Expression of genes related to long-chain (C_{18-22}) and very long-chain (>C_{24}) fatty acid biosynthesis in gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) larvae: Investigating early ontogeny and nutritional regulation

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Abstract

Long-chain polyunsaturated fatty acids (LC-PUFA) have been extensively studied in aquaculture due to their importance for survival and development in teleosts. However, very long-chain polyunsaturated fatty acids (VLC-PUFA) have been practically unexplored within the aquaculture scenario. VLC-PUFA, although always present in small amounts, can be pivotal for the correct development and function of tissues such as retina, brain and gonads of vertebrates including fish. This study aimed at determining the temporal expression patterns of genes involved in the biosynthesis of VLC-PUFA (*elovl4a*, *elovl4b*) and their precursors, LC-PUFA, (*fads2*, *elovl5*) during the early ontogeny of *Solea senegalensis* and *Sparus aurata*. Furthermore, we investigated the nutritional regulation of these genes in early life-cycle stages of fish fed low and high LC-PUFA diets consisting of non-enriched and enriched live preys (*Brachionus plicatilis* and *Artemia franciscana*), respectively. The effect of dietary LC-PUFA on growth and fatty acid composition was also examined. The results obtained during early development reveal that all genes studied are expressed before the hatching stage. There is a consistency between the timing at which retinogenesis occurs in both species and an increase of the expression of the two *elovl4* responsible for the synthesis of VLC-PUFA. The results obtained in nutritional assays for both species suggest that the expression of the two isoforms of *elovl4* (isoform *a* in early larvae, and *b* in late larvae) can be regulated positively according to the dietary content of LC-PUFA in early stages, which could activate the VLC-PUFA biosynthesis even during short-term feeding periods (seven days). The body part analysis in late larvae of both species revealed that both isoforms of *elovl4* are expressed preferentially in the head. This can be associated to their highest presence in the neural and visual tissues.

Keywords: *Solea senegalensis; Sparus aurata;* marine larvae; very long-chain polyunsaturated fatty acid; Elovl4.

Highlights

- There are differences between *Sparus aurata* and *Solea senegalensis* in the expression patterns of *fads2, elovl5, elovl4a* and *elovl4b* during early ontogeny.
- There is synchrony between the timing at which retinogenesis occurs in both species and an increase expression of the two *elovl4* responsible for VLC-PUFA biosynthesis.
- Both isoforms of *elovl4* present high specificity, showing high levels of expression in the head.
- Both isoforms of *elovl4* can be regulated in early life-cycle stages according to the dietary contents of LC-PUFA.

1. Introduction

One of the yet unresolved bottlenecks of intensive farming of many marine fish species is the lack of understanding of nutritional requirements of early life-cycle stages, where fish undergo dramatic morphological and physiological changes that determine their viability in later stages (Hamre *et al*., 2013; Izquierdo *et al*., 2015). Some lipids have been recognized as important nutritional components determining larval growth and development, and ultimately, survival (Izquierdo *et al*., 2000; Jobling, 2016; Tocher, 2010). Among them, long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA) are physiologically important nutrients for visual and cognitive development during early ontogeny, important for normal growth, as well as for tissue repair during injury (Bell and Tocher, 1989; Bell *et al*., 1995; Hamre *et al*., 2013; Jobling, 2016). Consequently, LC-PUFA such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are important nutrients for the normal growth and development of marine fish larvae, where neural tissues accumulating these compounds are rapidly forming.

Polyunsaturated fatty acids (PUFA) of 18 carbons, namely α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6), are dietary essential nutrients for all vertebrates since they cannot be synthesized *de novo* (Castro *et al*., 2016; Skov *et al.*, 2013). C_{18} PUFA do not play vital roles in vertebrates *per se* but are the precursors of the physiologically active C_{20-24} LC-PUFA (Monroig *et al*., 2018). Fish species vary in their capacity to convert C_{18} PUFA into C_{20-24} LC-PUFA, depending on the repertoire of fatty acyl elongase (*elovl*) and desaturase (*fads*) genes and the substrate specificities of their protein products (i.e., enzymes) (Li *et al*., 2010; Oboh *et al*., 2017a). Fads, with desaturase species-specific activity, and Elovl5 are limiting enzymes considered key in marine teleost LC-PUFA biosynthesis (Monroig *et al*., 2018).

Fads introduce double bonds into specific position within the fatty acyl chain, and desaturases with Δ4, Δ5, Δ6 and Δ8 activities have been demonstrated to play major roles in LC-PUFA biosynthesis in fish (Castro *et al*., 2016; Monroig *et al.*, 2018). Despite such a remarkable functional diversity, virtually all Fads-like desaturases from fish are *fads2* orthologs, with the exception of basal teleosts such as the Japanese eel (*Anguilla japonica*) possessing a Fads1 (Δ5 desaturase) (Lopes-Marques *et al*., 2018). Among Elovl, enzymes that catalyze the first reaction (condensation) of the elongation pathway resulting in the addition of two carbons to the preexisting fatty acyl chain (Castro *et al*., 2016; Monroig *et al*., 2018), three types, namely Elovl2, Elovl4 and Elovl5, participate in PUFA elongation (Jakobsson *et al*., 2006). All teleostean fish possess at least one Elovl5 and two Elovl4, the latter termed as “Elovl4a” and “Elovl4b” based on the nomenclature of the zebrafish *Danio rerio* orthologs (Monroig *et al*., 2010). However, Elovl2 has been lost during evolution of teleosts and this elongase is absent from many marine farmed species (Castro *et al*., 2016; Monroig *et al*., 2018).

Unlike C_{20-24} LC-PUFA, very long-chain (>C_{24}) PUFA (VLC-PUFA) have been barely investigated in fish, despite the key roles that these compounds have in vision, brain function, skin permeability and reproduction of mammals (Agbaga *et al*., 2008; Aldahmesh *et al*., 2011; Furland *et al*., 2007; Mandal *et al*., 2004; Poulos, 1995). Investigations of VLC-PUFA in fish have been mostly restricted to the characterization of Elovl4 enzymes involved in their biosynthesis (Monroig *et al*., 2010; Oboh *et al*., 2017b; Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017b; Kabeya *et al*., 2015). Fish Elovl4 proteins can actively elongate a range of PUFA substrates producing in some instances VLC-PUFA up to 36 carbons (Monroig *et al*., 2018). It is interesting to note that some fish Elovl4 enzymes have the ability to elongate 22:5n-3 to 24:5n-3 (Monroig *et al*., 2011, 2012), suggesting that these enzymes, in addition to their major role in VLC-PUFA biosynthesis, can contribute to the LC-PUFA biosynthesis thus denoting shared roles in both pathways (Jin *et al*., 2017a). Such enzymatic ability by fish Elovl4 has been also hypothesized to partly compensate for the above mentioned absence of Elovl2 in many fish species (Monroig *et al*., 2011, 2018). However, it is unknown which impacts might exist when supply of LC-PUFA, precursors of fish VLC-PUFA, is restricted in fish feeds. In the context of fish farming, dietary restriction of LC-PUFA is becoming an extended trend due to the scarce availability of marine (i.e. LC-PUFA rich) ingredients such as fish meal and fish oil (Shepherd *et al*., 2017; Ytrestøyl *et al*., 2015), and therefore it is important to investigate the molecular mechanisms underlying the biosynthetic pathways of VLC-PUFA, especially during early developmental stages undergoing central physiological processes in which these compounds are involved (Monroig *et al*., 2010). Moreover, it is interesting to understand how their expression patterns can be regulated through the diet at the onset of exogenous feeding with live preys varying in their contents of LC-PUFA.

This study aimed at determining the temporal expression patterns of genes involved in the biosynthesis of VLC-PUFA (*elovl4a*, *elovl4b*) and their precursors, LC-PUFA, (*fads2*, *elovl5*) during the early ontogeny of *Solea senegalensis* and *Sparus aurata*. These are the sole genes that participate in the biosynthetic pathways of LC and VLC-PUFA. Furthermore, we investigated the nutritional regulation of these genes in early life-cycle stages of fish fed low and high LC-PUFA diets consisting of non-enriched and enriched live preys, respectively. The species chosen as models in this study, namely *S. senegalensis* and *S. aurata*, are representative of marine fish species with different LC-PUFA biosynthesis strategies, particularly with regards to Fads2 functionality (Castro *et al*., 2016; Monroig *et al*., 2018). *S. aurata* possesses one sole Fads2 enzyme with Δ6, and to a lesser extent, Δ5 desaturase activities (Seiliez *et al*., 2003; Zheng *et al*., 2004). Moreover, *S. senegalensis* possess a Fads2 with Δ4 activity (Morais *et al*., 2012) enabling the culture of its larval stages on diets (non-enriched live preys) containing negligible DHA and low EPA levels without obvious detrimental effects on growth and survival (Morais *et al*., 2012; Villalta *et al*., 2005). These enzymatic differences in LC-PUFA biosynthesis, along to other characteristics, as their specific larval development, and the different feeding habits, i.e. pelagic or benthonic, are of special interest to study the nutritional regulation of *elovl4* genes in different marine teleosts fed diets with a similar LC-PUFA content.

2. Materials and methods

2.1 Larval culture

Fertilized eggs of *S. senegalensis* and *S. aurata* were obtained from naturally spawning captive broodstocks from Stolt Sea Farm S.A. (A Coruña, Spain) and Instituto Español de Oceanografía (IEO) (Murcia, Spain), and hatched at 18 °C in filtered seawater with continuous recirculation at a density of ~400 eggs l^{-1}. Once hatched, larvae were reared in a closed system in 11-litre aquaria at an initial density of 100 larvae l^{-1}, temperature of 18-19 °C, photoperiod 12L:12D, and salinity of 37.5 ± 0.5 g l^{-1}. From the start of exogenous feeding at 4 days after hatching (dah) until 8 dah, larvae of both species were fed rotifers three times daily (*Brachionus plicatilis* fed *Tetraselmis* sp. at ~ 9 x 10^5 cells ml^{-1}) at a density of 5-10 rotifers ml^{-1}. For nutritional regulation experiments, different diets were tested depending on early (16 dah) or late (40 dah) larvae.

2.2. Larval ontogeny

In order to study the temporal expression patterns of genes involved in the biosynthesis of LC- and VLC-PUFA (*elovl5, fads2*, *elovl4a* and *elovl4b*) during the early ontogeny of *S. senegalensis* and *S. aurata*, triplicate pools (~100 mg) of fertilized eggs, newly hatched larvae and larvae up to 7 dah were collected daily. Samples were immediately frozen and kept at -80 °C until further analysis.

2.3. Nutritional regulation

2.3.1. Experiment 1: Nutritional regulation in early larvae

A first experiment consisted of 9 dah larvae that were fed three times daily with rotifers (*Brachionus plicatilis*), which were obtained from cultures maintained at the facilities of IATS, enriched with Larviva Multigain (BioMar Iberia S.A., Palencia, Spain) with a proximate composition indicated by the supplier of 14% crude protein, 43% crude fats, 2.6% crude fiber and 7,7% crude ash (Rot E) or non-enriched (i.e., grown on the basal *Tetraselmis* sp. diet) (Rot NE) during 7 days in triplicate 11 l aquaria. Rot E were enriched according to the "short-term enrichment" protocol (Dhert *et al*., 2001) in 3 l cylindro-conical flasks during 3 h at 28 °C with aeration, at a density of 300-350 individuals ml^{-1} in 30 g l^{-1} salinity diluted seawater. Prey density began at 10 rotifers ml^{-1} and was increased with larval age up to 15 rotifers ml^{-1} three times daily.

2.3.2. Experiment 2: Nutritional regulation in late larvae

In a second experiment, *S. senegalensis* and *S. aurata* larvae (25 dah) were reared in a closed recirculation system in triplicate 20 l aquaria at 25 larvae per aquaria and fed *Artemia franciscana* metanauplii, obtained from cysts with a proximate composition indicated by the distributor (INVE Aquaculture, NV., Dendermonde, Belgium) of 54% crude protein, 11% crude fats and 8% crude ash, either enriched with Larviva Multigain (Art E) or bakery yeast (*Saccharomyces cerevisiae*) (Art NE) during 15 days. Enrichment was carried out in 3 l cylindro-conical flasks for 24 h at a density of 150-200 nauplii ml^{-1} in seawater at 28 °C and with strong aeration. Enrichment diets were supplied at 0.6 g l^{-1}, which were previously dispersed/homogenized in a known sea water volume using a stirrer. Prey density began at 5 nauplii ml^{-1} and was increased with larval age up to 15 nauplii ml^{-1} three times daily.

Fish samples (Experiments 1 and 2) were collected, weighed, measured and immediately frozen and kept at -80 °C until further analysis. For Experiment 2, due to their larger size, head, viscera and muscle body compartment were dissected and analyzed separately.

2.3.3. Larval growth

After the larval feeding trials, samples of 16 dah early larvae (Experiment 1) and 40 dah late larvae (Experiment 2) were collected, and their total lengths (TL) and weights measured. Larval TL was measured with the digital image processing software ImageJ (Rueden *et al*., 2017). Late larvae were measured manually under a binocular microscope Leica MZ6 coupled to Transmitted-Light Base TO ST (MDG 28) (Leica Microsistemas S.L.U., Barcelona, Spain) with an ocular micrometer. Wet weight (WW) was recorded using a Mettler Toledo XS105 semi-micro balance (Mettler-Toledo S.A.E., Barcelona, Spain), as a pool of five animals for early larvae from Experiment 1, and individually for late larvae from Experiment 2. In all cases, at least 15 fish were used. Specific growth rate (SGR) was calculated as SGR= log (TL_f)-log (TL_i)/T (experimental time)*100 (Lugert *et al*., 2016). Fulton's condition factor (K) of each late larvae was calculated as K=WW (g) / [TL (cm)]^3 x 100 (Froese, 2006). K was not calculated for early larval pools due to the impossibility of weighing each larva individually.

2.4. Fatty acid analysis

Total lipids of experimental diets (Rot E, Rot NE, Art E, and Art NE), and body compartments from larvae of Experiment 2, were extracted with chloroform/methanol (2:1, v/v) according to Folch *et al*. (1957) and quantified gravimetrically after evaporation of the solvent under nitrogen flow, followed by vacuum desiccation overnight. Total lipids were resuspended at 10 mg ml^{-1} in chloroform/methanol (2:1) containing 0.01 % (w/v) butylhydroxytoluene (BHT). Then, 100 µl of total lipids were subjected to an acid-catalyzed transesterification (Christie, 1982). Fatty acid methyl esters (FAME) were subsequently extracted using hexane/diethyl ether (1:1, v/v), and purified by TLC (Silica gel 60, VWR, Barcelona, Spain) as previously described (Christie, 1982). In the case of individual early larvae (Experiment 1), due to the small amount of sample, fatty acid (FA) profiles were obtained through an adapted direct transmethylation method (Garrido *et al*., 2016), and total lipid values are not available.

FA composition was determined using a Thermo Scientific TRACE GC Ultra gas chromatograph (Thermo Fisher Scientific, Madrid, Spain), equipped with a fused silica 30 m × 0.25 mm open tubular column (Tracer, TRB-WAX, film thickness: 0.25 µm, Teknokroma, Barcelona, Spain). Injections of 1 µl samples were carried out on-column, using helium as carrier gas (1.5 ml min^{-1} constant flow), and a thermal gradient from 50 (injection temperature) to 220 °C, and reported as % of total fatty acids. Methyl esters were identified by comparison with known standards.

2.5. RNA extraction and real time quantitative PCR (qPCR)

Total RNA was isolated from three pools of whole fertilized eggs and larvae at various stages of development (0 to 7, and 16 dah), using Maxwell 16 LEV simplyRNA Tissue Kit (Promega Biotech Ibérica S.L., Madrid, Spain) following the manufacturer's instructions. From Experiment 2, head, viscera and muscle body compartments of late larvae (40 dah) were differentiated and processed separately. RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-2000C, Thermo Fisher Scientific, Barcelona, Spain). Two micrograms of total RNA per sample was reverse transcribed into cDNA using the M-MLV reverse transcriptase first strand cDNA synthesis kit (Promega Biotech Ibérica S.L., Madrid, Spain) following manufacturer's instructions, using a mixture (3:1, mol/mol) of random primers and anchored oligo $(dT)_{15}$ primer (Promega Biotech Ibérica S.L., Madrid, Spain). Expression of fatty acyl desaturase (*fads2*) and elongases (*elovl5*, *elovl4a* and *elovl4b*) was quantified by qPCR using primers shown in Table 1. Primers were designed using Primer3 software (http://primer3.sourceforge.net) (Rozen and Skaletsky, 2000). The amplification efficiency of the primer pairs was assessed by serial dilutions of standard solutions of the studied genes with known copy numbers that helped to build a standard curve, which also allowed the conversion of threshold cycle (Ct) values to copy numbers. Amplifications were carried out in technical duplicates on a qPCR thermocycler (CFX Connect Real-Time System, Bio-Rad Laboratories S.A., Madrid, Spain) in reactions with a final volume of 20 µl, containing 5 µl diluted (1/20) cDNA problem samples for all genes, except for *S. senegalensis β-actin* (*actb*) gene (1/200), 0.5 µl of each primer and 4 µl Master Mix qPCR No-ROX PyroTaq EvaGreen 5x (CMB-Bioline, Madrid, Spain). All runs included a systematic negative control consisting of a non-template control (NTC). The qPCR program consisted of an initial activation step at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s, elongation at 72 °C for 15 s, and a final melt curve of 0.5 °C increments from 60 °C to 90 °C, enabling confirmation of the amplification of a single product in each reaction. Three potential housekeeping genes (*β-actin, elongation factor 1α and 18s rRNA*) were tested. Finally, next to check its stability using the Genorm software (Vandesompele *et al*., 2002), *β-actin* was used for normalization of the candidate gene expression. Gene expression results are given as mean normalized values (±SD) corresponding to the ratio between copy numbers of fatty acyl desaturase (*fads2*) and fatty acyl elongases (*elovl5*, *elovl4a* and *elovl4b*) transcripts and copy numbers of the reference gene *β-actin* (*actb*).

2.6. Statistical analysis

For each species, data from gene expression on different stages along larval development (0-7 dah) and different body compartments (viscera, muscle and head) in late larvae (Experiment 2) were checked for homogeneity of variances using Levene's test and analyzed by one-way analysis of variance (ANOVA) ($P \leq 0.05$) followed by Tukey HSD post-hoc test. To compare the effects of the two experimental diets tested in Experiments 1 and 2, WW, TL, K, FA and gene expression data were checked for homogeneity of variances using Levene's test and then analyzed by an independent sample t-Student test, at significance levels of $P \leq 0.05$, except where noted otherwise. The statistical software SPSS 24.0 (SPSS Inc., Chicago, USA) was used to analyze the data.

3. Results

3.1. Temporal expression of fads2, elovl5, elovl4a and elovl4b during early development of S. aurata and S. senegalensis

The results of the temporal expression of *fads2*, *elovl5*, *elovl4a* and *elovl4b* revealed differences in the expression patterns for *S. aurata* and *S. senegalensis*. In both species, the results showed that all the candidate genes were expressed before hatching stage, with transcripts detected throughout the entire developmental time frame studied (Fig. 1, 2).

For *S. aurata*, expression of *fads2*, *elovl5* and *elovl4a* showed a trend to increase until 5 dah (Fig. 1 A, B, C). *Elovl4b* showed an expression pattern similar to the other genes, although the highest expression was detected at 4 dah, point after which there was a decrease (Fig. 1 D). The lowest expression values shown in eggs for all the genes studied in comparison to post-hatching stages (1-7 dah) denoted a low transcriptional activity during early embryogenesis. For *S. senegalensis*, *fads2* showed an expression pattern characterized by the existence of two periods of high transcriptional activity. The first peak, at 1-2 dah, is consistent with those of *elovl4a*, *elovl4b*. Subsequently, *fads2* expression decreased and then increased showing a second peak at 5-6 dah (Fig. 2 A). *Elovl5* showed its highest expression in eggs (Fig. 2 B). After hatching, *S. senegalensis* presented a rapid increase in expression values for both *elovl4a* and *elovl4b*, showing a peak at 2 dah. Later, expression values decreased to remain relatively stable until the end of the period studied (Fig. 2 C; D).

3.2. Nutritional regulation experiments

3.2.1. Larval growth

Results obtained for larval growth are shown in Table 2. Generally, early and late larvae (Experiments 1 and 2) fed enriched diets (Rot E and Art E) presented higher growth performance at the end of the experimental periods, as fish show a higher TL and WW compared to non-enriched diets (Rot NE and Art NE).

In Experiment 1, *S. aurata* early larvae fed the Rot E diet presented higher WW than early larvae fed Rot NE diet. For *S. senegalensis,* early larvae fed Rot E diet presented higher TL than early larvae fed the Rot NE diet (Table 2).

In Experiment 2, *S. aurata* late larvae fed the Art E diet presented higher TL and WW than early larvae fed the Art NE diet. For *S. senegalensis*, late larvae fed the Art E diet presented higher WW than late larvae fed the Art NE diet. No significant differences were found between dietary regimes in the Fulton's K condition factor values for late larvae of both species (Table 2).

3.2.2. Fatty acid composition

Effects of dietary LC-PUFA during different windows of development (early larvae and late larvae) of *S. aurata* and *S. senegalensis* were investigated using enriched and non-enriched live preys (rotifers and *Artemia*). For Experiment 1, the Rot E diet consisted of rotifers enriched with Larviva Multigain containing high levels of n-6 docosapentaenoic acid (n-6 DPA; 22:5n-6) and DHA, while the Rot NE diet, i.e. rotifers grown on *Tetraselmis* sp., contained high levels of ALA, stearidonic acid (SDA; 18:4n-3) and eicosatetraenoic acid (ETA; 20:4n-3) (Table 3). For Experiment 2, the Art E diet had high levels of ARA, EPA, n-6 DPA and DHA, with the Art NE diet being characterized by high levels of ALA and LA (Table 3).

Fatty acid analyses for Experiment 1 (early larvae) denoted that *S. aurata* and *S. senegalensis* larvae fed Rot E diet showed the highest content of DHA and n-6 DPA, while *S. aurata* and *S. senegalensis* larvae fed Rot NE diet had the highest content of LA, ALA, and EPA (Table 4).

For Experiment 2 (late larvae), both species fed Art E diet showed the highest content of PUFA in the body part analyzed (muscle). *S. aurata* larvae fed Art E diet showed the highest content of EPA, n-6 DPA and DHA, while those fed Art NE diet contained the highest LA and ALA (Table 4). *S. senegalensis* larvae fed Art E diet showed the highest content of ARA, n-6 DPA, EPA and DHA, while those fed Art NE diet contained the highest LA and ALA (Table 4).

3.2.3. Gene expression

In Experiment 1, early larvae of both species showed an expression pattern characterized by an up-regulation of *elovl4a*, when fed enriched rotifers. However, only *S. aurata* larvae showed significant differences between different dietary treatments for *elovl4a* gene (Fig. 3 A). Regarding to *S. senegalensis*, larvae did not show significant differences between diets, but it is important to note that P values ($P < 0.07$, Fig. 3 B) close to the significance limit of 0.05 were obtained. No significant differences were found in the expressions of *fads2*, *elovl5*, and *elovl4b* genes for *S. aurata* (Fig. 3 A), or *S. senegalensis* (Fig. 3 B) in response to diet (E-NE).

In Experiment 2, for *S. aurata* late larvae, no significant differences were observed in the dietary regulation of *fads2*, *elovl5*, *elovl4a* and *elovl4b*, as a consequence of different dietary LC-PUFA content (Fig. 4). For *S. senegalensis* late larvae, differences were observed in the dietary regulation of *fads2*, whose expression was up-regulated in late larvae fed diet Art NE, i.e. low LC-PUFA diet. However, although no significant differences were observed in the expression of both isoforms of *elovl4* in response to dietary LC-PUFA, *elovl4b* ($P < 0.07$, Fig. 5 D) appeared to be up-regulated in the head of *S. senegalensis* late larvae fed diet Art E, denoting an opposite regulatory mechanism to that of *fads2* and *elovl5*, in response to dietary LC-PUFA (Fig. 5).

The results of body fraction analysis (viscera, muscle and head) revealed significant differences in the expression patterns of all genes studied (*fads2, elovl5, elovl4a* and *elovl4b*). For *S. aurata*, *fads2* showed the highest expression levels in the head (Fig. 4 A), whereas *elovl5* peaked in the viscera and head (Fig. 4 B). Besides, the head showed the highest expression levels for *elovl4a* and *elovl4b* (Fig. 4 C, D). For *S. senegalensis, fads2* and *elovl5* presented the highest expression levels in the visceral zone (Fig 5 A, B) and *elovl4a* and *elovl4b* in the head (Fig 5 C, D).

4. Discussion

Several studies on commercially important fish species have emphasized the importance of VLC-PUFA in aquaculture (Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017b; Monroig *et al*., 2012; Oboh *et al*., 2017b; Zhao *et al*., 2019). At present, the analysis of VLC-PUFA remains challenging due to the low presence of these compounds in tissues, their fragmentation during the chromatographic analysis and the lack of reference standards commercially available (Agbaga *et al*., 2010; Garlito *et al*., 2019). However, establishing the roles of Elovl4 in VLC-PUFA biosynthetic pathways and how their activity can be regulated through the diet has been identified central to understand the impacts that current feeding strategies, including the effects of a dietary reduction of VLC-PUFA precursors (i.e. LC-PUFA) can have on farmed fish. Physiological roles of Elovl4 products in vision and brain function make early development stages particularly vulnerable (Monroig *et al*., 2010), and this study aimed to investigate the metabolic and compositional responses of early life-cycle stages of *S. senegalensis* and *S. aurata* when fed diets with varying levels of LC-PUFA.

The results obtained in both species, for temporal expression of genes involved in the biosynthesis of LC-PUFA (*elovl5, fads2*) and VLC-PUFA (*elovl4a, elovl4b*) by qPCR, reveal the existence of inter- and intra-specific differences. On one hand, we observed a differential increase of the expression levels of the two *elovl4* genes in both species. On the other hand, peaks of expression of *elovl4* genes differed between species but, in each case, these were consistent with timing at which the most relevant processes involved in retinogenesis occurs during larval development of *S. senegalensis* (early after hatching) (Bejarano-Escobar *et al*., 2010) and *S. aurata* (late after hatching) (Pavón-Muñoz *et al*., 2016).

Generally, fishes have a well differentiated three-layered retina (Pavón-Muñoz *et al*., 2016). During early stages of development, however, the vertebrate neuroretina consists of a neuroepithelium composed of undifferentiated retinal progenitor cells (Pavón-Muñoz *et al*., 2016; Turner and Cepko, 1987). Later, altricial fish larvae, experience a process of retinal maturation where tissue differentiation is carried out until the development of a mature retina (Pavón-Muñoz *et al*., 2016). During this process, where fish undergo dramatic morphological and physiological changes, it is important to have an optimal reserve of nutrients that allows to face the changes that occur during larval ontogenesis. LC-PUFA, especially DHA, is a major component of biological membranes, particularly of immune cells and neural tissue, being vital for visual and cognitive development during early ontogeny (Bell and Tocher, 1989; Bell *et al*., 1995). Moreover, there are different studies that relate *elovl4* disarranges and an inefficient level of their biosynthesis products with the development of visual disorders in vertebrates (Barabas *et al*., 2013; Maugeri *et al*., 2004), since VLC-PUFA, although in small amounts, are present in retina, associated with the phosphatidylcholine from the outer membranes (Aveldaño and Sprecher, 1987). For this reason, we suggest that the synchrony between the timing at which retinogenesis occurs in both species and an increased expression of the two *elovl4* genes could highlight the importance of VLC-PUFA for the correct development of vision during early larval development of fish. There is a temporal decoupling in the expression of both *S. aurata elovl4* isoforms, since *elovl4a* showed a maximum activity at 5 dah, while *elovl4b* exhibited an advanced peak at 4 dah. This temporal decoupling could be indicative of differences existing at level of substrate specificity and/or tissue localization of both isoforms. Although the functional characterization of *S. aurata* and *S. senegalensis* Elovl4a and Elovl4b have not been yet published, the function of Elovl4 enzymes has been characterized in aquaculture species such as *Siganus canaliculatus, Clarias gariepinus, Salmo salar, Acanthopagrus schlegelii* and *Oncorhynchus mykiss* (Carmona-Antoñanzas *et al*., 2011; Jin *et al*., 2017b; Monroig *et al*., 2012; Oboh *et al*., 2017b; Zhao *et al*., 2019), and although in all cases Elovl4 participate in the biosynthesis of VLC-PUFA,the two isoforms do not have the same efficiency in converting the different substrates in all the species studied (Jin *et al*., 2017b; Monroig *et al*., 2010; Oboh *et al*., 2017b). As previously described in zebrafish (Monroig *et al*., 2010), *elovl4a* and *elovl4b* can present distinct substrate specificities, since Elovl4a has virtually no activity towards DHA itself, unlike Elovl4b. However, DHA in *Acanthopagrus schlegelii* was only elongated by Elovl4a isoform (Jin *et al*., 2017b). Moreover, Elovl4 isoforms have different tissue distribution patterns, with *elovl4a* being mostly expressed in brain tissues (brain and pituitary) (Monroig *et al*., 2010; Oboh *et al*., 2017b), while *elovl4b* is located mostly in retina and gonads (Monroig *et al*., 2010; Oboh *et al*., 2017b). These spatio-temporal differences in the pattern of expression of both isoforms of *elovl4*, could be pivotal in early stages of development, where important changes at the physiological level are carried out in short periods of time (Zambonino-Infante and

Cahu, 2001).

The rapid increase in expression values shown for *elovl4* (*elovl4a*, *elovl4b*) after hatching, besides the high *elovl5* transcript levels observed for *S. senegalensis* eggs, suggests that an over-expression of *elovl* genes is important to meet the high requirements of endogenous LC- and VLC-PUFA necessary for the optimal growth and development of neural tissue during early embryonic development independently of dietary supply (Morais *et al*., 2004). This pattern could be modified depending on the hypothetical requirements of VLC-PUFA associated to the larval development of each species, the conditions of the larval culture, as well as the physiological state of the fish. It is even possible that some maternal transference of target genes to the egg takes place to start the LC-PUFA biosynthesis in the embryo, thus the availability of PUFA for early neurogenesis could be ensured (Monroig *et al*., 2009; Morais *et al*., 2012).

Biometric parameters obtained for growth (TL, WW) of early and late larvae of both species showed higher growth performance for fish diets (live preys) containing high LC-PUFA (i.e., enriched). This may be due to a higher intake of the enriched live prey (rotifer and *Artemia*), since an intake of prey rich in LC-PUFA could activate the FA-detection system (hypothalamic mechanisms of lipid sensing that detect changes in plasma levels of LC-FA), positively regulating a higher food intake (Bonacic *et al*., 2016; Ibarra-Zatarain *et al*., 2015). Appetite and food intake are factors that greatly impact larval growth and development (Rønnestad *et al*., 2013), as they determine the amount of nutrients available to larvae for the high structural and energy demands for rapid growth and organogenesis (Bonacic *et al*., 2016; Hamre *et al*., 2013). It is known that lipids are an important source of metabolic energy, components of biological membranes and precursors of essential metabolites (Sargent *et al*., 1999). These properties are of particular importance in larvae of teleostean fish, which are characterized by extremely high growth rates coupled with high demands for energy and structural components (Conceição, 1997; Hamre *et al*., 2013; Tocher *et al*., 2010). The fatty acids released from lipid hydrolysis are used as energy substrates by the growing larvae, especially DHA (Hamre *et al*., 2013). Enriched diets used in our study were different both from a quantitative (higher lipid content) and qualitative (fatty acids) point of view than non-enriched diets, especially in ARA, EPA (*Artemia* E diet) n-6 DPA and DHA levels (rotifer and *Artemia* E diet). These differences could be associated with the dissimilar growth performance shown in *S. aurata* and *S. senegalensis* early and late larvae fed the two different diets in our study, since there are numerous evidences that relate high contents of essential fatty acids, especially EPA and DHA, with optimal growth, survival, behavior and biological functions and processes in marine fish larvae (Hamre *et al*., 2013).

Nutritional regulation of *fads2* and *elovl5* have been extensively studied in fish (Izquierdo *et al*., 2008; Kuah *et al*., 2015; Li *et al*., 2016; Li *et al*., 2017; Morais *et al*., 2012). However, except for the studies in the crab *Scylla paramamosain* (Lin *et al*., 2018), in the fish *Larimichthys crocea* (Li *et al*., 2017) and in *Oncorhynchus mykiss* (Zhao *et al*., 2019), there are no studies on the nutritional regulation of *elovl4* in marine vertebrates. Delta-6 and Δ5-desaturase activity (capacity to bioconvert C_{18} precursors into PUFA) in fish responds to levels of PUFA present in the diet, over-expressing these enzymes to compensate a deficient supply of dietary PUFA (Izquierdo *et al*., 2008; Ren *et al*., 2012; Seiliez *et al*., 2003). However, in contrast with the results reported by Izquierdo *et al*. (2008), where a significant effect of dietary lipids on the regulation of Δ6 desaturase expression in gilthead seabream larvae was observed, no nutritional effects on *S. aurata fads2* and *elovl5* was detected in our study. Our results are in agreement with results reported by Geay *et al*. (2010), where the comparison between the two dietary groups revealed that the use of a diet totally deprived of PUFA did not up-regulate the European sea bass *fads2* activity. Besides, these results are similar to those obtained in other marine fish species: e.g. Atlantic cod fed a PUFA free diet did not exhibit an increase of total desaturation/elongation activities (Tocher *et al*., 2006). This could be indicative of an insufficient Δ6 desaturase activity in the PUFA biosynthesis pathway to maintain the minimum requirements of EPA and DHA in *S. aurata* larvae (early and late larvae), which should be covered with a dietary supply of LC-PUFA. In agreement with results reported by Morais *et al*. (2012), our study showed that the *S. senegalensis fads2* but not *elovl5*, was up-regulated in response to low dietary LC-PUFA (non-enriched diet) in 40 dah larvae. These results are similar to those obtained in some freshwater fish species, e.g. silver barb, common carp and striped snakehead fed low PUFA diets, which exhibited an increase in total desaturation activity (Kuah *et al*., 2015; Nayak *et al*., 2017; Ren *et al*., 2012). This is probably due to the different desaturase activities shown by the Fads2 enzymes of each species, either Δ4 desaturase activity in *S. senegalensis*, or Δ6 activity in *S. aurata*, being the Δ4 desaturase activity the simplest and most direct pathway for the biosynthesis of DHA from EPA (Li *et al*., 2010; Morais *et al*., 2012). The up-regulation of Δ4 desaturase activity in visceral and head regions of *S. senegalensis* larvae as a consequence of a diet low in LC-PUFA could ensure that DHA levels remain constant under limited dietary DHA intake (Kuah *et al*., 2015; Morais *et al*., 2012). This could be indicative of the importance of DHA production from EPA via the Δ4 desaturation step in order to maintain an optimal reserve of DHA in key (neuronal) tissues of carnivore fish (Kuah *et al*., 2015), suggesting the biological importance of this pathway to reduce LC-PUFA dietary dependence in *S. senegalensis*, compared to other marine fish like *S. aurata* (Morais *et al*., 2012).

Analyzing the results concerning the nutritional regulation of *elovl4*a and *elovl4b* genes in 16 dah larvae in response to dietary LC-PUFA, differences were observed in the expression of *elovl4a*, although only at the verge of statistical significance for *S. senegalensis*. *Elovl4a* was up-regulated in 16 dah larvae fed the enriched diet, whereas no differences were observed in the expression of *elovl4b* in response to different dietary regimes. Conversely, in 40 dah larvae, the expression pattern differed from the previous stage, showing a trend towards an over-expression for *elovl4b,* but not for *elovl4a,* in fish fed the enriched diet. This opposite effect of the two isoforms at different development stages (16 dah and 40 dah) of both species could be indicative of the different substrate specificity and tissue localization of *elovl4* isoforms (Monroig *et al*., 2010; Zhao *et al*., 2019). *Elovl4* seems to experience an up-regulation in the expression of one isoform or another, attending to the different demands of PUFA (LC and VLC-PUFA) faced in function of the stage and the degree of fish tissue development. In contrast with the results observed in other fishes (Li *et al*., 2017; Zhao *et al*., 2019), this over-expression responds to a scenario (high levels of substrate) where there is enough dietary availability of LC-PUFA (essentially n-6 DPA, EPA and DHA), which could suggest that both isoforms respond positively to high levels of LC-PUFA activating its transcription to support the formation of specific tissues that have high requirements for VLC-PUFA (Monroig *et al*., 2011). In accordance with Li *et al*. (2017) and Zhao *et al*. (2019), the highest levels of *elovl4a* and *elovl4b* expression, shown in the head (probably in eyes and brain) where VLC-PUFA have a key biological function (Xue *et al*., 2014), are probably linked to this need. This up-regulation can be especially important in predatory fish that need excellent cognitive traits, especially those with a strong nocturnal activity, like *S. senegalensis* (Navarro *et al*., 2009). The lower expression of *elovl4b* in response to low levels of dietary LC-PUFA in late larvae may be associated to local (organ, tissue) synthesis of VLC-PUFA only if adequate levels of precursors (LC-PUFA) are reached, and deserves further exploration.

Although VLC-PUFA were not measured due to the analytical difficulty and the predicted low concentrations existing in the tissues of the species under study (Garlito *et al*., 2019) we can conclude that the presence of *elovl4a* and *elovl4b* mRNA transcripts in embryos and larval fish, including the eggs before hatching, suggests that VLC-PUFA biosynthesis can be important in early development. These findings highlight the importance that the study of VLC-PUFA and their biosynthesis might have in farmed fish in which altered visual acuity (critical in visual predators such as most cultured fish species, especially during larval stages) and disruptions of brain functioning can jeopardize their normal development (Monroig *et al*., 2010). Both isoforms of *elovl4* are expressed preferentially in the head, likely associated to the hypothetical abundance of VLC-PUFA in fish neural tissues including retina. Moreover, the results for both species suggest that the expression of *elovl4* (isoform *a* in early larvae, and *b* in late larvae) can be regulated positively according to the dietary content of LC-PUFA in early stages, including the potential activation of the VLC-PUFA biosynthesis during short-term feeding periods (seven days). These results can be very helpful in the design of diets for larvae (early and late stages) of *S. aurata* and *S. senegalensis*, opening the possibility to make feasible an early nutritional programming along the larval rearing including short periods, particularly for *S. senegalensis*, since the low LC-PUFA requirements attributed to this species could be reconsidered as a tool for the activation of *elovl4* genes, which can be necessary for the maintenance of optimal levels of VLC-PUFA at these stages.

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Tables

Table 1. Primers used for real-time quantitative PCR (qPCR) of *Sparus aurata* and *Solea senegalensis* genes. Sequences of the primer pairs used (Forward: F; Reverse: R), annealing temperatures (Ta) of the primer pairs, size of the fragments produced, and accession number of the sequences used for the primer design are shown.

		Sparus aurata			
Transcript	**Primer**	**Primer sequence**	**Ta**	**Fragment**	**Accession No**
elovl4a	F	5’-GCCCAAGTACATGAAGAACAGAG-3’	60°C	169 bp	MK610320
	R	3’-GGGGTCGTCTGAGTAGTCCA-5’			
elovl4b	F	5’-GTCAAGTACTCCAACGATGTCAA-3’	60°C	247 bp	MK610321
	R	3’-TGAGCACATGGATGGAAGAG-5’			
elovl5	F	5’-TCGTCCACGTCGTGATGTAT-3’	60°C	152 bp	Q68YU3
	R	3’-ACATGGCCATATGACTGCAA-5’			
fads2	F	5’-CACTCAGCCAGTCGAGTACG-3’	60°C	199 bp	GQ162822
	R	3’-ACAGCACAGGTAGCGAAGGT-5’			
actb	F	5’-TGCGTGACATCAAGGAGAAG-3’	60°C	190 bp	X89920
	R	3’-CAGGACTCCATACCGAGGAA-5’			
		Solea senegalensis			
Transcript	**Primer**	**Primer sequence**	**Ta**	**Fragment**	**Accession No**
elovl4a	F	5’-AGGTGAGGTAGGGCCTTGTT-3’	60°C	220 bp	MN164537
	R	3’-TGAAAACAGCCACCTTAGGC-5’			
elovl4b	F	5’-CCTCTGCCTTGTCCAGTTTC-3’	60°C	175 bp	MN164625
	R	3’-CAATTTGATGCCCAGTTCCT-5’			
elovl5	F	5’-CAAGTACATGCAGCACAGGC-3’	60°C	116 bp	JN793448
	R	3’-GCCACACAGCACTAACAAGC-5’			
fads2	F	5’-GTTCGTGTGGGTGACTCAGA-3’	60°C	121 bp	JN673546
	R	3’-GTCGTTGAAGGAGGACTGCT-5’			
actb	F	5’-ACAATGAGCTGAGAGTCGCC-3’	60°C	132 bp	DQ485686
	R	3’-CAACATACATGGCGGGGGTA-5			

Table 2. Growth of *S. aurata* and *S. senegalensis* larvae fed with different live preys: rotifer enriched (Rot E) *vs* non-enriched (Rot NE), and *Artemia* metanauplii enriched (Art E) *vs* non-enriched (Art NE). Length, weight, specific growth rate (SGR) and Fulton's K condition factor are presented as mean ± SD (n= 15). The symbol " * " shows significant differences (t-Student, $P \leq 0.05$) between the dietary regimes.

	S. aurata		***S. senegalensis***	
Diet	**Rot E**	**Rot NE**	**Rot E**	**Rot NE**
Total Length (mm)	4.33 ± 0.09	4.06 ± 0.18	4.23 ± 0.05 *	4.01 ± 0.07
Wet Weight (mg)	1.07 ± 0.17 *	0.55 ± 0.04	0.62 ± 0.07	0.58 ± 0.03
SGR	0.54 %	0.35 %	0.80 %	0.41 %
Diet	**Art E**	**Art NE**	**Art E**	**Art NE**
Total Length (mm)	21.17 ± 2.10*	17.37 ± 2.30	27.83 ± 2.45	24.33 ± 1.93
Wet Weight (mg)	94.83 ± 2.77 *	52.54 ± 2.23	171.20 ± 4.91*	106.10 ± 2.47
SGR	1.27 %	0.70 %	0.98 %	0.64 %
Fulton's K	0.98 ± 0.01	1.00 ± 0.05	0.69 ± 0.03	0.67 ± 0.03

Table 3. Selected fatty acids (% total fatty acids) of the experimental diets: enriched (E) *vs* non-enriched (NE) live preys. Results are expressed as mean ± SD (n=3). The symbol “ * ” indicates significant differences in fatty acid content of the two diets for each live prey (t-Student, $P \leq 0.05$).

Fatty acid	***Rotifers***		***Artemia* metanauplii**	
	E	NE	E	NE
18:2n-6 (linoleic acid**)**	3.06 ± 0.37	3.78 ± 0.32	4.14 ± 0.02*	5.52 ± 0.01
18:3n-6 (γ-linolenic acid**)**	0.14 ± 0.04	0.21 ± 0.02	-	-
20:2n-6 (eicosadienoic acid)	0.07 ± 0.00	0.13 ± 0.02	0.17 ± 0.01	0.25 ± 0.00
20:3n-6 (dihomo-γ-linolenic acid**)**	0.18 ± 0.06	0.14 ± 0.01	-	-
20:4n-6 (arachidonic acid**)**	0.89 ± 0.29	0.96 ± 0.03	2.73 ± 0.03*	1.14 ± 0.00
22:2n-6 (docosadienoic acid)	0.11 ± 0.00	0.14 ± 0.00	-	-
22:4n-6 (adrenic acid**)**	0.08 ± 0.00	0.11 ± 0.04	0.10 ± 0.01	-
22:5n-6 (n-6 docosapentaenoic acid**)**	4.08 ± 2.12*	0.40 ± 0.28	7.79 ± 0.02*	-
Total n-6 PUFA	8.61 ± 2.88	5.87 ± 0.73	14.94 ± 1.43*	6.91 ± 1.26
18 3n-3 (α-linolenic acid**)**	2.79 ± 0.32*	10.46 ± 0.68	13.90 ± 0.17*	23.81 ± 0.05
18 4n-3 (stearidonic acid**)**	1.82 ± 0.34*	4.38 ± 0.17	1.77 ± 0.04*	3.56 ± 0.05
20 3n-3 (docosatrienoic acid**)**	0.18 ± 0.02*	0.44 ± 0.03	0.48 ± 0.01*	0.76 ± 0.01
20:4n-3 (eicosatetraenoic acid**)**	1.85 ± 0.34*	3.67 ± 0.14	0.63 ± 0.01	0.69 ± 0.01
20:5n-3 (eicosapentaenoic acid**)**	1.30 ± 0.41*	2.72 ± 0.06	5.33 ± 0.05*	2.91 ± 0.01
22:3n-3 (docosatrienoic acid)	0.13 ± 0.02	0.23 ± 0.04	-	0.13 ± 0.01
22:5n-3 (n-3 docosapentaenoic acid**)**	0.30 ± 0.12	0.40 ± 0.01	0.33 ± 0.00	-
22:6n-3 (docosahexaenoic acid**)**	7.65 ± 0.55*	2.51 ± 0.03	17.82 ± 0.09*	-
Total n-3 PUFA	15.87 ± 2.10*	24.59 ± 1.13	40.25 ± 2.40*	31.96 ± 2.86
Total unsaturates	37.44 ± 7.24	52.90 ± 4.87	73.78 ± 1.05	75.78 ± 1.48
Total saturates	35.37 ± 3.81	24.88 ± 3.56	23.00 ± 1.99	19.50 ± 1.40
Total MUFA	12.34 ± 2.06	21.09 ± 2.77	18.09 ± 1.20*	36.02 ± 2.30
Total PUFA	25.10 ± 5.18	31.81 ± 2.10	41.87 ± 1.22*	39.76 ± 1.67
Total lipids (%)	15.22 ± 1.00	11.62 ± 0.60	21.60 ± 0.78*	13.77 ± 0.36

Totals include some components not shown. MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; (-): not detected; Total lipids (%): percentage of lipids with respect to the total dry weight of the sample analyzed.

Table 4. Selected fatty acids content (% total fatty acids) of *S. aurata* and *S. senegalensis* early larvae (fed enriched -Rot E- or non-enriched -Rot NE- rotifers) and late larvae (fed enriched -Art E- or non-enriched -Art NE- *Artemia* metanauplii) muscle. Results are expressed as mean ± SD (n=3). The symbol " * " indicates significant differences in selected fatty acids between larvae fed the two corresponding diets (t-Student, $P \leq 0.05$).

Fatty acid	*S. aurata early larvae*		*S. senegalensis early larvae*		*S. aurata late larvae*		*S. senegalensis late larvae*	
	Rot E	**Rot NE**	**Rot E**	**Rot NE**	**Art E**	**Art NE**	**Art E**	**Art NE**
18:2n-6 (linoleic acid**)**	3.35 ± 0.11*	4.03 ± 0.10	3.88 ± 0.11*	4.43 ± 0.11	3.17 ± 0.18*	4.90 ± 0.33	4.58 ± 0.08*	6.28 ± 0.06
18:3n-6 (γ-linolenic acid**)**	0.14 ± 0.04	0.15 ± 0.01	0.12 ± 0.01	0.15 ± 0.06	0.17 ± 0.02	0.15 ± 0.01	-	-
20:2n-6 (eicosadienoic acid)	0.22 ± 0.01	0.23 ± 0.01	0.44 ± 0.01	0.52 ± 0.03	0.10 ± 0.01*	0.16 ± 0.01	0.30 ± 0.02	0.38 ± 0.03
20:3n-6 (dihomo-γ-linolenic acid**)**	0.46 ± 0.01	0.48 ± 0.03	0.38 ± 0.02	0.29 ± 0.04	-	-	-	-
20:4n-6 (arachidonic acid**)**	4.75 ± 0.12	4.66 ± 0.27	4.68 ± 0.06	4.13 ± 0.18	4.75 ± 0.27	4.61 ± 0.26	5.11 ± 0.03*	2.75 ± 0.04
22:4n-6 (adrenic acid)	1.65 ± 0.05	1.74 ± 0.01	1.57 ± 0.14	1.82 ± 0.04	0.22 ± 0.03	0.15 ± 0.03	1.00 ± 0.06*	1.41 ± 0.03
22:5n-6 (n-6 docosapentaenoic acid**)**	3.48 ± 0.24*	0.95 ± 0.05	4.43 ± 0.12*	1.32 ± 0.04	4.86 ± 0.36*	0.44 ± 0.04	3.23 ± 0.14*	0.24 ± 0.02
Total n-6 PUFA	14.04 ± 0.57*	12.25 ± 0.48	15.50 ± 0.46*	12.67 ± 0.49	13.26 ± 0.86*	10.41 ± 0.67	14.22 ± 0.33*	11.07 ± 0.17
18:3n-3 (α-linolenic acid**)**	1.49 ± 0.25*	4.06 ± 0.52	1.73 ± 0.16*	4.34 ± 0.24	1.99 ± 0.07*	2.62 ± 0.10	6.87 ± 0.46*	10.30 ± 0.26
18:4n-3 (stearidonic acid**)**	0.84 ± 0.12	1.35 ± 0.15	0.88 ± 0.08*	1.71 ± 0.09	0.20 ± 0.02*	0.17 ± 0.01	0.66 ± 0.07	0.85 ± 0.04
20:3n-3 (eicosatrienoic acid**)**	0.31 ± 0.09	0.53 ± 0.02	0.32 ± 0.04*	0.56 ± 0.04	0.12 ± 0.01*	0.18 ± 0.01	0.37 ± 0.12	0.52 ± 0.12
20:4n-3 (eicosatetraenoic acid**)**	2.86 ± 0.29	4.20 ± 0.20	2.68 ± 0.15*	3.61 ± 0.14	0.23 ± 0.01*	0.27 ± 0.01	0.54 ± 0.10*	0.92 ± 0.08
20:5n-3 (eicosapentaenoic acid**)**	4.38 ± 0.16*	6.18 ± 0.09	2.11 ± 0.03*	3.48 ± 0.06	9.08 ± 0.46*	7.43 ± 0.03	3.42 ± 0.16*	2.66 ± 0.12
22:4n-3 (n-3 docosatetraenoic acid**)**	0.11 ± 0.01	0.17 ± 0.02	0.14 ± 0.01	0.18 ± 0.02	0.07 ± 0.02	0.06 ± 0.00	0.31 ± 0.02*	0.47 ± .020
22:5n-3 (n-3 docosapentaenoic acid**)**	2.75 ± 0.11	3.34 ± 0.09	2.21 ± 0.10	2.27 ± 0.05	0.71 ± 0.05	0.79 ± 0.07	2.28 ± 0.08*	1.32 ± 0.07
22:6n-3 (docosahexaenoic acid**)**	25.48 ± 1.16*	16.71 ± 1.15	25.09 ± 0.40*	18.85 ± 0.65	13.42 ± 1.58*	4.48 ± 0.36	10.43 ± 0.40*	3.10 ± 0.24
Total n-3 PUFA	38.22 ± 2.18	36.54 ± 2.14	35.16 ± 0.97	35.01 ± 1.28	24.11 ± 2.10*	17.59 ± 1.02	30.00 ± 1.45*	22.87 ± 0.98
Total unsaturates	68.95 ± 3.78	69.16 ± 3.70	68.56 ± 2.75	69.30 ± 2.56	59.99 ± 5.01*	54.43 ± 2.93	65.93 ± 2.49	63.42 ± 1.85
Total saturates	30.46 ± 0.86	30.42 ± 0.91	31.03 ± 1.28	30.11 ± 0.92	22.06 ± 0.90	22.99 ± 1.33	32.08 ± 1.71	29.23 ± 1.06
Total MUFA	15.91 ± 0.89*	19.64 ± 0.86	16.91 ± 0.95*	20.24 ± 0.71	21.05 ± 1.65	25.01 ± 1.01	25.83 ± 0.69*	30.95 ± 0.59
Total PUFA	53.04 ± 2.89*	49.52 ± 2.84	51.66 ± 1.80*	49.06 ± 1.85	37.44 ± 2.98*	28.06 ± 1.69	38.70 ± 1.74*	31.19 ± 1.12
Total lipids (%)	Not quantified	Not quantified	Not quantified	Not quantified	10.17 ± 0.94*	7.94 ± 0.92	13.54 ± 2.5	10.40 ± 1.97

Totals include some components not shown. MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; (-): not detected; Total lipids (%): percentage of lipids with respect to the total dry weight of the sample analyzed.

Figures

Figure 1. Expression pattern of *S. aurata* fatty acyl desaturase (*fads2*, A) and elongases (*elovl5*, B; *elovl4a*, C; *elovl4b*, D) genes during early ontogenetic development, determined by qPCR in whole eggs and larvae from 1 to 7 days after hatching (dah). The results shown as relative index, are β-actin normalized values (gene copy number/*β-actin* copy number) corresponding to the mean and standard deviation as error bars (n=3). Different letters above the columns show significant differences (ANOVA and Tukey test, $P \leq 0.05$) among time points for each gene.

Figure 2. Expression pattern of *S. senegalensis* fatty acyl desaturase (*fads2*, A) and elongases (*elovl5*, B; *elovl4a*, C; *elovl4b*, D) genes during early ontogenetic development, determined by qPCR in whole eggs and larvae from 1 to 7 days after hatching (dah). The results shown as relative index, are β-actin normalized values (gene copy number/*β-actin* copy number) corresponding to the mean and standard deviation as error bars (n=3). Different letters above the columns show significant differences (ANOVA and Tukey test, $P \leq 0.05$) among time points for each gene.

Figure 3. Expression pattern of *S. aurata* (A) and *S. senegalensis* (B) fatty acyl desaturase (*fads2*) and elongase (*elovl4a*, *elovl4b* and *elovl5*) genes in early larvae (16 days after hatching) fed rotifer diets: enriched (Rot E) and non-enriched (Rot NE). The results, shown as relative index, are β-actin normalized values (gene copy number / *β-actin* copy number) corresponding to the mean and standard deviation as error bars (n=3). Different letters above the columns show significant differences (t-Student, $P \leq 0.05$, except where noted) between the diets, for each gene.

Figure 4. Expression pattern of *fads2* (A), *elovl5* (B), *elovl4a* (C) and *elovl4b* (D) in *S. aurata* late larvae (40 days after hatching) fed *Artemia* diets: enriched (Art E) and non-

enriched (Art NE). The results, shown as relative index, are β-actin normalized values (gene copy number / *β-actin* copy number) corresponding to the mean and standard deviation as error bars (n=3). The symbol " * " above the columns shows significant differences (one way-ANOVA, $P \leq 0.05$) among body compartments for both diets pooled.

Figure 5. Expression pattern of *fads2* (A), *elovl5* (B), *elovl4a* (C) and *elovl4b* (D) in *S. senegalensis* late larvae (40 days after hatching) fed *Artemia* diets: enriched (Art E) and non-enriched (Art NE). The results shown as relative index, are β-actin normalized values (gene copy number / *β-actin* copy number) corresponding to the mean and standard deviation as error bars (n=3). Different letters above the columns represent significant differences (t-Student, $P \leq 0.05$, except where noted) between diets, for each gene. The symbol " * " above the columns shows significant differences (one way-ANOVA, $P \leq 0.05$) among body compartments for both diets pooled.