

Elovl4 genes in *Sparus aurata*

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Very long-chain ($>C_{24}$) fatty acids (VLC-FA) play critical roles during early development of vertebrates, since these compounds are accumulated in the rapidly forming neural tissues, ensuring their normal function. However, despite their putative importance, the study of VLC-FA in fish is scarce. Biosynthesis of VLC-FA is carried out by the so-called elongation of very long-chain fatty acid 4 (Elovl4) proteins and, consequently, the complement and function of these enzymes determine the capacity that a given species has for satisfying the physiological demands for VLC-FA, especially during its early development. The present study aimed to characterize *elovl4* genes from the marine teleosts *Sparus aurata* and *Solea senegalensis*, and determine the function of the corresponding encoded proteins. Moreover, the tissue expression pattern of *elovl4* genes was determined. The results confirmed that both fish species possess two distinct Elovl4 proteins termed as Elovl4a and Elovl4b based on their homology to the zebrafish orthologs. Functional assays in yeast denoted that both Elovl4a and Elovl4b from both species had the capability to elongate C_{20-24} fatty acid precursors to VLC-FA products. However, Elovl4b appeared to have a higher activity than Elovl4a elongating all the polyunsaturated fatty acid substrates assayed to longer chain polyunsaturated products, especially on the n-3 series. Gene expression results indicated that, although *elovl4* transcripts were detected in most tissues analyzed, *elovl4* genes were more strongly expressed in both species neural tissues such as brain and eyes, which showed the highest expression levels of *elovl4a* and *elovl4b*, respectively. These results are consistent with the functions of Elovl4 from other vertebrates. Importantly, these findings contribute to a better understanding of the VLC-FA biosynthetic pathway in marine teleosts, highlighting the crucial role that Elovl4 products carry out for the correct development and maintenance of neurophysiologic functions during early stages of the fish development.

Keywords: very long-chain polyunsaturated fatty acid ; Elovl4 ; fish

1. Introduction

Certain long-chain(C_{20-24}) polyunsaturated fatty acids (LC-PUFA), namely eicosapentaenoic acid (EPA; $20:5n-3$), arachidonic acid (ARA; $20:4n-6$) and docosahexaenoic acid (DHA; $22:6n-3$), are regarded as physiologically essential for the correct development of vertebrates, including fish ^[1]. These compounds can be obtained through the diet or, alternatively, biosynthesized from C_{18} polyunsaturated fatty acids (PUFA), such as α -linolenic acid ($18:3n-3$) and linoleic acid ($18:2n-6$) via enzymatic reactions carried out by fatty acyl desaturases (Fads) and elongation of very long-chain fatty acid (Elovl) proteins ^{[1][2]}. Fads are enzymes that introduce double bonds (unsaturations) into PUFA substrates. On the other hand, Elovl are considered pivotal components of fatty acid (FA) synthetic pathways ^{[3][4]}, being responsible for a condensation reaction which results in the extension of the pre-existing FA chain with two new carbon atoms ^[1]. The Elovl protein family contains several members (Elovl1-8) ^{[2][3][4][5][6]}, but only Elovl4 catalyzes the synthesis of very long-chain ($>C_{24}$) saturated (VLC-SFA) and polyunsaturated fatty acids (VLC-PUFA), which can have up to 36 or 38 carbons ^{[1][7]}. Furthermore, Elovl4 is additionally responsible for the production of very long-chain saturated fatty acids (VLC-SFA) ^[8].

Virtually all teleosts possess at least two Elovl4 isoforms termed as “Elovl4a” and “Elovl4b” ^{[2][9]}. However, their functions seem to vary among species, highlighting that the investigation of Elovl4 proteins in teleosts requires a species-specific approach. The gilthead seabream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*) are two commercially important species in marine finfish aquaculture. A recent study has highlighted a relationship between the expression of *elovl4* genes in both species and the formation of neural tissues during early life-cycle development ^[10]. Indeed, Elovl4 products, i.e. VLC-SFA and VLC-PUFA, play crucial roles during early-development of vertebrates by guaranteeing the correct development and functionality of the rapidly forming nervous system where these compounds accumulate ^{[8][10]}. Thus, due to the importance of very long-chain fatty acids (VLC-FA) during early development, it is crucial to understand the capacity that a given species has for endogenous production of these essential nutrients. Such ability is itself dependent on the complement of *elovl4* genes and the functions of their corresponding encoded enzymes ^[1]. Previous studies investigating the functions of *fads*- and other *elovl*-like genes confirmed that both fish species operate different

LC-PUFA biosynthesis mechanisms ^{[11][12][13][14]}, especially with regard to production of DHA. In particular, *Sa* operates the so-called “Sprecher pathway” ^{[11][15]} whereas *Ss* produces DHA via the more direct “ $\Delta 4$ pathway” ^[14] (Figure 1). Considering that both the LC-PUFA and VLC-PUFA biosynthetic pathways are interdependent, the aim of the present study was to characterize, both molecularly and functionally, *elovl4* paralogs from *S. aurata* (*Sa*) and *S. senegalensis* (*Ss*) and discuss the biosynthetic particularities of both species.

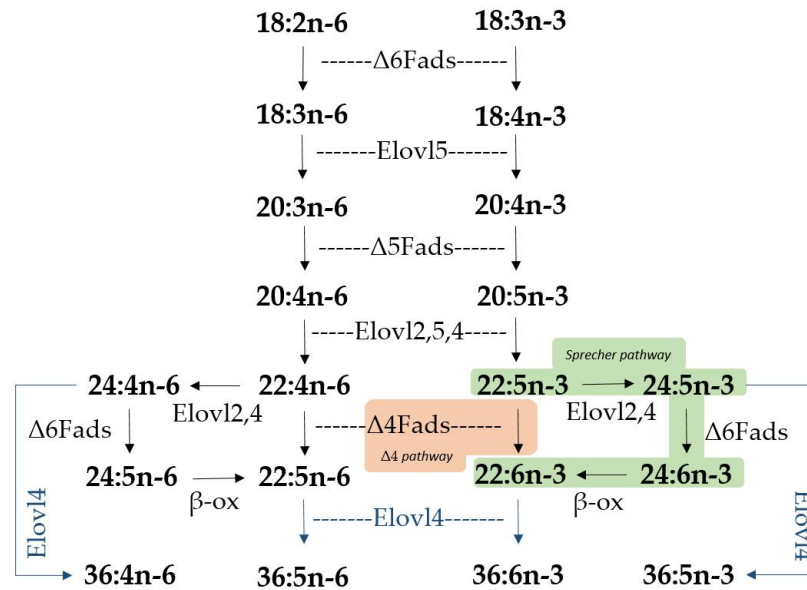


Figure 1. Biosynthetic pathways of long-chain (C₂₀₋₂₄) and very long-chain polyunsaturated fatty acids (VLC-PUFA; >C₂₄) in fish. Desaturation reactions are mediated by fatty acyl desaturases (Fads), whereas elongation reactions are catalyzed by elongation of very long-chain fatty acid (Elovl) proteins. Microsomal β -oxidation reactions are denoted as " β -ox". Two pathways for docosahexaenoic acid (22:6n-3) biosynthesis from docosapentaenoic acid (22:5n-3) are indicated, namely the Sprecher pathway (green background) and the $\Delta 4$ pathway (orange background). Elongation reactions leading to VLC-PUFA biosynthesis of up to C₃₆ are indicated with blue arrows. Note the fish species studied herein (*Sparus aurata* and *Solea senegalensis*) lack *elovl2* in their genomes ^[2].

2. Materials and Methods

2.1. Molecular Cloning of *elovl4* cDNA Sequences

Cloning of the *elovl4* full-length cDNA was carried out using PCR-based methodologies and brain-eye mix (1:1) cDNA as template. The PCR fragments were then purified and sequenced at least two times. In order to obtain the full-length ORF sequences, two-round (nested) Rapid Amplification of cDNA ends (RACE) PCR were performed. Potential positive fragments were cloned into pGEM-T Easy cloning vector and ligated with T4 DNA ligase. The plasmid preparations were purified and sequenced. The putative *elovl4* sequences were thus obtained and deposited in the GenBank database as gb|MK610320 (*Sa elovl4a*), gb|MK610321 (*Sa elovl4b*), gb|MN164537 (*elovl4a*) and gb|MN164625 (*elovl4b*).

2.2. Sequence and Phylogenetic Analysis

The BLAST sequence analysis of NCBI was used for sequence alignment and phylogenetic analysis. The aa sequences deduced from the nucleotide sequences of the *Sa* Elovl4a (gb|QES86604.1), *Ss* Elovl4a (gb|QGA31141.1), *Sa* Elovl4b (gb|QES86605.1), and *Ss* Elovl4b (gb|QGA31140.1), were aligned using the ClustalW tool (BioEdit v7.0.9). The phylogenetic tree was constructed using a total of 31 Elovl aa sequences from different species using MEGA X software.

2.3. Functional Characterization of *Sa* and *Ss* Elovl4 Isoforms

The *Sa* and *Ss* putative Elovl4 elongases were functionally characterized by determining the FA profiles of *Saccharomyces cerevisiae* transformed with pYES2 yeast expression vector containing the putative *elovl4* as inserts, and grown in the presence of potential FA substrates.

2.4. Fatty Acid Analysis

The elongation of endogenous saturated FA with 24 carbons or longer was assessed by comparison of the areas of the fatty acids of control yeast with those of yeast transformed with each of the pYES2-*elovl4* plasmid constructs ($n=3$). In the case of VLC-PUFA, the elongation conversions of exogenously added PUFA were calculated as (area of first product and

longer chain products/(area of first product and longer chain products + substrate area)) × 100.

2.5. Tissue Expression of elovl4 Genes in Gilthead Seabream and Senegalese Sole

Expression of elovl4 isoforms in each tissue from one specimen of gilthead seabream and Senegalese sole was analyzed by reverse transcriptase PCR (RT-PCR), using 18s ribosomal RNA (18s) as a reference gene. Expression of elovl4a and elovl4b in selected tissues that showed a strong signal in RT-PCR analyses (brain, eye and gonad) was analyzed by qPCR using β -actin (actb) as reference gene ($n=3$).

2.6. Statistical Analysis

The homogeneity of variances of the data associated to VLC-SFA (%) and tissue gene expression values, determined by qPCR, were checked using Levene's test. Statistical differences were analyzed by one-way analysis of variance (ANOVA) ($P \leq 0.05$) followed by Tukey HSD *post-hoc* tests using SPSS 26.0 software.

3. Results

3.1. Elovl4 Sequence and Phylogenetic Analysis

The *Sa* and *Ss* *elovl4a* ORF sequences have 969 base pairs (bp) and 960 bp, encoding putative proteins of 322 amino acids (aa) and 319 aa, respectively. On the other hand, *Sa* and *Ss* *elovl4b* ORF sequences contain 918 bp, encoding proteins of 305 aa. The phylogenetic analysis showed that both Elovl4 sequences from each species form two separate clusters that include either Elovl4a or Elovl4b sequences from a range of teleosts (Figure 2).

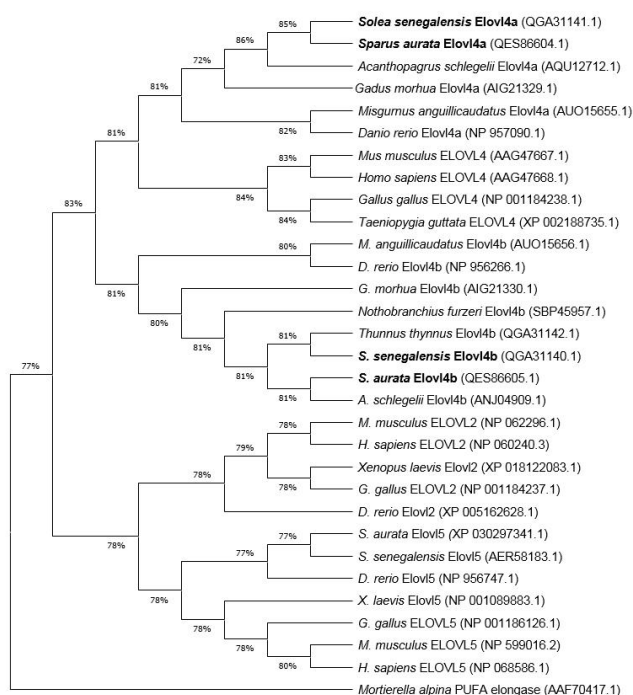


Figure 2. Phylogenetic tree comparing *Sparus aurata* and *Solea senegalensis* Elovl4a and Elovl4b proteins (highlighted in bold) with Elovl2, Elovl4 and Elovl5 proteins from other vertebrates. The tree was constructed using the Maximum Likelihood method and JTT matrix-based model. The numbers in branches represent the frequencies (%) of each node after 1000 iterations by bootstrapping. The *Mortierella alpina* PUFA elongase was included in the analysis as an outgroup, to construct the rooted tree.

3.2. Functional characterization of Elovl4a and Elovl4b

The results confirmed that both *Sa* and *Ss* Elovl4 isoforms are involved in the biosynthesis of VLC-SFA (Table 1A-B). These results suggest that 26:0 is an important substrate for both *Sa* Elovl4 proteins (Table 1A). Moreover, *Ss* Elovl4 proteins are involved in the biosynthesis of VLC-SFA up to 26:0 for which they use C_{24} fatty acids as elongation substrates (Table 1B). Furthermore, the chromatographic analyses of Elovl4-transformed yeast revealed that *Sa* Elovl4a

elongated all n-6 PUFA substrates, as well as n-3 PUFA substrates (Table 2). *Sa* Elovl4b also elongated n-6 PUFA substrates, but showed particularly high affinity towards n-3 PUFA substrates, especially to elongate 22:6n-3 (DHA) substrates to 32:6n-3 (Table 2). For Senegalese sole, both Elovl4 elongases presented the capability to elongate PUFA substrates from the n-3 and n-6 series, to longer chain FA of up to C₃₄ (Table 2). It is noteworthy that *Ss* Elovl4b, and to a lesser extent Elovl4a, showed high capacity to elongate 20:5n-3 (EPA) to 22:5n-3, a key step required for DHA synthesis via the $\Delta 4$ pathway.

A				B			
FA	Elovl4a	Elovl4b	Control	FA	Elovl4a	Elovl4b	Control
24:0	14.6 ^a	11.3 ^a	12.1 ^a	24:0	9.2 ^b	9.5 ^b	6.1 ^a
26:0	49.5 ^b	68.2 ^a	75.0 ^a	26:0	72.1 ^b	81.2 ^b	58.3 ^a
28:0	20.8 ^c	14.1 ^b	8.6 ^a	28:0	11.9 ^b	5.7 ^c	21.7 ^a
30:0	11.0 ^b	4.5 ^a	2.7 ^a	30:0	5.5 ^b	2.9 ^b	11.5 ^a
32:0	3.3 ^b	1.5 ^a	1.0 ^a	32:0	1.4 ^{ab}	0.7 ^b	2.4 ^a
34:0	0.7 ^a	0.3 ^a	0.3 ^a	34:0	0.0 ^a	0.0 ^a	0.0 ^a

Table 1. Functional characterization of *Sparus aurata* (A) and *Solea senegalensis* (B) Elovl4 elongases: role in the biosynthesis of very long-chain saturated fatty acids (FA). Results are expressed as area percentage (%) of total saturated FA \geq C₂₄ found in yeast transformed with either pYES2 containing the *elovl4* coding regions or empty pYES2 vector (control) ($n=3$). Different superscripts denote significant differences in each row, among area percentages of each saturated FA (one way-ANOVA and Tukey test, $P \leq 0.05$).

		Sa Elovl4a	Sa Elovl4b	Ss Elovl4a	Ss Elovl4b
FA substrate	Product	% Conversion			
18:4n-3	20:4n-3	2.5	2.7	4.5	8.1
	22:4n-3	9.7	12.5	19.6	41.2
	24:4n-3	5.6	49.9	39.5	79.0
	26:4n-3	n.d.	65.6	39.6	95.3
	28:4n-3	n.d.	n.d.	100	96.8
	30:4n-3	n.d.	n.d.	100	98.7
	32:4n-3	n.d.	n.d.	65.4	65.7
	34:4n-3	n.d.	n.d.	n.d.	1.7

18:3n-6	20:3n-6	2.6	2.1	4.6	6.2
	22:3n-6	21.6	9.6	38.6	40.8
	24:3n-6	52.5	n.d.	66.2	66.0
	26:3n-6	57.1	n.d.	65.3	89.1
	28:3n-6	64.8	n.d.	100	91.9
	30:3n-6	90.0	n.d.	55.0	90.4
	32:3n-6	84.1	n.d.	62.7	17.8
	34:3n-6	41.3	n.d.	n.d.	n.d.
20:5n-3	22:5n-3	5.8	9.1	12.1	30.9
	24:5n-3	17.2	33.3	31.8	75.1
	26:5n-3	20.0	57.8	35.7	87.4
	28:5n-3	n.d.	86.8	100	96.9
	30:5n-3	n.d.	97.7	50.0	98.9
	32:5n-3	n.d.	72.7	33.7	82.9
	34:5n-3	n.d.	8.1	38.2	14.5

20:4n-6	22:4n-6	10.9	8.9	18.1	33.1
	24:4n-6	31.0	30.2	49.9	73.4
	26:4n-6	37.1	55.9	56.7	85.1
	28:4n-6	39.0	81.0	65.2	94.3
	30:4n-6	88.6	37.8	95.2	95.9
	32:4n-6	83.6	n.d.	84.9	51.8
	34:4n-6	73.7	n.d.	25.3	2.7
	36:4n-6	11.4	n.d.	n.d.	n.d.
22:5n-3	24:5n-3	3.4	12.6	7.8	44.3
	26:5n-3	19.8	52.2	33.9	87.9
	28:5n-3	26.0	86.3	51.2	97.0
	30:5n-3	85.6	96.5	92.3	99.0
	32:5n-3	74.2	64.4	27.4	82.5
	34:5n-3	63.0	5.3	32.4	16.2

22:4n-6	24:4n-6	8.2	10.4	13.5	37.2
	26:4n-6	35.1	43.1	58.3	85.5
	28:4n-6	45.5	71.8	71.8	94.5
	30:4n-6	90.8	83.0	94.5	96.3
	32:4n-6	78.7	19.5	21.6	53.9
	34:4n-6	54.6	n.d.	25.9	5.0
	36:4n-6	7.2	n.d.	n.d.	n.d.
	24:6n-3	0.4	1.8	0.6	5.1
22:6n-3	26:6n-3	n.d.	100	n.d.	100
	28:6n-3	n.d.	100	n.d.	100
	30:6n-3	n.d.	40.2	n.d.	100
	32:6n-3	n.d.	61.3	n.d.	22.3
	34:6n-3	n.d.	n.d.	n.d.	n.d.

n.d.: not detected.

Table 2. Functional characterization of the *Sparus aurata* (Sa) and *Solea senegalensis* (Ss) *Elov14a* and *Elov14b* elongases by heterologous expression in the yeast *Saccharomyces cerevisiae*. Data are presented as the percentage conversions of polyunsaturated fatty acid (FA) substrates (n=1). Individual conversions were calculated according to the formula (area of first product and longer chain products/(area of first product and longer chain products + substrate area)) × 100.

3.3. Tissue expression of *elov14* genes

RT-PCR analyses denoted that, in both species, *elov14a* and *elov14b* transcripts appear to be present in most of the analyzed tissues (Figure 3A-B). RT-PCR analyses indicated that for both species, highest expression values were found in brain for *elov14a*, and eye for *elov14b* (Figure 3C-F).

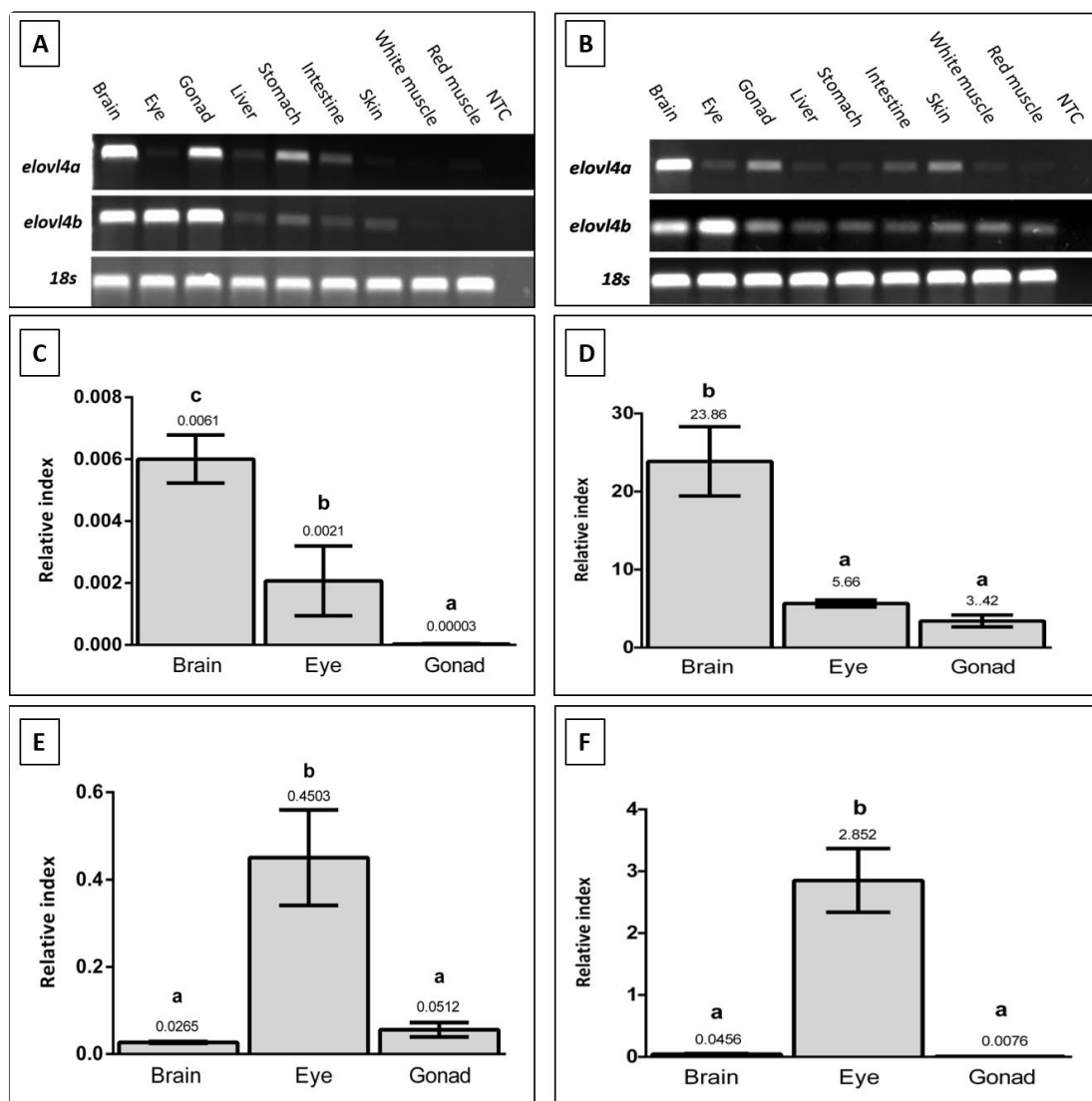


Figure 3. Tissue distribution of *elovl4a* and *elovl4b* transcripts in *Sparus aurata* (A) and *Solea senegalensis* (B) determined by RT-PCR (n=1 fish). Expression of housekeeping gene 18s is also shown. Expression in selected tissues of *Sparus aurata* *elovl4a* (C) *Solea senegalensis* *elovl4a* (D), *Sparus aurata* *elovl4b* (E) and *Solea senegalensis* *elovl4b* (F) transcripts was also determined by qPCR. The results, shown as relative index, are β -actin normalized values (gene copy number/ β -actin copy number). Bars represent means and standard deviations (n=3 fish). Different letters denote significant differences (ANOVA and Tukey HSD test, $P \leq 0.05$) among tissues.

4. Discussion

Sequence analyses revealed that the investigated predicted Elov4 proteins contain all characteristic domains of vertebrate Elov4 family members [16][17]. Moreover, phylogenetic analysis confirmed that the described Elov4 isoforms are true orthologs of the Elov4a and Elov4b proteins present in teleosts [2]. So, the conservation of both Elov4 isoforms in fish genomes [2][9][18][19][20][21] and their clear segregation into separate clusters points towards a likely functional specialization of these proteins in teleosts [22][23], which we aimed to further elucidate in this study by functionally characterizing the two isoforms in two new fish species with diverse life histories, dietary habits, and notably different LC-PUFA biosynthesis mechanisms [11][12][13][14].

As suggested in previous studies with other fish species, including *Danio rerio* [9], *Clarias gariepinus* [18] and *Salmo salar* [24], our results support the notion that both isoforms can participate in VLC-SFA elongation. Nevertheless, similarly to what was reported in *D. rerio* [9], Elov4a seems to be more efficient than Elov4b at elongating VLC-SFA. Notably, the functional characterization of Ss Elov4 enzymes showed some differences with respect to Sa, particularly concerning the preferred fatty acid substrates. Moreover, functional analyses of Elov4a and Elov4b confirmed that both proteins participate actively in the biosynthesis of either n-6 or n-3 VLC-PUFA, from n-6 and n-3 PUFA substrates, in the two studied fish species. However, intra- and inter-specific differences were found in the efficiency of the different Elov4 isoforms to biosynthesize VLC-PUFA. This differences could be related with different VLC-SFA and VLC-PUFA requirements between the two fish species [10], but further studies are necessary to clearly establish this. It is also noteworthy that, similar to what has been described in zebrafish [9], both Sa and Ss Elov4a elongases showed low elongation activity from DHA to 24:6n-3. This could suggest that, as described in rat retinas [25], EPA and not DHA might

be the preferred substrate for VLC-PUFA biosynthesis in fish. Similarly to what has been found in other teleosts [18][19][26], Elovl4b proteins in both species were able to elongate 24:6n-3 up to 32:6n-3, a VLC-PUFA found in retinal phosphatidylcholine in fish [27][28]. Thus, this specific activity of Elovl4b proteins, along with the above mentioned presence of 32:6n-3 in fish retina, is coherent with the tissue expression results obtained for both species, in which Elovl4b transcripts were mostly found in the eye suggesting that, similarly to what has been described in other teleosts like *Thunnus. thynnus* [26], *D. rerio* [9], *Acanthopagrus schlegelli* [19], *Oncorhynchus mykiss* [23], *S. salar* [24] or *Epinephelus coioides* [29], this is a major tissue for VLC-PUFA biosynthesis.

The quantitative expression results confirmed previous evidences of a differential *elovl4a* and *elovl4b* tissue-specific expression pattern [10][30], with *elovl4a* being mostly expressed in fish brain [9][18][19][23], and *elovl4b* in eye [9][19][23][24][26][29]. This results suggest a role of these enzymes in the local biosynthesis and incorporation of VLC-FA in fish neural tissues. This is in agreement with what is known in mammals [8], in which VLC-PUFA are key functional components, essential for the development and cell protection, of neural tissues such as those found in retina or brain [7][31][32]. Moreover, as previously described [10], retinogenesis in gilthead seabream and Senegalese sole larvae is clearly synchronized with an increase in expression of both *elovl4* genes. Consequently, as described in mammals [33], alterations in VLC-PUFA biosynthesis could negatively impact visual acuity and disrupt brain functionality, jeopardizing the normal development of fish. This is particularly relevant in visual predators such as gilthead seabream and Senegalese sole, which previously showed a differential *elovl4* expression in larvae and postlarvae according to the VLC-PUFA putative needs associated with each life-stage and LC-PUFA dietary availability [10][30]. Thus, the application of this knowledge is of special relevance during early larval development, particularly in species with high commercial interest for aquaculture production, as is the case of gilthead seabream and Senegalese sole, and should be kept in mind in feeding protocols during hatchery rearing.

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