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Tissue-expression pattern of *elovl4* genes in *Sparus aurata* and *Solea senegalensis*: from larvae to adult

Patrón de expresión tisular de los genes *elovl4* en *Sparus aurata* y *Solea senegalensis*: de larva a adulto

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RESUMEN | Los ácidos grasos de cadena muy larga (> C24) (VLC-FA) juegan un papel crítico durante el desarrollo temprano de los vertebrados, ya que estos compuestos se acumulan en los tejidos neurales que se forman rápidamente, asegurando su función normal. La funcionalidad de los VLC-FA ha despertado el interés científico, centrándose en el estudio de las proteínas elongasas 4 de ácidos grasos de cadena muy larga (Elovl4), que son responsables de su biosíntesis a partir de ácidos grasos más cortos (precursores). Para comprender mejor el metabolismo y los potenciales requisitos a nivel tisular de los VLC-FA en teleósteos marinos, el presente estudio tuvo como objetivo determinar el patrón de expresión tisular de los genes que codifican las isoformas de Elovl4, es decir, *elovl4a* y *elovl4b*, en diferentes ventanas del desarrollo (larvas y adultos) de dorada (*Sparus aurata*) y lenguado senegalés (*Solea senegalensis*). Los resultados indicaron que en las larvas de *S. aurata*, *elovl4a* se expresa ampliamente en la región cerebral, mientras que *elovl4b* se expresa intensamente en los ojos. Curiosamente, en las larvas de *S. senegalensis* se observó un patrón de expresión tisular opuesto, siendo *elovl4a* y *elovl4b* fuertemente expresado en ojos y región cerebral, respectivamente. En adultos de ambas especies, aunque se detectaron transcritos de *elovl4* en la mayoría de los tejidos analizados, los mayores valores de expresión de *elovl4a* y *elovl4b* se observaron en el cerebro y en los ojos, respectivamente. Es importante destacar que el diferente patrón de expresión tisular observado para ambos genes *elovl4* asociados a la etapa pre y posmetamórfica de *S. senegalensis* podría ser indicativo de las necesidades particulares de VLC-FA vinculadas a la funcionalidad de los tejidos neurales en cada etapa de desarrollo. Estos hallazgos pueden contribuir a una mejor comprensión del metabolismo de VLC-FA específico en función de la especie de teleósteo marino.

Keywords
Very long-chain fatty acids
Marine fish
Special-distribution

ABSTRACT | Very long-chain (> C24) 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. The functionality of VLC-FA has aroused scientific interest, focusing on the study of elongases protein 4 of very long chain fatty acid (Elovl4), which are responsible for their biosynthesis from shorter fatty acids (precursors). For a better understanding of the metabolism and the potential tissue-specific requirements of VLC-FA in marine teleosts, the present study aimed to determine the tissue-expression pattern of the genes that encode for Elovl4 isoforms, i.e. *elovl4a* and *elovl4b*, in different windows of development (larval and adult stages) of Gilthead seabream *Sparus aurata* and Senegalese sole *Solea senegalensis*. The results indicated that in *S. aurata* larvae, *elovl4a* is widely expressed in the head, while *elovl4b* is strongly focused in the eyes. Interestingly, in *S. senegalensis* larvae an opposite tissue-expression pattern was observed for both *elovl4* isoforms. In adults of both fish, although *elovl4* transcripts were detected in most tissues analyzed, *elovl4a* and *elovl4b* genes were strongly expressed in brain and eyes, respectively. Importantly, the differential tissue-expression pattern of both *elovl4* isoforms associated to the pre- and post-metamorphic stage of *S. senegalensis* could be indicative of the VLC-FA particular needs linked to neural tissues functionality in each development stage. These findings can contribute to a better understanding of the species-specific VLC-FA metabolism in marine teleosts.

INTRODUCTION

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. Their biosynthesis is carried out by the so-called elongation of very long-chain fatty acid 4 (Elovl4) proteins through the successive elongation reactions from pre-existing long-chain fatty acids (LC-FA) (Monroig *et al*., 2018). 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 (Deák *et al*., 2019; Torres *et al*., 2020a, b).

Virtually, all teleost fish possess at least two Elovl4 isoforms termed as Elovl4a and Elovl4b (Castro *et al*., 2016; Monroig *et al*., 2010). Both Elovl4 are associated to VLC-FA biosynthesis (Monroig *et al*., 2018. Morais *et al*., 2020). However, the capability of each isoform to efficiently participate in the VLC-FA production can differ in function of the fish species studied (Betancor *et al*., 2020; Jin *et al*., 2017; Monroig *et al*., 2010; Morais *et al*., 2020; Oboh *et al*., 2017). Thus, Elovl4a is mostly involved in the very long-chain saturated fatty acids (VLC-SFA) biosynthesis, while Elovl4b mainly participates in the very long-chain polyunsaturated fatty acids (VLC-PUFA) production (Deák *et al*., 2019; Monroig *et al*., 2010).

VLC-SFA and VLC-PUFA, are strongly connected with correct development and functionality of nervous system (Deák *et al*., 2019; Morais *et al*., 2020), being synthesized and incorporated into more complex lipids in a tissue-specific manner (Cameron *et al*., 2007; Deák *et al*., 2019). Generally, VLC-PUFA are incorporated into phosphatidylcoline in the photoreceptor cells that make up the retina (Agbaga *et al*., 2010) participating in the photoreceptor protection (Bazan, 2018; Deák *et al*., 2019). VLC-SFA are mainly incorporated into sphingolipids in the brain (Deák *et al*., 2019) taking part in the membrane fusion of synaptic vesicles carried out during neurotransmission process (Hopiavuori *et al*., 2018, 2019). Moreover, Elovl4 seem to play also a significant role in the LC-PUFA biosynthesis pathway, elongating actively C_{18-20} PUFA up to DHA (Morais *et al*., 2020; Xie *et al*., 2016; Yan *et al*., 2018), which is the most abundant fatty acid in brain and retinal cells (Mourente, 2003).

Resulting from the essential neurophysiological role and the tissue-specific generation/incorporation of their biosynthesis products (Agbaga *et al*., 2010; Aldahmesh *et al*., 2011; Dyall, 2015), *elovl4* is considered a crucial gene strongly connected to neuronal function of vertebrates (Agbaga *et al*., 2010; Deák *et al*., 2019; Monroig *et al*., 2011; Morais *et al*., 2020; Torres *et al*., 2020a). Thus, the knowledge of the *elovl4* tissue-specific expression along fish development can be essential to understand the metabolism and the potential role that VLC-FA play in fish. With this in mind, the present study aimed to determine the tissue-expression pattern of genes that encode for both Elovl4 isoforms, *elovl4a* and *elovl4b*, in different windows of development (larvae and adults) of *S. aurata* and *S. senegalensis*. Both species display different LC-PUFA biosynthesis strategies, since *S. aurata* possesses one sole Fads2 enzyme with Δ6 activity (Seiliez *et al*., 2003; Zheng *et al*., 2004), while *S. senegalensis* possess a Fads2 with Δ4 activity (Morais *et al*., 2012). These enzymatic differences in LC-PUFA biosynthesis, along to other characteristics, like their specific larval development, and the different post-larval feeding habits, i.e. pelagic or benthonic, are of special interest to assess the tissue-expression pattern of *elovl4* genes from a comparative point of view.

MATERIALS AND METHODS

Spatial expression of *elovl4* genes in *S. aurata* and *S. senegalensis* early larvae

Larvae: sample preparation

In order to study the spatial expression of *elovl4* genes (*elovl4a* and *elovl4b*) during the early larvae development of *S. aurata* and *S. senegalensis* by whole mount *in situ* hybridization (WISH) analyses, triplicate pools of 24 hours post hatching (hph) larvae were collected (~50 individuals per sample). Larvae were fixed overnight at 4 ºC in 4 % paraformaldehyde (PAF) in 1x phosphate buffered saline (PBS),

washed in PBS, and dehydrated by immersion in methanol series to subsequently be frozen at -20 ºC in pure methanol.

Cloning and spatial expression of elovl4 genes by whole-mount in situ hybridization (WISH)

To determine the spatial expression of *S. aurata* and *S. senegalensis elovl4a* and *elovl4b*, WISH was performed on 24 hph larvae using Digoxygenin (DIG)-labelled antisense riboprobes as described in Rotllant *et al*. (2008) and Thisse and Thisse (2008). Antisense riboprobes were made from linearized partial length *S. aurata* and *S. senegalensis elovl4a* and *elovl4b* plasmids, using pGEM®- T Easy Vector Systems I (Promega Biotech Ibérica S.L., Madrid, Spain). To prepare in vitro mRNA synthesis, a Riboprobe® Combination System - SP6/T7 RNA polymerase was used (Promega Biotech Ibérica S.L.). Finally, digoxigenin-tagged RNA probes were isolated using mini Quick Spin RNA Columns (Roche Diagnostics GmbH, Mannheim, Germany) and stored to -80 ºC until required. Primers used for RNA probe synthesis in spatial expression of *S. aurata* and *S. senegalensis elovl4* genes by WISH are shown in Table 1.

Table 1. Sequences of the primer pairs used, size of the fragment produced and accession number of the sequences utilized for RNA probe synthesis employed in spatial expression of *S. aurata* and *S. senegalensis elovl4* genes by whole-mount *in situ* hybridization.

Sparus aurata				
Transcript	**Primer**	**Primer sequence**	**Fragment**	**Accession No**
elovl4a	F	5'-GCCCAAGTACATGAAGAACAGAG-3'	563 bp	MK610320
	R	3'- GGTGACCTACGTGATGAGGG -5'		
elovl4b	F	5'-GTCAAGTACTCCAACGATGTCAA-3'	394 bp	MK610321
	R	3'- TGTGTCCGACGGGTAAGG -5'		
Solea senegalensis				
Transcript	**Primer**	**Primer sequence**	**Fragment**	**Accession No**
elovl4a	F	5'-CTTTCCAGCTCCGCAAAACC-3'	645 bp	MN164537
	R	3'-GAGGAGGAGGTTTCGTTCGT -5'		
elovl4b	F	5'-GATCGCCAGGCCTACACA-3'	559 bp	MN164625
	R	3'-TCACCCGAGACTAACCAATGC -5'		

Gene expression analysis by reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qPCR) in adult fish

The expression of *elovl4* isoforms in each tissue from one specimen of gilthead seabream and Senegalese sole was analyzed by reverse transcription PCR (RT-PCR) using GoTag Polymerase (Promega Biotech Ibérica S.L.). *18s ribosomal RNA* (*18s*) was used a reference gene. A random set of RT-PCR samples were purified and sequenced to confirm the identity of the amplicons.

The expression of *elovl4a* and *elovl4b* was analyzed by qPCR in selected tissues, i.e. brain, eye, and gonad from three individuals of each species. Table 2 shows the primers used in PCR analyses. The efficiency of the primer pairs was assessed through a standard curve that was obtained by serial dilutions of standard solutions of the studied genes with known copy numbers, which also allowed for the conversion of threshold cycle (Ct) values to copy numbers. The amplification was carried out, as previously described in (Torres *et al*., 2020a) using *β-actin* as gene for expression normalization.

Table 2. Primers used for reverse transcription PCR (RT-PCR) and real-time quantitative PCR (qPCR) of *Sparus aurata* and *Solea senegalensis* genes. Sequences of primer pairs used (Forward: F; Reverse: R), annealing temperatures (Ta) of primer pairs, size of fragments produced, and accession number of the sequences used for primer design are showed.

Sparus aurata						
Aim	**Transcript**	**Primer**	**Primer Sequence (5'-3')**	**Ta**	**Fragment**	**Accession No**
RT-PCR	*elovl4a*	F	GCCCAAGTACATGAAGAACAGAG	60 °C	563 bp	MK610320
		R	GGGAGTAGTGCATCCAGTGG			
	elovl4b	F	GTCAAGTACTCCAACGATGTCAA	60 °C	394 bp	MK610321
		R	GGAATGGGCAGCCTGTGT			
	18s	F	TCCTTTGATCGCTCTACCGT	60 °C	460 bp	AY993930.1
		R	TGCCCTCCAATTGATCCTCG			
qPCR	*elovl4a*	F	GCCCAAGTACATGAAGAACAGAG	60 °C	169 bp	MK610320
		R	ACCTGATGAGTCTGCTGGGG			
	elovl4b	F	GTCAAGTACTCCAACGATGTCAA	60 °C	247 bp	MK610321
		R	GAGAAGGTAGGTACACGAGT			
	Actb	F	TGCGTGACATCAAGGAGAAG	60 °C	190 bp	X89920
		R	AAGGAGCCATACCTCAGGAC			
Solea senegalensis						
Aim	**Transcript**	**Primer**	**Primer Sequence (5'-3')**	**Ta**	**Fragment**	**Accession No**
RT-PCR	*elovl4a*	F	TGCACTACTCCCTCATCTGC	60 °C	497 bp	MN164537
		R	TGAAAACAGCCACCTTAGGC			
	elovl4b	F	CCTCTGCCTTGTCCAGTTTC	60 °C	175 bp	MN164625
		R	TCCTTGACCCGTAGTTTAAC			
	18s	F	TCAGACCCAAAACCCATGCG	60 °C	464 bp	EF126042.1
		R	CCCGAGATCCAACTACGAGC			
qPCR	*elovl4a*	F	AGGTGAGGTAGGGCCTTGTT	60 °C	220 bp	MN164537
		R	CGGATTCCACCGACAAAAGT			
	elovl4b	F	CCTCTGCCTTGTCCAGTTTC	60 °C	175 bp	MN164625
		R	TCCTTGACCCGTAGTTTAAC			
	Actb	F	ACAATGAGCTGAGAGTCGCC	60 °C	132 bp	DQ485686
		R	ATGGGGGCGGTACATACAAC			

Statistical analysis

The homogeneity of variances of the data associated to gene expression values determined by qPCR was 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 the statistical software SPSS 26.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Spatial expression of *elovl4* genes in 24 hph larvae

Sparus aurata

WISH results for 24 hph *S. aurata* larvae revealed that *elovl4a* was widely distributed in the head region (Fig. 1B). Moreover, *elovl4b* was specifically expressed in the eyes (Fig. 2C), showing a strong signal in the retinal epithelium (Fig. 2D). No signal was detected for sense control probes of *elovl4a* (Fig. 1A) and *elovl4b* (Fig. 2A, B) genes.

Figure 1. WISH showing the tissue-expression pattern of *S. aurata elovl4a* in 24 hph larvae. Larvae were hybridized with either sense (A) or antisense (B) probes.

Figure 2. WISH showing the tissue-expression pattern of *S. aurata elovl4b* in 24 hph larvae. Larvae were hybridized with either sense (A, B) or antisense (C, D) probes.

Solea senegalensis

WISH results for 24 hph *S. senegalensis* larvae denoted that, curiously, *elovl4a* expression signal was located in the eyes (Fig. 3C, D). Contrary to the tissue-expression pattern shown by *S. aurata* larvae, *elovl4b* expression signal was widely distributed in the cephalic region (Fig. 4B). As expected, no signal was detected for sense control probes of *elovl4a* (Fig. 3A, B) and *elovl4b* (Fig. 4A) genes.

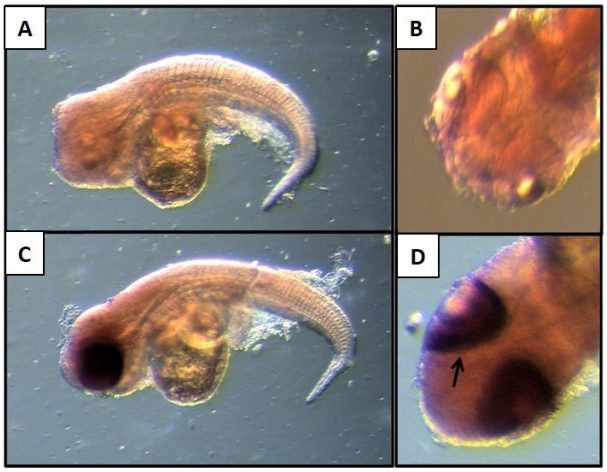

Figure 3. WISH showing the tissue-expression pattern of *S. senegalensis elovl4a* in 24 hph larvae. Larvae were hybridized with either sense (A, B) or antisense (C, D) probes. Black arrow denotes a strong expression signal in retinal epithelium.

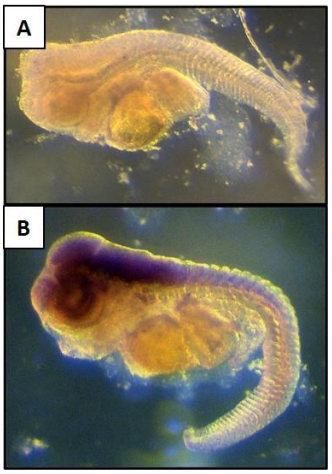

Figure 4. WISH showing the tissue-expression pattern of *S. senegalensis elovl4b* in 24 hph larvae. Larvae were hybridized with either sense (A) or antisense (B) probes.

Tissue expression of *elovl4* genes in adult fish

In adults of both fish, RT-PCR results denoted a differential *elovl4a* and *elovl4b* tissue-specific expression pattern (Fig. 5A, B). As expected, qPCR results confirmed a similar *elovl4* expression pattern between *S. aurata* larvae and adults, with *elovl4a* being mostly expressed in brain (Fig. 5C), and *elovl4b* in eye (Fig. 5E). For *S. senegalensis*, in contrast with what was found in 24 hph larvae, *elovl4a* was highly expressed in brain (Fig. 5D), and *elovl4b* in eye (Fig. 5F).

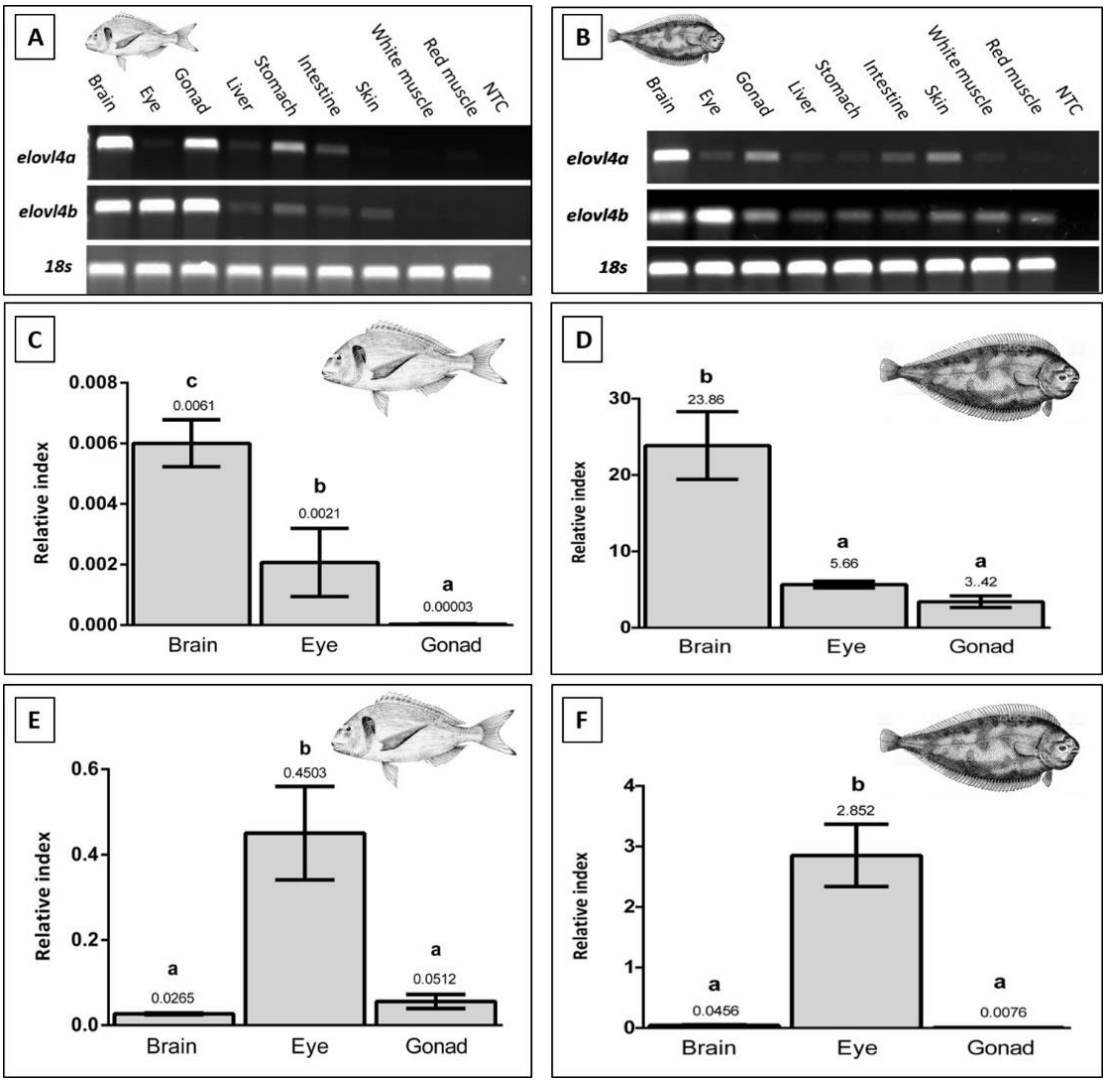

Figure 5. Tissue-expression pattern of *elovl4a* and *elovl4b* genes in adults of gilthead seabream (A) and Senegalese sole (B) determined by RT-PCR (n=1 fish). Expression of housekeeping gene *18s* is also shown. Expression of gilthead seabream *elovl4a* (C) Senegalese sole *elovl4a* (D), gilthead seabream *elovl4b* (E) and Senegalese sole *elovl4b* (F) transcripts in brain, eye and gonads determined by q-PCR. 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 (a, b, c) denote significant differences (ANOVA and Tukey HSD test, $P \leq 0.05$) among tissues.

DISCUSSION

WISH results denoted the existence of a differential *elovl4a* and *elov4b* tissue-expression pattern between *S. aurata* and *S. senegalensis* 24 hph larvae. Remarkably, an opposite tissue-expression pattern was observed between these 24 hph marine fish larvae for *elov4* genes. Thus, *elovl4* spatial expression results found in *S. aurata* larvae are in agreement with those observed in zebrafish larvae (Monroig *et al*., 2010), which denoted that *elovl4a* expression signal was extensively distributed in the head region, while *elovl4b* was specifically detected in the eye. These results agree with the *elovl4a* and *elovl4b* expression values detected in adults of both species (Fig. 5), and with the results described in adults of other fish (Betancor *et al*., 2020; Carmona-Antoñanzas *et al*., 2011; Monroig *et al*., 2010), whose *elovl4a* and *elovl4b* results showed a strong signal expression in brain and eye, respectively. Curiously, for *S. senegalensis*, an opposite *elovl4* tissue-expression pattern was observed between the pre- (Fig. 3, 4) and post-metamorphic stages (Fig. 5). Why in *S. senegalensis* larvae both *elovl4a* and *elovl4b*, unlike to what it was observed in *S. aurata*, presented an opposite tissue-expression pattern with respect to those found in adult fish, is a question that requires further exploration. It is tempting to hypothesize linking these specific-tissue expression patterns, associated to developmental events, with the important neural tissue remodeling carried out during the metamorphosis process, after which, the cognitive system and feeding habits of *S.*

senegalensis are consequently adapted to the strong nocturnal activity developed in the post-metamorphic stage (Sarasquete *et al*., 2019). These neurophysiological changes could modify the VLC-FA requirements associated to neural tissues and, consequently, remodel the *elovl4* expression pattern to be adapted to the new conditions.

Different assays, carried out in larval and post-larval stages of both species (Torres *et al*., 2020a; 2020b), suggest that *elovl4* isoforms show a specific-expression pattern in function of the different VLC-FA requirements associated to each species, developmental stage and precursors availability, highlighting the independent expression pattern and regulation of both *elovl4* isoforms. So, our results are in agreement with those obtained in recent studies carried out in late larvae (40 days after hatching) and post-larvae of both fish (Torres *et al*; 2020a; 2020b), where both *elovl4* isoforms were strongly and preferentially expressed in the head. Independently to the species-specific expression differences observed, these results suggest a role of Elovl4a/b enzymes in the local biosynthesis and incorporation of VLC-FA in fish neural tissues, especially during their early development (Morais *et al*., 2020; Torres *et al*., 2020a).

Thus, due to the cell-specific incorporation of VLC-FA in vertebrates (Agbaga *et al*., 2010; Deák *et al*., 2019; Hopiavuori *et al*., 2018, 2019), their focused tissue functionality, and the specific *elovl4a* and *elovl4b* tissue-expression pattern detected in fish, we can conclude that, similarly to what it has been described in mammals, neural tissues are the major site of *elovl4* expression in marine teleosts. Moreover, in contrast to what is found for *S. aurata*, the *elovl4a* and *elovl4b* tissue-expression pattern seems to be stage-specific in *S. senegalensis*. These results suggest that the investigation of *elov4* genes, and consequently that of their encoded Elovl4 proteins in teleosts, requires a species-specific approach.

Conflict of interests: There is no conflict of interest.

Declaration of good practices in the use of animals: All of the experimental procedures were conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes, at the Instituto de Acuicultura de Torre de la Sal (IATS-CSIC). The Animal Welfare and Bioethical Committee of IATS-CSIC approved all experimental conditions and sampling protocols under the code 015/2013 on 24 January 2014 according to Royal Decree RD53/2013.

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