*) [3]. On the other hand, a study with Atlantic cod (*Gadus morhua*) found that a diet with 4% ARA, increased the production of estradiol and extended the length of the spawning season [60]. However, negative effects on steroid production, fecundity, egg and larval quality have been observed when fish were fed high ARA levels. Japanese flounder fed 7.3% ARA exhibited a significant reduction in egg and larval quality [3] whereas Atlantic halibut fed 3.2% ARA showed a delay in the spawning season [4]. Other effects such as an earlier estradiol peak in Senegalese sole [32], and an earlier peak in estradiol and vitellogenesis in Atlantic cod [60] were also observed. More research is required to establish the effects of dietary ARA on sperm and oocyte quality in *S. senegalensis* and the feeding time required to incorporate ARA into the reproductive organs, but the present study suggests that dietary levels between 2.3% and 3.2%, depending on the duration of the feeding, might improve the reproductive performance of Senegalese sole G1.

The results obtained in the present study also showed that dietary ARA was preferentially transferred to and accumulated in the gonads (testis and ovary), followed by the liver and muscle, similarly to what had been previously observed in Atlantic halibut, white seabream, black seabream and silver pomfret [4,22,23,61,62].

A positive correlation between the level of ARA and the concentrations of 22:4n-6 and 22:5n-6 in testis, liver and muscle of wild fish had been shown previously [17], and it was suggested that this probably occurred from metabolism (elongation and desaturation) of ARA. A similar pattern

of 22:4n-6 and 22:5n-6 concentration in the tissues after graded dietary ARA was observed in the testis and liver of males in the present study. Accumulation of these fatty acids in the muscle of wild black seabream [23] and Senegalese sole [17], in the gonads of seabass [63], seabream [21], silver pomfret [62] and in the sperm of seabass [29] and rainbow trout [28,29] have been reported previously, with the accumulation of 22:5n-6 being suggested as deriving from local production and uptake from the circulatory system [64-66]. These fatty acids are found in storage lipids in testis of mammals [38] and are considered indicators of normal testicular and sperm condition [36-42]. Although the physiological function of these LC-PUFA in sperm is not well understood, Lenzie et al. [36] suggested that they are involved in sperm formation and in fertilization and, in mammals, there is an increase in the degree of fatty acid metabolism and desaturation during spermatogenesis and sperm maturation [42]. However, the function of 22:4n-6 and 22:5n-6 in fish and their effects on reproduction and spermatogenesis have not been established. It has been suggested that 22:5n-6 is accumulated in the testis as an ARA reservoir, being retro-converted into ARA by hydrogenation and subsequent oxidation [37,64,65,67]. Cultured fish fed commercial extruded diets show a significantly lower accumulation of 22:5n-6 and 22:4n-6 compared to wild fish [17]. Nonetheless, in the present study, when fish were fed increasing ARA levels, a parallel increase in these ARA-derived fatty acids was observed, especially in the liver and testis. Recently, the cloning and characterization of *elovl5* and *d4fad* transcripts in Senegalese sole revealed that this species is able to elongate ARA to 22:4n-6 and then directly desaturate this substrate to 22:5n-6 [45]. Although the activity of this pathway could not be assessed *in vivo*, results from this experiment suggest that it is physiologically relevant given that ARA induced a marked dose-dependent up-regulation in the expression of both *elovl5* and *d4fad* in liver, with *elovl5* transcripts increasing 5.3-fold in males fed 6.0% ARA in relation to those fed 0.7%, and *d4fad* being up-regulated 3.2- and 6.8-fold in fish fed diets containing 2.3% and 6.0% ARA, respectively. However, an interesting observation was that the up-regulation of these two genes was only observed in males, which was also clearly reflected in the fatty acid composition of the tissues, where significant changes in 22:4n-6 and 22:5n-6 contents between treatments were only observed in testis, liver and flesh of males whereas in female tissues only ARA levels were significantly different between fish fed the experimental diets. Gender differences in liver desaturase expression have also been observed in Wistar rats fed an n-3 PUFA enriched diet [68]. In that case, desaturation activity was significantly increased

in the females compared to males. It was also shown that female rats have higher plasma DHA concentrations than males [69]. It is conceivable that the LC-PUFA biosynthesis pathway in female fish liver is also more directed to the provision of DHA for later incorporation into eggs and, at least in post-larvae, the expression of *d4fad* was up-regulated at lower dietary levels of n-3 LC-PUFA (mainly DHA and EPA) [45]. However, in the present experiment only the levels of ARA varied significantly between diets and hence *elovl5* and *d4fad* expression was associated with the conversion of ARA into 22:4n-6 and subsequently to 22:5n-6 in G1 male fish, both of which are important fatty acids involved in testis and sperm composition in mammals [39-42]. Further studies are necessary to understand the importance of these two fatty acids and their physiological function in male fish reproduction, but the present results suggest that they may be as important in Senegalese sole as in higher vertebrates.

5. Conclusion

Based on previous data on ARA content in wild Senegalese sole [17], and considering the results presented here, fish fed either 2.3% or 3.2% ARA enriched diets showed levels of ARA in testis, ovary and liver compared to those of wild fish. Thus, diets with 3.2% ARA for feeding periods up to nine months or 2.3% ARA diets for prolonged feeding periods are suggested. The ARA was preferentially transferred and conserved in the gonads (testis and ovary), followed by the liver and muscle. The increase in the expression of *elovl5* and *d4fad* transcripts in liver in response to dietary ARA content and a parallel increase in tissue 22:4n-6 and 22:5n-6 levels suggest the ability of Senegalese sole to elongate ARA to 22:4n-6 followed by desaturation to 22:5n-6, suggesting an important role of these ARA-derived fatty acids in male fish reproduction. Further studies are required to establish the dietary ARA effect on reproductive performance of Senegalese sole and the time required for effective incorporation of ARA into reproductive organs.

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Fig. 1 *Arachidonic acid content (20:4n-6, ARA) in a) gonads, b) liver and c) muscle of wild fish (Norambuena et al., 2012a) compared with cultured fish fed with different dietary ARA levels (A= 0.7% ARA, B=1.6% ARA, C= 2.3% ARA,D= 3.2% ARA, E= 5.0% ARA and F= 6.0% ARA. Different letters indicate significant differences (ANOVA, P<0.05, N=6) between fish groups. Capital letters are used for males and small letters for females. (*) Indicate significant differences (P<0.05) between males and females.*

Fig. 2 *Relative expression (RT-qPCR) of fatty acyl (a) elongase (elovl5) and (b) desaturase (d4fad) in the liver of female and male Senegalese sole in relation to group A (control), normalized by the expression of UBQ and RPS4 (reference genes). (*) Denote significant differences of groups C and F with respect to control (group A), calculated by REST (P<0.05, N=6).*

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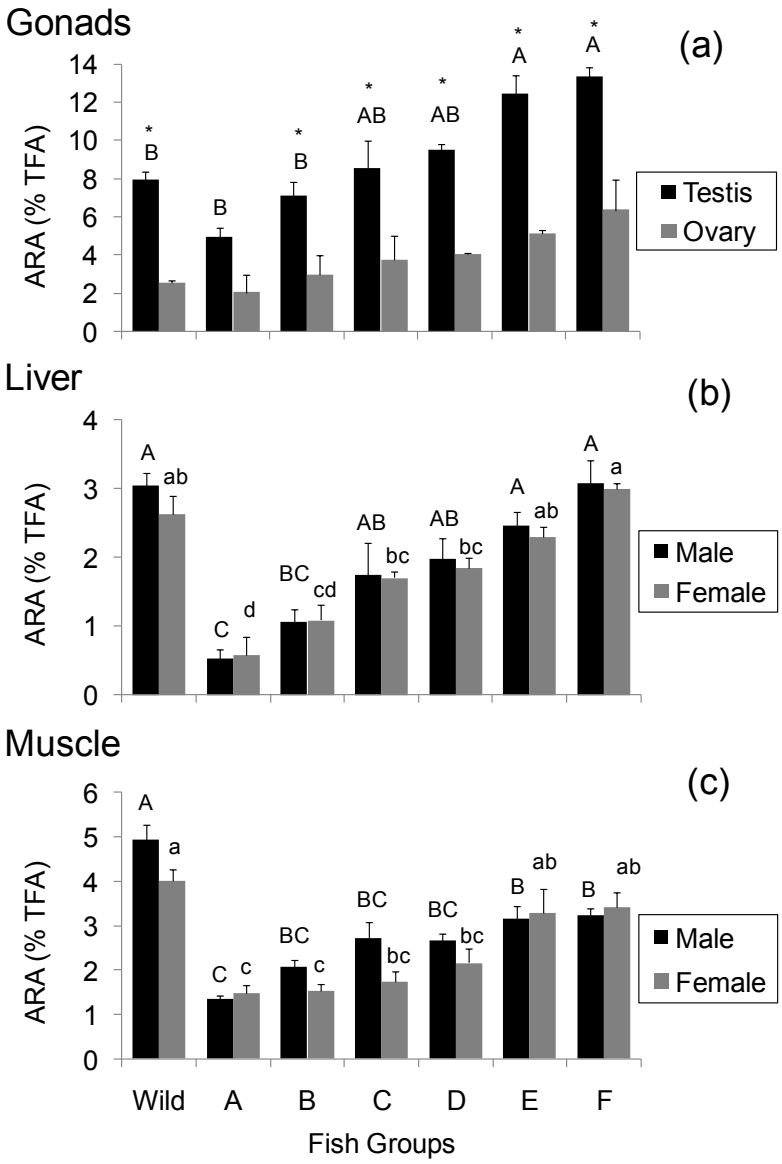

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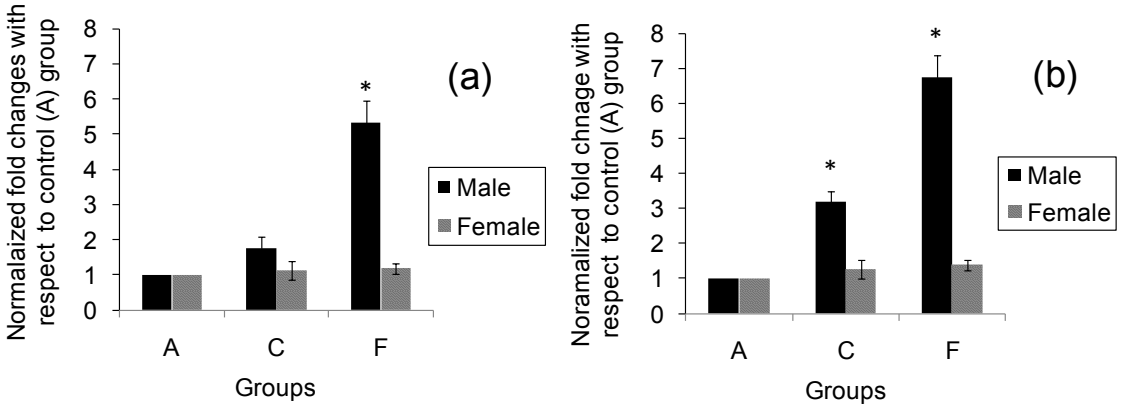

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620 ***Table 1.*** *Ingredients and proximate composition of the experimental diets (A, B, C, D, E and F)*

Ingredients (g/Kg)	A	B	C	D	E	F
Fish meal[1]	645.0	645.0	645.0	645.0	645.0	645.0
Wheat gluten[2]	120.0	120.0	120.0	120.0	120.0	120.0
Wheat[3]	125.8	125.8	125.8	125.8	125.8	125.8
Fish oil[4]	80.0	76.0	71.8	67.6	63.2	59.0
Vevodar[5]	0.0	4.0	8.2	12.4	16.8	21.0
Premixes[6]	29.2	29.2	29.2	29.2	29.2	29.2
Analysed values						
Moisture, %	8.0	7.8	8.3	8.4	8.6	8.3
Crude protein, % DM[7]	61,2	61,4	61,6	61,7	61,8	62,2
Crude fat, % DM	13.8	14.1	14.4	13.7	14.1	14.3

[1] LT fish meal, Skretting, Stavanger, Norway
[2] Cargill Nordic, Charlottenlund, Denmark
[3] Skretting, Stavanger, Norway
[4] Scandinavian fish oil, Skretting, Stavanger, Norway
[5] Contains 35% arachidonic acid, DSM Food Specialities, Delft, The Netherlands
[6] Include micronutrients, vitamin and mineral supplementation. Trouw Nutrition, Boxmeer, Netherlands, proprietary composition Skretting ARC
[7] Dry matter

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Table 2. *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of the diets used (A, B, C, D, E and F) for feeding G1 Senegalese sole (Solea senegalensis). Columns assigned different letters were significantly different (ANOVA, P<0.05, N=3)*

	A		B		C		D		E		F	
TFA(µg mg^{-1} L)	880 ± 87		702 ± 34		820 ± 74		861 ± 82		961 ± 19		911 ± 94	
Fatty acid composition (%TFA)												
14:0	2.5 ± 1.2		3.6 ± 2.8		3.8 ± 2.1		3.6 ± 2.3		3.7 ± 2.8		4.0 ± 2.9	
16:0	14.9 ± 1.1		15.3 ± 3.7		17.6 ± 2.6		15.8 ± 1.8		15.0 ± 2.8		16.1 ± 2.7	
18:0	1.8 ± 0.9		2.4 ± 0.9		2.6 ± 0.7		2.6 ± 0.6		3.0 ± 0.7		3.2 ± 0.4	
Total SFA	19.4 ± 1.5		21.7 ± 5.7		24.4 ± 4.2		22.3 ± 3.6		21.9 ± 4.8		23.4 ± 5.2	
16:1n-7	4.9 ± 0.8		4.8 ± 2.0		5.0 ± 0.8		4.9 ± 0.8		4.3 ± 1.8		4.4 ± 1.2	
18:1n-9	15.0 ± 1.3		15.7 ± 2.4		16.9 ± 1.9		15.9 ± 2.0		15.1 ± 2.7		15.7 ± 0.6	
18:1n-7	1.2 ± 2.0		0.9 ± 1.6		1.0 ± 1.7		0.8 ± 1.4		0.8 ± 1.4		1.0 ± 1.7	
20:1n-9	7.2 ± 1.5		7.1 ± 0.3		7.2 ± 1.1		6.3 ± 0.5		6.9 ± 0.7		6.6 ± 0.4	
22:1n-9	3.5 ± 6.0		3.9 ± 6.7		2.9 ± 5.0		3.0 ± 5.2		3.9 ± 6.7		2.9 ± 5.1	
Total MUFA	32.3 ± 10.5		32.7 ± 4.7		33.4 ± 4.4		31.3 ± 4.9		31.4 ± 5.5		31.1 ± 6.0	
18:2n-6	5.9 ± 0.7		6.4 ± 0.7		6.0 ± 0.9		6.6 ± 0.4		5.9 ± 0.1		7.2 ± 0.7	
20:4n-6, ARA	0.7 ± 0.3	c	1.6 ± 0.6	c	2.3 ± 0.8	bc	3.2 ± 0.7	b	5.0 ± 0.6	a	6.0 ± 0.1	a
22:4n-6	0.1 ± 0.1		0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0	
22:5n-6	0.3 ± 0.6		0.3 ± 0.2		0.2 ± 0.3		0.2 ± 0.3		0.2 ± 0.2		0.3 ± 0.4	
Total n-6 PUFA	9.3 ± 3.4		8.4 ± 1.3		8.9 ± 1.5		10.6 ± 0.8		12.4 ± 1.0		14.0 ± 1.6	
18:3n-3	1.3 ± 0.2		1.4 ± 0.2		1.3 ± 0.3		1.3 ± 0.2		1.2 ± 0.1		1.2 ± 0.1	
18:4n-3	2.2 ± 0.3		2.0 ± 0.1		1.8 ± 0.3		2.0 ± 0.1		1.8 ± 0.2		1.7 ± 0.2	
20:4n-3	0.7 ± 0.1		0.7 ± 0.1		0.6 ± 0.1		0.7 ± 0.0		0.6 ± 0.1		0.6 ± 0.1	
20:5n-3, EPA	13.0 ± 8.4		16.8 ± 5.3		15.9 ± 5.4		16.4 ± 5.4		14.8 ± 6.0		14.7 ± 4.9	
22:5n-3, DPA	1.6 ± 0.4		3.0 ± 2.3		2.0 ± 1.0		2.3 ± 1.3		4.6 ± 5.7		2.0 ± 0.9	
22:6n-3, DHA	14.4 ± 2.0		13.0 ± 2.1		11.3 ± 2.5		13.0 ± 1.6		11.1 ± 0.6		11.3 ± 2.3	
Total n-3 PUFA	39.0 ± 11.0		37.1 ± 0.7		33.4 ± 2.9		35.8 ± 2.1		34.3 ± 1.9		31.5 ± 1.4	
Total PUFA	48.3 ± 11.2		45.5 ± 1.4		42.3 ± 2.6		46.4 ± 1.4		46.6 ± 2.0		45.5 ± 1.7	
EPA/ARA	23.6 ± 18.2	a	12.4 ± 6.6	a	7.8 ± 4.2	ab	5.5 ± 2.5	ab	3.0 ± 1.3	b	2.4 ± 0.8	b
EPA/DHA	0.9 ± 0.5		1.3 ± 0.6		1.5 ± 0.8		1.3 ± 0.5		1.3 ± 0.6		1.4 ± 0.7	
DHA/ARA	23.5 ± 9.0	a	8.8 ± 1.6	b	5.1 ± 1.1	bc	4.1 ± 0.5	bc	2.2 ± 0.3	bc	1.9 ± 0.4	c
n-3/n-6	4.6 ± 2.3		4.5 ± 0.7		3.8 ± 0.8		3.4 ± 0.4		2.8 ± 0.3		2.3 ± 0.3	

L: lipids, DW: dry weight, TFA: total fatty acids, ARA: arachidonic acid, DPA: docosapentaenoic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

Table 3. *Sequences of PCR primers utilized in this study [45,52]*

Transcript	Primer name	Sequence	Amplicon	Tm[4]	Accession no.
d4fad	Δ4fad -Solea-F8	AAGCCTCTGCTGATTGGAGA	131 bp[3]	60	JN673546
	Δ4fad-Solea-R5	GGCTGAGCTTGAAACAGACC			
Elovl5	Elovl5-Solea-F3	TTTCATGTTTTTGCACACTGC	161 bp	60	JN793448
	Elovl5-Solea-R3	GACACCTTTAGGCTCGGTTTT			
UBQ[1]	qUBQ-F	AGCTGGCCCAGAAATATAACTGCGACA	93 bp	70	AB291588
	qUBQ-R	ACTTCTTCTTGCGGCAGTTGACAGCAC			
RPS4[2]	qRPS4-F	GTGAAGAAGCTCCTTGTCGGCACCA	83 bp	70	AB291557
	qRPS4-R	AGGGGGTCGGGGTAGCGGATG			

[1] UBQ: Ubiquitin
[2] RPS4: 40S ribosomal protein S4
[3] bp: base pairs
[4] Tm: annealing temperature.

655 ***Table 4.*** *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of testis of*
656 *Senegalese sole fed with six different diets (A, B, C, D, E and F) for nine months (ANOVA,*
657 *P<0.05, N=6)*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	78 ± 7	68 ± 22	66 ± 20	81 ± 3	76 ± 22	81 ± 17
TFA(μg mg^{-1} L)	503 ± 46	533 ± 52	546 ± 39	561 ± 56	538 ± 34	520 ± 13
Fatty acid composition (%TFA)						
14:0	0.7 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
16:0	17.8 ± 0.9	17.8 ± 0.6	18.9 ± 0.6	19.8 ± 0.4	17.7 ± 1.1	19.0 ± 0.9
18:0	7.0 ± 0.5	6.4 ± 0.4	6.3 ± 0.8	6.7 ± 0.7	6.8 ± 0.6	6.4 ± 0.3
Total SFA	26.3 ± 0.8	26.3 ± 1.7	26.9 ± 1.2	28.0 ± 0.8	25.4 ± 1.7	26.5 ± 0.9
16:1n-7	3.3 ± 0.4	2.6 ± 0.1	3.2 ± 0.4	3.2 ± 0.3	2.4 ± 0.3	3.6 ± 0.3
18:1n-9	15.8 ± 1.4	14.2 ± 1.6	15.1 ± 1.8	14.9 ± 0.6	13.0 ± 1.0	17.3 ± 1.2
18:1n-7	6.1 ± 1.7	6.5 ± 0.6	6.7 ± 0.7	5.2 ± 1.5	7.4 ± 0.6	6.0 ± 1.6
20:1n-9	1.9 ± 0.3	1.7 ± 0.4	1.8 ± 0.2	1.3 ± 0.2	1.8 ± 0.2	1.5 ± 0.1
22:1n-9	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Total MUFA	27.8 ± 1.8	24.9 ± 2.5	26.1 ± 1.1	24.2 ± 1.3	24.6 ± 0.9	28.9 ± 1.1
18:2n-6	8.5 ± 0.5	8.0 ± 0.2	6.9 ± 0.2	8.1 ± 0.3	7.0 ± 0.4	7.0 ± 0.4
20:4n-6, ARA	5.0 ± 0.5 ^{d}	7.1 ± 0.8 ^{cd}	8.6 ± 1.4 ^{c}	9.5 ± 0.3 ^{bc}	12.5 ± 1.0 ^{ab}	13.4 ± 0.5 ^{a}
22:4n-6	0.2 ± 0.1 ^{d}	0.4 ± 0.1 ^{c}	0.8 ± 0.1 ^{bc}	0.8 ± 0.1 ^{b}	1.1 ± 0.1 ^{ab}	1.5 ± 0.1 ^{a}
22:5n-6, DPA	0.4 ± 0.1 ^{d}	0.5 ± 0.1 ^{cd}	0.6 ± 0.0 ^{bcd}	0.7 ± 0.1 ^{abc}	0.9 ± 0.1 ^{ab}	1.0 ± 0.1 ^{a}
Total n-6 PUFA	16.1 ± 0.5 ^{c}	16.6 ± 0.8 ^{c}	18.8 ± 1.5 ^{bc}	19.6 ± 0.5 ^{ab}	22.2 ± 0.6 ^{ab}	22.3 ± 0.2 ^{a}
18:3n-3	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1
18:4n-3	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	1.5 ± 0.7	0.8 ± 0.3	0.4 ± 0.1	0.1 ± 0.1	0.4 ± 0.0	0.2 ± 0.0
20:5n-3, EPA	4.1 ± 0.5	5.1 ± 0.6	3.6 ± 0.2	3.4 ± 0.8	3.9 ± 0.4	2.9 ± 0.3
22:5n-3	3.6 ± 0.3	4.4 ± 0.5	3.7 ± 0.4	3.9 ± 0.4	3.5 ± 0.3	2.9 ± 0.2
22:6n-3, DHA	16.5 ± 1.2	18.8 ± 0.5	18.9 ± 1.7	19.2 ± 0.5	17.8 ± 0.5	15.1 ± 0.8
Total n-3 PUFA	27.4 ± 1.7 ^{a}	30.7 ± 1.6 ^{a}	27.3 ± 2.0 ^{a}	27.4 ± 0.4 ^{a}	26.1 ± 0.6 ^{a}	20.7 ± 0.8 ^{b}
Total PUFA	43.5 ± 1.5 ^{ab}	47.3 ± 2.3 ^{ab}	46.1 ± 0.7 ^{ab}	46.9 ± 0.6 ^{ab}	48.4 ± 0.6 ^{a}	43.0 ± 0.8 ^{b}
EPA/ARA	0.8 ± 0.1 ^{a}	0.7 ± 0.1 ^{ab}	0.5 ± 0.1 ^{bc}	0.4 ± 0.1 ^{c}	0.3 ± 0.1 ^{c}	0.2 ± 0.0 ^{c}
EPA/DHA	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
DHA/ARA	3.5 ± 0.5	2.3 ± 0.6	3.0 ± 1.3	2.0 ± 0.1	1.5 ± 0.1	0.9 ± 0.2
n-3/n-6	1.7 ± 0.1 ^{a}	1.8 ± 0.1 ^{a}	1.5 ± 0.3 ^{ab}	1.4 ± 0.0 ^{ab}	1.2 ± 0.1 ^{b}	0.9 ± 0.0 ^{c}

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659 TL: Total lipids, L: lipids, DW: dry weight, TFA: total fatty acids, ARA: arachidonic acid, DPA: docosapentaenoic
660 acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid. Diet A= 0.7, B= 1.6, C= 2.3, D= 3.2, E= 5.0 and F=
661 6.0% TFA. Data within a row assigned different letters were significantly different (ANOVA, $P<0.05$, N=6).

662 ***Table 5.*** *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of ovary of*
663 *Senegalese sole fed with six different diets (A, B, C, D, E and F) for nine months (ANOVA,*
664 *P<0.05, N=6)*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	108 ± 20	100 ± 22	125 ± 10	127 ± 6	131 ± 9	130 ± 8
TFA(μg mg^{-1} L)	503 ± 46	533 ± 52	546 ± 39	561 ± 56	538 ± 34	520 ± 13
Fatty acid composition (%TFA)						
14:0	2.0 ± 0.2	1.8 ± 0.3	1.7 ± 0.4	2.0 ± 0.2	2.0 ± 0.1	1.9 ± 0.4
16:0	19.1 ± 1.0	19.8 ± 1.5	18.2 ± 0.9	19.0 ± 1.0	18.7 ± 0.7	19.6 ± 0.5
18:0	4.2 ± 0.4	4.3 ± 1.4	4.4 ± 0.8	3.2 ± 0.3	3.5 ± 0.1	4.8 ± 0.6
Total SFA	25.5 ± 0.9	28.1 ± 2.9	25.3 ± 1.1	24.5 ± 1.4	24.9 ± 1.0	27.4 ± 0.2
16:1n-7	5.3 ± 0.5	4.8 ± 0.7	4.6 ± 0.6	6.2 ± 0.4	5.0 ± 0.2	4.6 ± 0.7
18:1n-9	16.5 ± 1.1	14.9 ± 1.0	15.7 ± 1.3	13.3 ± 2.8	14.9 ± 0.8	16.2 ± 0.8
18:1n-7	3.8 ± 0.4	3.8 ± 0.3	4.4 ± 0.7	3.1 ± 0.1	3.8 ± 0.4	3.3 ± 1.1
20:1n-9	2.9 ± 0.3	2.5 ± 0.5	2.6 ± 0.5	2.9 ± 0.2	3.1 ± 0.3	2.6 ± 0.4
22:1n-9	1.6 ± 0.3	1.8 ± 0.2	1.8 ± 0.6	1.6 ± 0.2	1.4 ± 0.5	1.4 ± 0.4
Total MUFA	30.7 ± 1.4	26.6 ± 2.1	28.9 ± 1.8	25.1 ± 2.7	28.4 ± 1.1	27.4 ± 1.2
18:2n-6	7.8 ± 0.4	8.4 ± 0.4	7.1 ± 0.7	7.9 ± 0.5	7.1 ± 0.3	7.5 ± 0.5
20:4n-6, ARA	2.0 ± 0.9 [b]	2.9 ± 1.0 [b]	3.8 ± 1.3 [ab]	4.1 ± 0.1 [ab]	5.1 ± 0.2 [ab]	6.4 ± 1.6 [a]
22:4n-6	0.3 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.2	0.7 ± 0.2
22:5n-6, DPA	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.1
Total n-6 PUFA	11.3 ± 1.3	12.9 ± 1.1	14.1 ± 2.0	13.2 ± 0.8	14.1 ± 0.5	15.9 ± 1.8
18:3n-3	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	1.0 ± 0.1	0.9 ± 0.0	0.8 ± 0.1
18:4n-3	0.6 ± 0.1	0.9 ± 0.4	0.5 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.6 ± 0.1
20:4n-3	1.4 ± 0.7	1.1 ± 0.5	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
20:5n-3, EPA	2.8 ± 0.5	2.0 ± 0.8	2.9 ± 0.7	2.8 ± 0.2	2.3 ± 0.0	2.7 ± 0.4
22:5n-3	4.1 ± 0.4	4.3 ± 0.6	3.9 ± 0.3	4.3 ± 0.4	4.3 ± 0.1	4.0 ± 0.3
22:6n-3, DHA	20.5 ± 1.5	21.2 ± 3.4	22.8 ± 2.9	23.3 ± 1.3	22.7 ± 0.8	20.0 ± 0.8
Total n-3 PUFA	31.0 ± 1.5	30.8 ± 2.0	31.1 ± 2.0	35.8 ± 1.4	31.6 ± 0.8	28.0 ± 1.2
Total PUFA	42.4 ± 1.6	43.6 ± 1.7	45.2 ± 1.9	49.0 ± 1.4	45.7 ± 0.9	43.9 ± 1.3
EPA/ARA	1.9 ± 0.0 [a]	0.9 ± 0.2 [b]	0.8 ± 0.1 [b]	0.7 ± 0.0 [b]	0.4 ± 0.0 [b]	0.5 ± 0.0 [b]
EPA/DHA	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
DHA/ARA	17.3 ± 0.2	12.3 ± 5.1	12.0 ± 5.8	6.0 ± 0.2	4.5 ± 0.3	3.4 ± 0.9
n-3/n-6	2.9 ± 0.2	2.5 ± 0.3	2.5 ± 0.4	2.8 ± 0.2	2.3 ± 0.1	1.9 ± 0.3

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666 Abbreviations as in Table 4.

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668 ***Table 6.*** *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of liver of male*
669 *Senegalese sole fed with six different diets (A, B, C, D, E and F) for nine months (ANOVA,*
670 *P<0.05, N=6)*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	485 ± 39	472 ± 41	477 ± 29	455 ± 43	457 ± 36	450 ± 22
TFA(μg mg^{-1} L)	562 ± 60	538 ± 109	555 ± 59	566 ± 66	504 ± 69	539 ± 50
Fatty acid composition (%TFA)						
14:0	3.9 ± 0.7	2.8 ± 0.8	3.8 ± 0.5	2.2 ± 1.1	2.9 ± 0.7	3.4 ± 0.7
16:0	19.9 ± 0.4	17.8 ± 0.8	18.6 ± 0.5	19.4 ± 1.2	16.6 ± 0.9	16.7 ± 1.5
18:0	3.7 ± 0.7	3.5 ± 0.5	2.7 ± 0.2	2.5 ± 0.4	2.5 ± 0.3	3.0 ± 0.3
Total SFA	28.8 ± 1.1	24.8 ± 1.0	25.7 ± 1.1	24.4 ± 1.7	22.5 ± 1.1	23.5 ± 2.3
16:1n-7	7.3 ± 0.6	6.5 ± 1.0	8.8 ± 0.5	7.4 ± 0.4	6.2 ± 0.7	6.1 ± 1.7
18:1n-9	20.9 ± 1.3	17.1 ± 2.2	21.9 ± 1.3	21.1 ± 1.4	18.3 ± 1.0	20.4 ± 1.2
18:1n-7	3.7 ± 0.0	3.3 ± 0.3	3.6 ± 0.3	3.8 ± 0.1	3.3 ± 0.2	3.5 ± 0.9
20:1n-9	3.5 ± 0.6	3.2 ± 0.7	3.7 ± 0.4	3.8 ± 0.6	3.8 ± 0.4	2.7 ± 0.3
22:1n-9	3.0 ± 0.4	2.9 ± 0.4	2.9 ± 0.5	3.1 ± 0.8	3.4 ± 0.6	2.8 ± 0.4
Total MUFA	29.9 ± 5.8	30.7 ± 3.8	40.5 ± 2.6	45.8 ± 4.5	33.7 ± 2.1	33.7 ± 2.0
18:2n-6	6.7 ± 0.5	6.7 ± 1.0	6.4 ± 0.5	6.8 ± 0.7	7.9 ± 0.4	7.5 ± 0.4
20:4n-6, ARA	0.5 ± 0.1 ^{d}	1.2 ± 0.2 ^{c}	1.5 ± 0.1 ^{c}	1.8 ± 0.1 ^{bc}	2.4 ± 0.2 ^{ab}	2.8 ± 0.4 ^{a}
22:4n-6	0.1 ± 0.0 ^{c}	0.1 ± 0.1 ^{c}	0.5 ± 0.0 ^{bc}	0.5 ± 0.2 ^{bc}	0.9 ± 0.1 ^{ab}	1.2 ± 0.1 ^{a}
22:5n-6, DPA	0.2 ± 0.1 ^{b}	0.3 ± 0.1 ^{b}	0.6 ± 0.1 ^{ab}	0.6 ± 0.1 ^{ab}	0.9 ± 0.1 ^{ab}	1.1 ± 0.1 ^{a}
Total n-6 PUFA	7.8 ± 0.6	8.4 ± 1.1	9.0 ± 0.6	9.0 ± 1.1	12.6 ± 0.6	12.8 ± 0.8
18:3n-3	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.6 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
18:4n-3	0.4 ± 0.0	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	0.3 ± 0.1
20:4n-3	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
20:5n-3, EPA	2.7 ± 0.7	2.2 ± 0.8	2.7 ± 0.8	1.7 ± 0.8	2.3 ± 0.6	3.2 ± 0.7
22:5n-3	4.3 ± 0.4	4.8 ± 0.8	4.2 ± 0.7	4.0 ± 0.8	5.2 ± 0.2	4.7 ± 0.4
22:6n-3, DHA	20.2 ± 3.7	23.8 ± 4.1	18.8 ± 1.9	17.3 ± 2.5	22.0 ± 2.0	23.0 ± 4.8
Total n-3 PUFA	29.3 ± 3.3	33.5 ± 3.3	24.2 ± 2.2	24.4 ± 4.3	30.7 ± 2.1	29.6 ± 3.8
Total PUFA	39.4 ± 3.0	41.7 ± 3.6	34.8 ± 2.4	29.9 ± 2.6	42.5 ± 1.8	42.5 ± 3.9
EPA/ARA	7.4 ± 0.9 ^{a}	2.6 ± 0.9 ^{b}	1.7 ± 0.7 ^{b}	1.4 ± 0.7 ^{b}	1.3 ± 0.2 ^{b}	1.4 ± 0.1 ^{b}
EPA/DHA	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
DHA/ARA	32.9 ± 2.5 ^{a}	20.0 ± 5.6 ^{b}	7.1 ± 2.9 ^{b}	5.8 ± 2.3 ^{b}	6.2 ± 1.7 ^{b}	6.0 ± 1.9 ^{b}
n-3/n-6	3.8 ± 0.5	3.5 ± 1.1	2.7 ± 0.2	2.2 ± 0.1	2.3 ± 0.2	2.3 ± 0.4

671
672 Abbreviations as in Table 4.
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675 ***Table 7.*** *Lipid, fatty acid content and fatty acid composition (% TFA ± SEM) of liver of female*
676 *Senegalese sole fed with six different diets (A, B, C, D, E and F) for nine months (ANOVA,*
677 *P<0.05, N=6)*

	A	B	C	D	E	F
TL (mg g^{-1} DW)	442 ± 83	436 ± 35	419 ± 27	365 ± 30	361 ± 40	406 ± 33
TFA(μg mg^{-1} L)	482 ± 94	417 ± 85	542 ± 20	534 ± 27	456 ± 25	427 ± 94
Fatty acid composition (%TFA)						
14:0	4.3 ± 0.6	4.2 ± 0.7	3.7 ± 0.7	4.3 ± 0.7	3.9 ± 0.6	3.9 ± 1.0
16:0	21.9 ± 0.7	19.6 ± 1.4	18.5 ± 1.3	21.0 ± 0.9	21.5 ± 0.6	19.8 ± 0.8
18:0	3.7 ± 1.0	2.8 ± 0.2	1.9 ± 0.1	2.1 ± 0.2	2.9 ± 0.2	3.2 ± 0.5
Total SFA	31.1 ± 1.5	26.9 ± 2.0	24.2 ± 1.7	28.3 ± 1.3	28.8 ± 1.0	27.0 ± 2.3
16:1n-7	9.5 ± 1.7	7.8 ± 0.6	7.3 ± 1.6	9.8 ± 0.4	7.8 ± 0.4	7.3 ± 1.3
18:1n-9	18.2 ± 4.4	19.4 ± 2.0	21.0 ± 2.0	21.4 ± 0.6	22.5 ± 0.1	20.2 ± 1.8
18:1n-7	4.2 ± 0.4	4.1 ± 0.3	4.7 ± 0.2	4.7 ± 0.4	5.1 ± 0.2	4.9 ± 0.2
20:1n-9	2.9 ± 0.5	3.5 ± 0.8	3.2 ± 0.6	2.5 ± 0.2	3.8 ± 0.4	2.6 ± 0.5
22:1n-9	2.2 ± 0.7	2.3 ± 0.3	2.4 ± 0.6	2.0 ± 0.7	2.2 ± 0.5	2.5 ± 0.3
Total MUFA	32.2 ± 5.9	35.2 ± 3.4	39.8 ± 2.8	38.8 ± 1.1	39.7 ± 0.2	33.7 ± 3.7
18:2n-6	8.0 ± 1.1	8.0 ± 0.1	9.6 ± 0.5	9.7 ± 0.6	9.1 ± 0.2	8.7 ± 0.6
20:4n-6, ARA	0.6 ± 0.3 ^{c}	1.1 ± 0.2 ^{c}	1.7 ± 0.1 ^{b}	1.8 ± 0.2 ^{b}	2.3 ± 0.1 ^{ab}	3.0 ± 0.1 ^{a}
22:4n-6	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.5 ± 0.1
22:5n-6, DPA	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
Total n-6 PUFA	9.0 ± 0.9 ^{b}	9.6 ± 0.4 ^{b}	12.6 ± 0.4 ^{a}	12.6 ± 0.6 ^{a}	13.9 ± 0.4 ^{a}	13.0 ± 0.6 ^{a}
18:3n-3	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
18:4n-3	0.8 ± 0.2	0.5 ± 0.0	0.6 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	0.7 ± 0.1
20:4n-3	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.0
20:5n-3, EPA	1.2 ± 0.3	1.9 ± 0.4	1.2 ± 0.2	1.2 ± 0.2	0.8 ± 0.3	1.1 ± 0.1
22:5n-3	2.8 ± 0.4	3.8 ± 0.4	3.7 ± 0.5	3.0 ± 0.7	3.5 ± 0.4	3.7 ± 0.7
22:6n-3, DHA	16.9 ± 3.9	14.4 ± 1.4	15.7 ± 2.3	13.4 ± 1.9	12.0 ± 1.3	16.4 ± 6.0
Total n-3 PUFA	22.6 ± 4.0	22.9 ± 1.7	23.1 ± 3.0	19.5 ± 1.9	17.2 ± 0.8	22.3 ± 5.8
Total PUFA	31.7 ± 4.6	37.3 ± 5.4	35.7 ± 3.3	32.1 ± 2.3	31.1 ± 1.2	38.9 ± 5.9
EPA/ARA	3.3 ± 1.0 ^{a}	2.0 ± 0.6 ^{ab}	0.7 ± 0.2 ^{b}	0.6 ± 0.1 ^{b}	0.4 ± 0.1 ^{b}	0.3 ± 0.0 ^{b}
EPA/DHA	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
DHA/ARA	45.1 ± 17 ^{a}	13.4 ± 3.6 ^{ab}	9.6 ± 2.0 ^{b}	7.5 ± 1.3 ^{b}	5.4 ± 1.1 ^{b}	4.5 ± 2.1 ^{b}
n-3/n-6	2.5 ± 0.3	2.0 ± 0.5	1.8 ± 0.2	1.5 ± 0.1	1.2 ± 0.0	0.9 ± 0.3

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679 Abbreviations as in Table 4.
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