Functional characterization and differential nutritional regulation of putative Elovl5 and Elovl4 elongases in large yellow croaker (Larimichthys crocea)

Songlin Li1, Óscar Monroig2, Tianjiao Wang1, Yuhui Yuan1, Juan Carlos Navarro3, Francisco Hontoria3, Kai Liao1, Douglas R. Tocher2, Kangsen Mai1, Wei Xu1 & Qinghui Ai1,4

In the present study, two elongases, Elovl4 and Elovl5, were functionally characterized and their transcriptional regulation in response to n-3 LC-PUFA administration were investigated in vivo and in vitro. We previously described the molecular characterization of croaker elovl5. Here, we report the full-length cDNA sequence of croaker elovl4, which contained 1794 bp (excluding the polyA tail), including 909 bp of coding region that encoded a polypeptide of 302 amino acids possessing all the characteristic features of Elovl proteins. Functional studies showed that croaker Elovl5 displayed high elongation activity towards C18 and C20 PUFA, with only low activity towards C22 PUFA. In contrast, croaker Elovl4 could effectively convert both C20 and C22 PUFA to longer polyenoic products up to C34. n-3 LC-PUFA suppressed transcription of the two elongase genes, as well as srebp-1 and lxrα, major regulators of hepatic lipid metabolism. The results of dual-luciferase reporter assays and in vitro studies both indicated that the transcriptions of elovl5 and elovl4 elongases could be regulated by Lxrα. Moreover, Lxrα could mediate the transcription of elovl4 directly or indirectly through regulating the transcription of srebp-1. The above findings contribute further insight and understanding of the mechanisms regulating LC-PUFA biosynthesis in marine fish species.

Long chain (C20–24) polyunsaturated fatty acids (LC-PUFA), especially the omega-3 (n-3) fatty acids eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), play pivotal roles in promoting cognitive development, enhancing immune function and minimizing the risk of cardiovascular disease1–3. The limited biosynthetic capacity for LC-PUFA in humans makes consumption of fish, especially oily fish, a key component of the diet to guarantee adequate supply of these physiologically essential nutrients4. Farmed fish that now contribute significantly to seafood intake in the human diet have been traditionally produced with diets containing high levels of marine fishmeal (FM) and fish oil (FO), ingredients that ensured good growth rates and high levels of n-3 LC-PUFA in the flesh. However, the ever-increasing use of vegetable oils (VO) in aquafeeds has led to reduced deposition of n-3 LC-PUFA in farmed fish and, consequently, to a decrease in their nutritional value for human consumers4–6. This has prompted interest in elucidating the mechanisms underlying the endogenous LC-PUFA biosynthetic pathways in farmed finfish species7, 8.

Elongases of very long-chain fatty acids (Elovl) catalyze the rate-limiting condensation step in the elongation of fatty acids including LC-PUFA biosynthesis9–10. The crucial role of Elovl enzymes has also been confirmed in fish species11–14, and the overexpression of fish elongases elevated the endogenous production of LC-PUFA in transgenic zebrafish and nibe croaker15–17. Three fatty acid elongases, Elovl2, Elovl4 and Elovl5, have been

1 Key Laboratory of Aquaculture Nutrition and Feed (Ministry of Agriculture) and Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, Qingdao, 266003, People’s Republic of China. 2 Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling, FK9 4LA, Scotland, UK. 3 Instituto de Acuicultura Torre de la Sal (IATS-CSIC), Ribera de Cabanes, 12595, Castellón, Spain. 4 Laboratory for Marine Fisheries and Aquaculture, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266003, People’s Republic of China. Correspondence and requests for materials should be addressed to Q.A. (email: qhai@ouc.edu.cn)
identified and functionally characterized as crucial enzymes involved in the biosynthetic pathway of LC-PUFA\textsuperscript{11}. While Elovl2 appears to be lost in Acanthopterygii, a phylogenetic group that includes the vast majority of the most important farmed marine fish species, Elovl4 and Elovl5 are present in virtually all fish species\textsuperscript{11}. Importantly, functional studies have shown that Elovl5 can effectively elongate both C\textsubscript{18} and C\textsubscript{20} PUFA, whereas Elovl4 is mainly involved in the elongation of C\textsubscript{22}-LC-PUFA producing polyenes up to 36 carbons\textsuperscript{7,11}.

Dietary fatty acids can regulate the transcription of elovl5 and elovl4 in fish and, generally, expression of these elongase genes is suppressed by dietary n-3 LC-PUFA\textsuperscript{14-20}. Additionally, PUFA and their metabolites regulate lipid metabolism, including inhibition of lipogenesis and activation of fatty acid oxidation, through several transcription factors such as the nuclear receptors liver X receptor (Lxr) and sterol regulatory element-binding proteins (Srebp)\textsuperscript{21}. Srebp-1c can stimulate expression of target genes directly through binding to sterol response elements (SREs) in the promoter region of target genes\textsuperscript{22}. In addition to the direct regulation mechanism, Lxrα can also indirectly mediate the expression of target genes through regulating the transcription of srebp-1 and certain other transcription factors\textsuperscript{23}. However, the role of these transcription mediators on the expression of elongases Elovl4 and Elovl5 remains poorly understood in fish species. It has been demonstrated that grouper elovl5 promoter activity was elevated by over-expression of lrxα, but not when srebp-1 was over-expressed, indicating the direct regulation role of Lxrα on elovl5 expression\textsuperscript{26}. However, mouse elovl5 was activated by Srebp-1c directly, and also by Lxrα indirectly elevating the transcription of elovl5 via Srebp-1c\textsuperscript{24}. Elovl4 is the member of Elovl family that has been investigated most recently in fish and no information is available on the underlying regulation mechanism of elovl4\textsuperscript{22}.

Large yellow croaker (Larimichthys crocea) is an important carnivorous marine fish species widely cultured in southeast China. Recently, low retention of n-3 LC-PUFA has been observed after a large proportion of dietary FO was replaced by VO, which seriously affected the nutritional quality of the fillet of farmed croaker\textsuperscript{26}. Recently an elovl5-like gene (cDNA) was cloned and its mRNA expression was investigated in response to dietary fatty acids, although no functional data was reported\textsuperscript{29}. The aims of the present study were to characterize the function of the previously cloned elovl5, and the molecular cloning and functional characterization of an elovl4 cDNA from L. crocea. In addition, we also investigated the underlying mechanisms regulating the expression of these two elongases. The results may contribute to increased insight into the potential regulatory mechanisms involved, which will be key to understanding and applying strategies for enhancing the biosynthesis of LC-PUFA in large yellow croaker.

Results

Molecular cloning and phylogenetics of the L. crocea Elovl4. The full-length cDNA of croaker elovl4 encompassed 1794 bp including a 262 bp 5′ untranslated terminal region (UTR), a 909 bp coding region that encoded 302 aa and a 909 bp 3′ UTR (GenBank Accession No. KP681700). BLAST analysis revealed that the croaker elovl4 shared high sequence identity with elovl4 sequences from other teleosts including the orange-spotted grouper (Epinephelus coioides, 94%), rabbitfish (Siganus canaliculatus, 94%), cobia (Rachycentron canadum, 92%) and Atlantic salmon (Salmo salar, 86%). Additionally, the croaker elovl4 cDNA was 40% identical to croaker elovl5.

Characteristically, the croaker elovl4 deduced protein contained one histidine box (HXHHH), five putative membrane-spanning domains, and single lysine and arginine residues (RXXXX) at the carboxyl terminus (Fig. 1). Phylogenetic tree analysis showed that the croaker Elovl4 clustered with several other Elovl4-like sequences from fish and mammals, and more distantly with Elovl2 and Elovl5 from these vertebrate lineages (Fig. 1). The results strongly suggested that the newly cloned cDNA encoded an Elovl4 elongase.

Functional characterization of the Elovl4 and Elovl5 elongases from L. crocea. The functions of croaker Elovl4 and Elovl5 were characterized by determining the FA profiles of S. cerevisiae transformed with pYES2-Elo4 or pYES2-Elo5, respectively, and grown in the presence of potential FA substrates. For Elovl4, its role in the biosynthesis of saturated VLC-FA was studied by comparing the saturated FA (> C\textsubscript{18}) profiles of yeast transformed with pYES2-Elo4 and pYES2 (control) (Table 1). No significant differences were found in the content of 24:0, 26:0, 28:0, 30:0 or 32:0, indicating that the croaker Elovl4 does not have a role in the biosynthesis of saturated VLC-FA. In order to investigate the role of the L. crocea Elovl4 in the biosynthesis of VLC-PUFA, pYES2-Elo4 transformed yeast were grown in the presence of C\textsubscript{18} (18:4n-3 and 18:3n-6), C\textsubscript{20} (20:5n-3 and 20:4n-6) and C\textsubscript{22} (22:5n-3, 22:4n-6 and 22:6n-3) substrates. The results confirmed that croaker Elovl4 was able to elongate all PUFA substrates producing in most cases polyenes of up to 32 or 34 carbons, except for DHA that was only elongated to 24:6n-3 (Table 2). Interestingly, the elongation towards C\textsubscript{24} and C\textsubscript{26} substrates showed the highest conversion values (%) regardless of the initial PUFA substrate. However, despite limited activity towards DHA, the croaker Elovl4 could elongate both 20:5n-3 and 22:5n-3 to 24:5n-3, the C\textsubscript{24} substrate for DHA synthesis.

The function of L. crocea Elovl5 was characterized by growing the transgenic yeast expressing its coding region in the presence of C\textsubscript{18} (18:3n-3, 18:2n-6, 18:4n-3 and 18:3n-6), C\textsubscript{20} (20:5n-3 and 20:4n-6) and C\textsubscript{22} (22:5n-3 and 22:4n-6) PUFA substrates. The results from the yeast assays revealed that the L. crocea Elovl5 exhibited high conversion towards C\textsubscript{20} PUFA, particularly 18:4n-3 (86.7%) and 18:3n-6 (77.9%), followed by C\textsubscript{22} substrates including 20:5n-3 (7.6%) and 20:4n-6 (70.0%) (Table 3). The L. crocea Elovl5 showed relatively low activity towards C\textsubscript{22} substrates such as 22:5n-3 (7.6%) and 22:4n-6 (2.3%) (Table 3). Fatty acid composition of yeast transformed with the empty pYES2 vector and grown in the presence of PUFA substrates used in both Elovl4 and Elovl5 functional assays primarily comprised of the four major endogenous FA, namely 16:0, 16:1n-7, 18:0 and 18:1 (including 18:1n-9 and 18:1n-7 isomers), together with any exogenously added FA (data not shown).
Tissue distribution of elovl4. The distribution of elovl4 mRNA in L. crocea was detected in several tissues, including eye, brain, testis, heart, liver, kidney, spleen, stomach, intestine and muscle. Gene expression analysis revealed that the highest levels of elovl4 transcript were found in eye, follow by brain and testis (Fig. 3).

Nutritional regulation of the L. crocea elovl4 and elovl5 and transcription factors: in vivo and in vitro trials. The expression of elovl4 and elovl5 from fish fed the experimental diets containing varying levels of LC-PUFA is shown in Fig. 4A. The results showed that the expression of both genes was down-regulated in fish fed diets containing both high and moderate n-3 LC-PUFA levels in comparison to diets with low n-3 LC-PUFA levels.
In vitro assays performed with hepatocyte primary cultures prepared from fish fed a standard diet indicated that the expression of \( \text{elovl}4 \), but not \( \text{elovl}5 \), was down-regulated by DHA supplementation \((P < 0.05)\) (Fig. 4B). Additionally, supplementation of EPA to hepatocytes suppressed the expression of both elongases \((P < 0.05)\) (Fig. 4C).

With regards to the regulation of transcription factors, dietary n-3 LC-PUFA significantly decreased the expression of \( \text{srebp-1} \) and \( \text{lxr}\alpha \) \((P < 0.05)\) (Fig. 5A). The in vitro studies clearly showed that both DHA and EPA significantly suppressed the transcription of \( \text{srebp-1} \) and \( \text{lxr}\alpha \) \((P < 0.05)\) (Fig. 5B and C).

Transcriptional regulation of the \( \text{L. crocea elovl}4 \) and \( \text{elovl}5 \) by \( \text{lxr}\alpha \) and \( \text{srebp-1} \). Over-expression of \( \text{lxr}\alpha \) significantly increased the transcription of \( \text{elovl}4 \) \((P < 0.05)\), but the expression of \( \text{elovl}4 \) was significantly suppressed through the inhibition of \( \text{srebp-1} \) \((P < 0.05)\) in yellow croaker hepatocytes (Fig. 6A). These results indicated that both \( \text{lxr}\alpha \) and \( \text{srebp-1} \) up-regulated \( \text{elovl}4 \). The addition of DHA or EPA significantly reduced the abovementioned up-regulation effects of \( \text{lxr}\alpha \) and \( \text{srebp-1} \) \((P < 0.05)\) (Fig. 6A). In addition, the inhibition of \( \text{srebp-1} \) significantly decreased the regulatory role of LXR\( \alpha \) on the transcription of \( \text{elovl}4 \) \((P < 0.05)\) (Fig. 6A).

Table 1. Functional characterization of the large yellow croaker \( \text{elovl}4 \): Role in biosynthesis of very long-chain saturated fatty acids (FA). Results are expressed as an area percentage of total saturated FA \( \geq \text{C}_{24} \) found in yeast transformed with either the empty pYES2 vector (Control) or the large yellow croaker \( \text{elovl}4 \) ORE. Results are means ± standard deviations \((N = 3)\). No statistical differences were observed between treatments (Students\( t\)-test, \( P \leq 0.05)\). nd, not detected.
Regarding the transcription of *elovl5*, over-expression of *lxr*α significantly increased the transcription of *elovl5* (*P* < 0.05), while the inhibition of Srebp-1 had no significant effect on *elovl5* expression (*P* > 0.05) (Fig. 6B). Moreover, the addition of DHA or EPA suppressed the up-regulation of *elovl5* by Lxrα (*P* < 0.05) (Fig. 6B). Nevertheless, inhibition of Srebp-1 did not affect the regulation of *elovl5* by Lxrα (*P* < 0.05) (Fig. 6B).

**Dual-luciferase reporter assays.** In order to investigate the molecular mechanisms involved in the regulation of *L. crocea elovl4* and *elovl5*, regions of 1921 (GenBank Accession No. KY863452) and 2291 bp (GenBank Accession No. KY863451), respectively, upstream of the initiation codon of the genes were cloned to perform dual-luciferase reporter assays. For *elovl4*, the reporter activity was 2.41-fold than the control (Fig. 7A). The croaker *elovl4* reporter activity was significantly elevated by over-expression of *lxr* and *srebp*-1 (Fig. 7A). For *elovl5*, the reporter activity was 1.66-fold than the control (Fig. 7B). Similarly, the *L. crocea elovl5* reporter activity was significantly elevated by over-expression of *lxr* (Fig. 7B). However, the over-expression of *srebp*-1 had no significant effect on the activity of the *elovl5* promoter (Fig. 7B).

| FA substrate | Product | % Conversion |
|--------------|---------|--------------|
| 18:4n-3      | 20:4n-3 | 8.5          |
|              | 22:4n-3 | 32.6         |
|              | 24:4n-3 | 71.5         |
|              | 26:4n-3 | 100.0        |
|              | 28:4n-3 | 100.0        |
|              | 30:4n-3 | 94.5         |
|              | 32:4n-3 | 72.2         |
| 18:3n-6      | 20:3n-6 | 6.1          |
|              | 22:3n-6 | 36.4         |
|              | 24:3n-6 | 67.1         |
|              | 26:3n-6 | 90.7         |
|              | 28:3n-6 | 93.2         |
|              | 30:3n-6 | 89.7         |
|              | 32:3n-6 | 21.9         |
| 20:5n-3      | 22:5n-3 | 14.7         |
|              | 24:5n-3 | 47.0         |
|              | 26:5n-3 | 69.2         |
|              | 28:5n-3 | 92.7         |
|              | 30:5n-3 | 98.2         |
|              | 32:5n-3 | 81.9         |
|              | 34:5n-3 | 11.1         |
| 20:4n-6      | 22:4n-6 | 21.1         |
|              | 24:4n-6 | 50.7         |
|              | 26:4n-6 | 65.7         |
|              | 28:4n-6 | 85.0         |
|              | 30:4n-6 | 91.4         |
|              | 32:4n-6 | 41.1         |
| 22:5n-3      | 24:5n-3 | 23.5         |
|              | 26:5n-3 | 59.8         |
|              | 28:5n-3 | 90.4         |
|              | 30:5n-3 | 97.6         |
|              | 32:5n-3 | 75.5         |
| 22:4n-6      | 24:4n-6 | 35.8         |
|              | 26:4n-6 | 71.0         |
|              | 28:4n-6 | 89.7         |
|              | 30:4n-6 | 94.2         |
|              | 32:4n-6 | 49.4         |
| 22:6n-3      | 24:6n-3 | 1.2          |

**Table 2.** Functional characterization of the large yellow croaker Elovl4: conversions of polyunsaturated fatty acid (FA) substrates. Conversions were calculated for each stepwise elongation according to the formula [areas of first product and longer chain products/areas of all products with longer chain than substrate + substrate area] × 100. The substrate FA varies as indicated in each step-wise elongation.
Discussion

The large yellow croaker is widely cultured in China due to its great commercial value. Recently, a study investigating the effects of FO replacement in diets for *L. crocea* showed that low retention of LC-PUFA in muscle occurred, thus reducing the nutritional quality of the product. This was possibly due to the low LC-PUFA biosynthetic ability of large yellow croaker as generally reported for most marine species. Elongases play key roles in the biosynthesis of LC-PUFA in fish and thus a better understanding of the underlying mechanisms regulating the transcription of elongases would contribute to strategies designed to enhance the endogenous LC-PUFA synthetic ability of the large yellow croaker that may lead to increased nutritional value of farmed products for human consumers.

Seven members of the Elovl protein family have been described in vertebrates. Typically, Elovl2 and Elovl5 play roles in the biosynthesis of LC-PUFA in vertebrates, having somewhat overlapping functions possibly related to their common evolutionary origin. Whereas Elovl5 is present in virtually all teleosts, Elovl2 appears to be lost during evolution and is absent in Acanthopterygii that encompass the majority of farmed marine fish. Fish generally possess two Elovl4-like elongases, but only the so-called “isoform b” has PUFA as preferred substrates. In the present study, we conducted the molecular cloning of an elo4 and functionally characterized the functions of the herein reported Elovl4 and the previously cloned elo5. In addition, we investigated regulatory mechanisms of transcription factors in both elongases and the influence of “dietary” LC-PUFA in both *in vivo* and *in vitro* conditions.

### Table 3. Functional characterization of the large yellow croaker Elovl5: conversions of polyunsaturated fatty acid (FA) substrates.

Conversions were calculated for each stepwise elongation according to the formula: 
\[
\text{Area of first product and longer chain products} / \text{(area of all products with longer chain than substrate + substrate area)} \times 100
\]

| FA Substrate | FA Product | % Conversion |
|--------------|------------|--------------|
| 18:3n-3      | 20:3n-3    | 48.3         |
|              | 22:3n-3    | 6.3          |
|              | 24:3n-3    | 7.4          |
| 18:2n-6      | 20:2n-6    | 24.1         |
|              | 22:2n-6    | 6.9          |
| 18:4n-3      | 20:4n-3    | 86.7         |
|              | 22:4n-3    | 28.0         |
|              | 24:4n-3    | 3.1          |
| 18:3n-6      | 20:3n-6    | 77.9         |
|              | 22:3n-6    | 21.8         |
|              | 24:3n-6    | 4.8          |
| 20:5n-3      | 22:5n-3    | 74.6         |
|              | 24:5n-3    | 11.1         |
|              | 26:5n-3    | 6.2          |
| 20:4n-6      | 22:4n-6    | 70.0         |
|              | 24:4n-6    | 4.6          |
|              | 26:4n-6    | 4.8          |
| 22:5n-3      | 24:5n-3    | 7.6          |
|              | 26:5n-3    | 6.3          |
| 22:4n-6      | 24:4n-6    | 2.3          |

Figure 3. Tissue expression of elo4 in large yellow croaker. Values (means ± standard error of the mean, SEM) in bars that have the same letter are not significantly different (*P* > 0.05; Tukey’s test) among treatments (n = 3).
The isolated croaker Elovl4 possessed all the features of the elongase family including transmembrane domains, a histidine box (HXXHH), and an arginine residue and single lysine residue (RXKXX) in the canonical C-terminal, indicating its role in LC-PUFA biosynthesis\(^30\). Zebrafish possessed two Elovl4 enzymes, Elovl4a and Elovl4b, with different functions\(^25\). Both the Elovl4 in zebrafish had roles in the biosynthesis of saturated VLC-FA, while Elovl4b was also able to operate on PUFA substrates producing polyenes of up to 36 carbons and thus participated in the biosynthesis of VLC-PUFA\(^25\). Phylogenetic analysis of the newly cloned \(L.\) crocea elovl4 showed that it was an orthologue of zebrafish Elovl4b and not Elovl4a. Consistent with these genes being orthologues of the zebrafish Elovl4b, previously cloned elovl4-like elongases isolated from cobia\(^{26}\), Atlantic salmon\(^{31}\), rabbitfish\(^{32}\) and orange-spotted grouper\(^{15}\), had similar functions compared to zebrafish Elovl4b and they were all capable of elongating both saturated and polyunsaturated FA. In contrast, the \(L.\) crocea Elovl4-b like elongase did not show any activity towards endogenous saturated FA in yeast and thus does not appear to have a role in the biosynthesis of saturated VLC-FA as its previously characterized counterparts. The reasons why Elovl4 in \(L.\) crocea does not play a role in the biosynthesis of saturated VLC-FA remain uncertain, but a recent study reporting the functional characterization of Elovl4 in nibe croaker \(Nibea\) mitsukurii did not report any activity towards saturated FA using a similar expression system as that used herein. While it is possible that the activity of the nibe croaker Elovl4

**Figure 4.** Effects of dietary n-3 LC-PUFA (A), DHA (B) and EPA (C) contents on the expression of elovl4 and elovl5. Values (means ± SEM) in bars that have the same letter are not significantly different (\(P > 0.05\); Tukey’s test) among treatments (\(n = 3\)).
towards endogenous saturated FA in yeast was simply not measured and/or reported, the close phylogenetic relationship between *L. crocea* and *N. mitsukurii*, both from the Sciaenidae family, possibly indicated that some Elovl4 from certain species or families within teleosts have subfunctionalized during evolution, as has occurred in the fatty acyl desaturase Fads233, a protein family that also plays key roles in LC-PUFA biosynthesis in vertebrates11, 28.

In agreement with the functional data obtained with fish Elovl415, 26, 31, 32, the *L. crocea* Elovl4 showed activity towards PUFA substrates and thus confirmed its role in the biosynthesis of VLC-PUFA. Thus, the *L. crocea* Elovl4 exhibited high elongation efficiency towards a range of PUFA substrates, namely C 20 (20:5n-3 and 20:4n-6) and C 22 (22:5n-3 and 22:4n-6) PUFA, with C 18 substrates (18:4n-3 and 18:3n-3) being elongated to a lesser extent. The functions of VLC-PUFA are not fully understood, but studies in mammals have suggested that these compounds play pivotal roles in vision34 and reproduction 35. Importantly, the large yellow croaker Elovl4 could produce 24:5n-3 from directly supplemented 22:5n-3 or by step-wise elongation of 20:5n-3. Such an ability of Elovl4 has been noted as an important feature by which this enzyme can contribute to DHA biosynthesis through the Sprecher pathway in species that, like most marine farmed species including the large yellow croaker, might have lost Elovl2. Thus, the elongation product of Elovl4, namely 24:5n-3, can be desaturated to 24:6n-3 and subsequently chain shortened to 22:6n-3 by partial β-oxidation36. Similar activities were also demonstrated.

**Figure 5.** Effects of dietary n-3 LC-PUFA (A), DHA (B) and EPA (C) contents on the expression of the transcription factors lxrα and srebp-1. Values (means ± standard error of the mean, SEM) in bars that have the same letter are not significantly different (*P* > 0.05; Tukey's test) among treatments (n = 3).
for Elovl4 from zebrafish (isoform b), Atlantic salmon, cobia, rabbitfish, nibe croaker and orange-spotted grouper.

The large yellow croaker Elovl5 could efficiently elongate C₁₈ (18:4n-3 and 18:3n-6) and C₂₀ (20:5n-3 and 20:4n-6) substrates, although C₂₂ PUFA including 22:5n-3 and 22:4n-6 were only elongated to a small degree. Such substrate preference as observed here with the L. crocea Elovl5 is highly conserved among teleost Elovl5.

Furthermore, the L. crocea Elovl5 was also able to elongate 18:3n-3 and 18:2n-6, FA substrates not only for Δ6 desaturases to initiate LC-PUFA biosynthesis through the classical “Δ6 pathway” but also for elongases like Elovl5 to initiate biosynthesis through the alternative “Δ8 pathway”. The ability of the L. crocea Elovl5 to actively elongate 18:3n-3 and 18:2n-6 has also been reported in Elovl5 elongases characterized from the southern bluefin tuna (Thunnus maccoyii), Japanese eel (Anguilla japonica), striped snakehead (Channa striata) and orange-spotted grouper (E. coioides). While further studies are required to elucidate the desaturase gene and function repertoire, the results from the present study clearly indicated that L. crocea expressed genes encoding elongases with all the activities required for the biosynthesis of C₂₀–₂₂ LC-PUFA from C₁₈ precursors supplied in the diet.

Aquafeeds are currently formulated with decreasing levels of the marine ingredients FM and FO due to increased demand and unpredictable availability, leading to high price volatility. Nutritional regulation of genes encoding desaturases and elongases has been proposed as a possible strategy to enhance the endogenous production on n-3 LC-PUFA and optimise their retention in farmed fish. Additionally, previous studies found that LC-PUFA and their metabolites can regulate transcription of lipid metabolism related genes through modulation of transcription factors including, among others, Lxrα and Srebp-1. However, the regulatory mechanisms of these transcription factors on the two herein studied elongases has been barely investigated. In mammals, Srebp-1 positively regulates transcription via interacting with sterol response elements (SRE) in the promoter region of target genes. Lxrα can activate transcription directly and/or indirectly via regulating Srebp-1 or other transcription factors. Dual-luciferase reporter assays were conducted in the present study to clarify the regulatory mechanisms by which Lxrα and Srebp-1 modulate the expression of Elovl4 and Elovl5.
elovl5. The results showed that the large yellow croaker Elovl5 reporter activity was induced by over-expression of lxrα, but not by over-expression of srebp-1. This may indicate that the transcription of elovl5 was up-regulated by Lxrα directly, but not dependent on Srebp-1. This observation is consistent with the results of studies carried out in Atlantic salmon and orange-spotted grouper16, 44. The results of the in vitro experiment conducted in the present study to confirm the above findings showed that the expression of elovl5 in L. crocea hepatocytes was induced by over-expression of lxrα, while inhibition of the expression of srebp-1 had no effect on the expression of elovl5 and could not repress the activation effect of Lxrα on elovl5. This confirmed the direct stimulatory role of Lxrα on elovl5, and suggested that such regulatory mechanisms operated differently compared to mammals24. Promoter studies showed that the large yellow croaker Elovl4 reporter activity was induced by over-expression of both lxrα and srebp-1. This observation indicated that the expression of the L. crocea elovl4 can be up-regulated through Lxrα and Srebp-1. Additionally, the in vitro results showed that the transcription of elovl4 in hepatocyte was significantly induced by over-expression of lxrα, but significantly repressed by inhibiting the expression of srebp-1. The inhibition of the expression of srebp-1 would significantly repress the activation effect of Lxrα on elovl4. These results suggested that Lxrα can promote the expression of croaker elovl4 via a Srebp-dependent pathway. The above observations indicated differential regulation of Lxrα and Srebp-1 on Elovl4 and Elovl5 in the large yellow croaker. Knowledge of the underlying regulation mechanism of these two elongases can contribute to the development of novel practical approaches to enhance endogenous LC-PUFA production via both nutritional strategies and genetic modification.

In summary, the large yellow croaker Elovl4 exhibited all the structural features of Elovl proteins. Functional studies showed that Elovl4 and Elovl5 of L. crocea had complementary functions since the latter could effectively elongate both C18 and C20 PUFA substrates, while Elovl4 was more effective in the elongation of C20 and C22 FA substrates. Moreover, the functional characterization of the L. crocea Elovl4 demonstrated its role in the biosynthesis of VLC-PUFA. In addition, n-3 LC-PUFA can regulate the transcription of elovl5 and elovl4 elongases through Lxrα. Moreover, Lxrα could mediate the transcription of elovl4 directly or indirectly via regulating the transcription of srebp-1.
Materials and Methods

Cloning and phylogenetic analysis of the *L. crocea* full-length *elovl4* cDNA. Total RNA was extracted from isolated croaker liver using Trizol Reagent (Takara, Japan). Thereafter, first strand cDNA was synthesized using Prime Script™ RT reagent Kit (Takara). Two degenerate primers (*Elo4-F* and *Elo4-R*) (Supplementary Table) were designed based on highly conserved regions of *elovl4* cDNA sequences of other fish and used for PCR (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg) using PrimeSTAR® HS DNA Polymerase (Takara, Japan). The amplified products were purified (SK8131, Sangon Biotech, Shanghai), ligated into the pEASY-T1 vector (TransGen Biotech, China), transformed into competent Trans1-T1 cells (TransGen Biotech, China) and sequenced (Biosune Biotech, Shanghai, China), followed by blasting on GenBank to confirm its high similarity with other *Elov4* proteins. Gene specific primers (Supplementary Table) were designed to obtain the full-length *elovl4* cDNA sequence by 5’- and 3’-rapid amplification of cDNA ends (RACE) PCR according to the manufacturer’s instructions (SMARTer™ RACE cDNA Amplification Kit, Clontech, CA, USA). RACE PCR products were purified, cloned into pEASY-T1 vector, and sequenced as described above.

The deduced amino acid (aa) sequence of the newly cloned croaker *elovl4* cDNA was aligned with other orthologues including human (*Homo sapiens*, NP_073563), mouse (*Mus musculus*, NP_688743), rat (*Rattus norvegicus*, NP_001178725), zebrafish (*Danio rerio*, NP_957090 and NP_956266), cobra (*Rachycentron canadum*, ACG59898), and Atlantic salmon (*Salmo salar*, NP_001182481). Multiple sequence alignment was performed with Mega 6.0. A phylogenetic tree was constructed on the basis of aa sequences using the neighbor-joining method and including the newly cloned croaker *Elov4* sequence and those from other vertebrate *Elolv4* proteins. Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping the data through 1000 iterations.

Functional characterization of the *L. crocea* *Elolv5* and *Elolv4* elongases. PCR fragments corresponding to the open reading frames (ORF) of the *Elolv5* and *Elolv4* elongases were amplified from croaker liver cDNA, using High Fidelity PrimeScript® RT-PCR Kit with primers containing restriction sites for HindIII and XhoI (Supplementary Table). The DNA fragments containing the croaker *elolv5* or *elolv4* ORF were digested with corresponding restriction endonucleases (Takara, Japan) and then ligated into a similarly restricted pYES2 vector (Invitrogen, UK) to yield the resulting plasmid constructs pYES2-El5o and pYES2-Elo4. *Saccharomyces cerevisiae* competent cells were transformed (S.c. EasyComp Transformation Kit, Invitrogen, USA) with the purified recombinant plasmid constructs pYES2-El5o or pYES2-El4o. Transformation and selection of yeast with recombinant plasmids, and yeast culture were conducted as described in detail previously. A single colony of transgenic yeast was grown in *S. cerevisiae* minimal medium supplemented with potential fatty acid (FA) substrates. For *Elolv5*, each culture was supplemented with one of the following substrates: 18:4n-3, 18:2n-6, 18:4n-3, 18:3n-6, 20:5n-3, 22:5n-3 and 22:6n-3. For *Elolv4*, each culture was supplemented with one of the following substrates: 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 and 22:6n-3. In order to compensate for differences in FA update efficiency by yeast, final concentrations of FA substrates varied according to their fatty acyl chain lengths, 0.5 mM (C18), 0.75 mM (C20) and 1.0 mM (C22). *Elolv4* plays a role in the biosynthesis of very-long-chain (>C22) saturated FA (VLC-FA) and thus pYES2-El4o transformed yeast were also grown in the absence of exogenously added fatty acids to enable comparison with saturated FA profiles of yeast transformed with the empty pYES2. For both *Elolv5* and *Elolv4* assays, yeast were grown for 2 days and then harvested, washed twice with 5 mL ice-cold HBSS (Invitrogen, UK) and freeze dried for 24 h for further analyses. Yeast transformed with empty pYES2 were additionally grown in the presence of all PUFA substrates tested in both assays for *Elolv5* and *Elolv4* as further control treatments.

Fatty acid analysis. Total lipids were extracted from yeast samples and fatty acids derivatized to methyl esters (FAME) as described in detail previously. FAME were identified and quantified after splitless injection and run with temperature programming in an Agilent 6850 Gas Chromatograph system equipped with a Sapiens-5MS (30 m × 0.25 μm × 0.25 μm) capillary column (Teknokroma, Barcelona, Spain) coupled to a 5975 series MSD (Agilent Technologies, Santa Clara, CA, USA) as described by Monroig et al. The elongation of exogenously added FAME substrates (18:3n-3, 18:2n-6, 18:4n-3, 18:3n-6, 18:4n-3, 20:5n-3, 20:4n-6, 22:5n-3 and 22:6n-3) was calculated by the step-wise proportion of substrate FA converted to elongated product as [areas of first product × 100] / [areas of substrate area] + [areas of first product × 100] / [areas of second product area] + ... + [areas of last product × 100] / [areas of last substrate area] × 100. 1000 iterations.

Animal experiments. The present study was carried out in strict accordance with the recommendations in the Guide for the Use of Experimental Animals of Ocean University of China. The protocols for animal care and handling, methods and experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Ocean University of China. A 10-week feeding trial was conducted to investigate the effect of dietary n-3 LC-PUFA on expression of *elolv4* and *elolv5*, and the regulation of certain transcription factors on the two enzymes. DHA-enriched oil (DHA content: 40.6% of total fatty acid in the form of DHA-methyl ester, Jiangsu Tiankai Biotechnology Company Limited, China) and EPA-enriched oil (EPA and DHA at 45.9% and 23.8%, respectively, of total fatty acids both in the form of TAG; Hebei Haiyuan Health Biological Science and Technology Company Limited, China) were utilized in different ratios to form three isoprotein (43% crude protein) and isolipidic (18% crude lipid) diets with low (0.46%), moderate (1.05%) and high (2.44%) n-3 LC-PUFA content (the ratio of DHA to EPA was approximately 2.0). Large yellow croaker were obtained from a local farm located in Xiangshan Bay, Ningbo, China. At the beginning of the experiment, the fish were fasted for 24 h and weighed after being anesthetized with eugenol (1:10,000) (Shanghai Reagent, China). Triplicate groups of croaker (individual weight of 10.0 ± 0.6 g) were distributed into each sea cage (1 m × 1 m × 1.5 m) at a stocking density of 60 individuals, and fed twice daily (05:00 and 17:00 h) to apparent satiation for 70 days. At the end of the experiment, croaker were fasted for 24 h and
anesthetized and culled by an overdose of MS222 (ethyl 3-aminobenzoate methanesulfonic acid salt, Aldrich, USA). Livers of three fish per cage were isolated, frozen in liquid nitrogen and stored at −80 °C for subsequent gene expression analysis of elongases (elovl4 and elovl5) and transcription factors (lxrx and srebp-1). In addition, various tissues (eye, brain, testis, heart, liver, kidney, stomach, intestine and muscle) were isolated from nine croaker individuals fed diets with moderate n-3 LC-PUFA levels (1.05%) to investigate the tissue distribution of elovl4 mRNA.

**Primary hepatocyte isolation and incubation.** The above feeding study provided preliminary data on the in vivo nutritional regulation of elovl4 and elovl5. To confirm and support these data, an in vitro study was conducted to further investigate the role of certain transcription factors on the regulation of elovl4 and elovl5 elongases in *L. crocea*. Hepatocytes were isolated from five yellow croaker individuals (~50 g) starved for 24 h according to the published protocols with slight modification. Briefly, croaker were anesthetized with MS 222 and the branchial arch cut followed by immersion in 70% ethanol for 3 min to sterilize the external surface. Liver tissues were excised aseptically and rinsed twice with phosphate buffered saline (PBS, pH 7.4 at 4 °C) supplemented with amphotericin-B (25 μg/mL), streptomycin (100 μg/mL) and penicillin (100 U/mL). Thereafter, the liver was aseptically minced into 1 mm³ pieces, followed by digestion in 0.25% sterile trypsin at room temperature for 15 min. Trypsin was neutralized with Dulbecco’s Modified Eagle Medium (DMEM, Gibco, USA) containing 20% fetal bovine plasma (FBS, Invitrogen, USA). The cell suspension was collected and filtered through sterile 75 μm mesh, followed by centrifugation at low speed (100 g, 5 min) and the supernatant discarded. Isolated cells were washed in red blood cell lysis buffer (Boyetime Institute of Biotechnology, Haimen, China) for 2 min at 4 °C. Cell viability was evaluated using a hemocytometer under an inverted microscope after the cells were stained with 0.4% Trypan Blue. The hepatocytes were re-suspended in DMEM medium containing 1 mM glutamine, 20% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells suspensions with more than 95% cell viability were used for the subsequent experiments.

Croaker primary hepatocyte suspensions were incubated with fatty acid (DHA or EPA) to confirm the influence of n-3 LC-PUFA on the transcription of elovl4 and elovl5 and transcription factors (lxrx and srebp-1) in vivo. Fatty acid (DHA and EPA, Cayman Chemical Co., USA) was supplemented to cells in the form of BSA/fatty acid complexes that were prepared at 10 mM concentration according to Ou et al. and stored at −20 °C. Additionally, inhibitors/agonists of transcription factors were used to clarify the role of the transcription factors on the regulation of elovl4 and elovl5 elongases in *L. crocea*. GW3965 HCl (Selleckchem, Shanghai, China) was used as an LXRα agonist whereas FGH10019 (MCE, USA) was used as a SREBP-1 inhibitor, respectively. Cells were seeded in 6-well plates with a density of 2 × 10⁶ viable cells per well in DMEM/F12 ( Gibco) containing 20% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, followed by incubation for 24 h. The hepatocytes were then washed and incubated for 1 h in FBS-free DMEM/F12 medium prior to incubation with EPA, DHA and the above inhibitors or agonists in triplicate wells. After incubation, cells were lysed in the wells and harvested for RNA extraction.

**Real-time quantitative PCR (RT-qPCR) analysis.** Specific primers for RT-qPCR were designed by Primer Premier 5.0 (Premier Biosoft) based on the cloned nucleotide sequences. The stability of β-actin was verified and confirmed and used as the reference gene. The amplification was performed in a quantitative thermal cycler (Mastercycler EP Realplex, Eppendorf, Germany). The program was 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 57 °C for 10 s, and 72 °C for 20 s. At the end of each reaction, melting curve analysis of amplification products was carried out to confirm that a single PCR product was present in these reactions. The amplification efficiencies of the target and reference genes were determined from the slope of the log-linear portion of the calibration curve and PCR efficiency = 10−1/Slope − 1. The expression levels of the target genes were calculated following the 2−ΔΔCt method described by Livak & Schmittgen.

**Cloning of the elovl4 and elovl5 promoters.** Genomic DNA was extracted from croaker liver using the SQ Tissue DNA Kit (OMEGA, USA) according to the manufacturer’s instructions. Then, the genomic DNA was digested with four restriction enzymes (DraI, EcoRV, PvuII and StuI), purified and ligated to Genome Walker Adaptors following the instructions of Universal Genome Walker 2.0 Kit user manual (Clontech, USA). The promoter region of elovl5 and elovl4 was amplified through a nested (two round) PCR combining the kit supplied primers Ap1 and Ap2 with Advantage 2 PCR Kit (Clontech, USA) and the gene specific primers designed on elovl5 and elovl4 cDNA (see Supplementary Table for primer details). The conditions for the primary round PCR were: 7 cycles of 25 s at 94 °C, 3 min at 72 °C, followed by 32 cycles of 25 s at 94 °C, 3 min at 65 °C, then with an additional 7 min at 67 °C after the final cycle. The conditions for the secondary round PCR were: 5 cycles of 25 s at 94 °C, 3 min at 72 °C, followed by 20 cycles of 25 s at 94 °C, 3 min at 65 °C, then with an additional 7 min at 67 °C after the final cycle. The PCR products were purified, cloned into pEASY-T1 and sequenced as described above.

**Expression and reporter plasmids constructs.** For expression plasmids, PCR fragments corresponding to the ORF of the croaker srebp-1 (GenBank accession number: KP342262) were amplified with primers containing restriction sites for EcoRI and XhoI, respectively, and the ORF of croaker lrxr (GenBank accession number: XM_019273432) was amplified with primers containing restriction sites for ClaI and EcoRI (Supplementary Table). The DNA fragments were digested with corresponding restriction endonucleases (Takara) and ligated into a similarly restricted pCS2+ vector (Invitrogen, USA) to yield the expression plasmid constructs pCS2-srebp1 and pCS-lrxr. The reporter plasmid Elovl5-Luc and Elovl4-Luc were engineered to contain a fragment of the large yellow croaker Elovl5 and Elovl4 promoters cloned into the
Cell culturing, transfection and luciferase assay. HEK 293 T cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine plasma (Invitrogen) at 37 °C in a humidified incubator under 5% CO₂. For DNA transfection, cells were seeded in 24-well plates until they were 90–100% confluent at the time of transfection. Then plasmids were transfected by using the Lipofectamine™ 2000 Reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, 0.6μg expression plasmid, 0.2μg reporter gene plasmid, 0.02μg pRLTK renilla luciferase plasmid, and 2.0μL Lipofectamine™ 2000 were co-transfected into the cells in each well in the 24-well plate. All assays were performed with three independent transfections. Firefly and renilla luciferase activities were measured using Dual Luciferase Reporter Assay System (Promega, USA) according to the manufacturer’s instructions. Briefly, after 48 h transfection, HEK293T cells in the 24-well plates were washed twice with 100 μL PBS, then lysed with 100 μL 1x passive lysis buffer (PLB) at room temperature for 10 min. Cell lystate (20 μL) was transferred to a clean plate and 50 μL of luciferase assay reagent II and 1x Stop & Glo substrate were added in sequence, then firefly and renilla luciferase activities were measured using a InfiniTE200 plate reader (Tecan, Switzerland).

Statistical analysis. The results were presented as means ± standard error of the mean (SEM). Data from each treatment were subjected to one-way analysis of variance (ANOVA) and correlation analysis where appropriate using SPSS 19.0 for Windows. Tukey’s multiple range test was chosen as a multiple comparison test with a significance level of 0.05. For the Elov14 functional characterization, the saturated VLC-FA profiles from yeast expressing the elov14 were compared to the control by a Student’s t-test (P < 0.05).

References

1. Calder, P. C. Immunomodulation by omega-3 fatty acids. Prostag. Leukot. Ess. 77, 327–335 (2007).
2. Fleeth, M. & Clandinin, M. T. Dietary PUFA for preterm and term infants: review of clinical studies. Crit. Rev. Food Sci. 45, 205–229 (2005).
3. Nugent, A. P. The metabolic syndrome. Nutr. Bull. 29, 36–43 (2004).
4. Sprague, M., Dick, J. R. & Tocher, D. R. Impact of sustainable feeds on omega-3 long-chain fatty acid levels in farmed Atlantic salmon, 2006–2015. Sci. Rep. UK 621892 (2016).
5. Bell, J. G. et al. Substituting fish oil with crude palm oil in the diet of Atlantic salmon (Salmo salar) affects muscle fatty acid composition and hepatic fatty acid metabolism. J. Nutr. 132, 222–230 (2002).
6. Henriquez, J., Dick, J. R., Tocher, D. R. & Bell, J. G. Nutritional quality of salmon products available from major retailers in the UK: content and composition of n-3 long-chain PUFA. Brit. J. Nutr. 112, 964–973 (2014).
7. Monroig, O., Navarro, J. C. & Tocher, D. R. Long-chain polyunsaturated fatty acids in fish: recent advances on desaturases and elongases involved in their biosynthesis. Avances en Nutrición Acuícola. 11, 257–283 (2011a).
8. Tocher, D. R. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. Aquaculture 439, 94–107 (2015).
9. Jakobsson, A., Westerberg, R. & Jacobsson, A. Fatty acid elongases in mammals: their regulation and roles in metabolism. Prog. Lipid Res. 45, 237–249 (2006).
10. Nugteren, D. H. The enzymic chain elongation of fatty acids by rat-liver microsomes. BBA-Derivation and Lipid Metab. 1801, 1145–1154 (2010).
11. Li, S. et al. Molecular cloning and functional characterization of a putative elov4 gene and its expression in response to dietary fatty acid profiles in orange-spotted grouper Epinephelus coioides. Aquac. Res. 48, 537–552 (2017).
12. Li, S. et al. Molecular cloning, functional characterization and nutritional regulation of the putative elov5 gene in the orange-spotted grouper (Epinephelus coioides). Plos One 11, e0150544 (2016).
13. Torroño, V., Sotoño, S., Takeuchi, T. & Yotsumaki, G. Cloning and over-expression of a masu salmon (Onchorhynchus masou) fatty acid elongase-like gene in zebrafish. Aquaculture 282, 13–18 (2008).
14. Kabeya, N. et al. Modification of the n-3 HUFA biosynthetic pathway by transgenesis in a marine teleost, nibe croaker. J. Biotechnol. 172, 46–54 (2014).
15. Kuh, M. K., Jaya-Ram, A. & Shu-Chien, A. C. The capacity for long-chain polyunsaturated fatty acid synthesis in a carnivorous vertebrate: functional characterisation and nutritional regulation of a Fads2 fatty acyl desaturase with Δ4 activity and an Elov5 elongase in striped snakehead (Channa striata). BBA-Mol. Cell Biol. 1851, 248–260 (2015).
16. Li, S. et al. Molecular cloning and functional characterization of a putative elov4 gene and its expression in response to dietary fatty acid profiles in orange-spotted grouper Epinephelus coioides. Plos One 11, e0150544 (2016).
17. Moraes, S., Moura, G., Martinez, A., Gjas, N. & Tocher, D. R. Docosahexaenoic acid biosynthesis via fatty acyl elongase and Δ4-desaturase and its modulation by dietary lipid level and fatty acid composition in a marine vertebrate. BBA-Mol. Cell Biol. L. 1851, 588–597 (2015).
18. Ren, H. T., Xu, J. H., Xu, P. & Tang, Y. K. Influence of dietary fatty acids on muscle fatty acid composition and expression levels of Δ6 desaturase-like and elov5-like elongase in common carp (Cyprinus carpio var. Jian). Comp. Biochem. Physiol. B 163, 184–192 (2012).
19. Yamamoto, Y. et al. Cloning and nutritional regulation of polyunsaturated fatty acid desaturase and elongase of a marine teleost, the nibe croaker Nibea mitsukurii. Fisheries Sci. 76, 463–472 (2010).
20. Zuo, R., Mai, K., Xu, W., Dong, X. & Ai, Q. Molecular cloning, tissue distribution and nutritional regulation of a fatty acyl elov5-like elongase in large yellow croaker, Larimichthys crocea. Aquac. Res. 47, 445–459 (2016).
21. Sampath, H. & Ntambl, J. M. Polysaturated fatty acid regulation of genes of lipid metabolism. Annu. Rev. Nutr. 25, 317–340 (2005).
22. Horton, J. D., Goldstein, J. L. & Brown, M. S. SREBP activators: the complete program of cholesterol and fatty acid synthesis in the liver. J. Clin. Invest. 109, 1125–1131 (2002).
23. Cha, J. Y. & Repa, J. J. The liver X receptor (LXR) and hepatic lipogenesis The carboxyhydrate-response element-binding protein is a target gene of LXR. J. Biol. Chem. 282, 743–751 (2007).
24. Qin, Y., Dahlen, K. T., Gustafsson, J. A. & Nebb, H. L. Regulation of hepatic fatty acid elongase 5 by LXRα–SREBP-1c. BBA-Mol. Cell Biol. L. 1791, 140–147 (2009).
25. monroig, O. et al. Expression and role of elov4 elongases in biosynthesis of very long-chain fatty acids during zebrafish Danio rerio early embryonic development. BBA-Mol. Cell Biol. L. 1801, 1145–1154 (2010).
26. Duan, Q. et al. Replacement of dietary fish oil with vegetable oils improves the growth and flesh quality of large yellow croaker (Larimichthys crocea). J. Ocean U. China 13, 445–452 (2014).
27. Tocher, D. R. Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquac. Res.* **41**, 717–732 (2010).
28. Guillou, H., Zadravec, D., Martin, P. G. & Jacobsson, A. The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. *Prog. Lipid Res.* **49**, 186–199 (2010).
29. Monroig, Ó. et al. Evolutionary functional elaboration of the Elav2/5 gene family in chordates. *Sci. Rep.* **6**, 20510 (2016).
30. Cook, H. W. & McMaster, R. C. R. Fatty acid desaturation and chain elongation in eukaryotes. In (Eds) Vance, D. E., Vance, J. E., *Biochemistry of Lipids, Lipoproteins and Membranes*, Elsevier, Amsterdam, pp. 181–204 (2004).
31. Carmona-Antonanzaz, G., Monroig, Ó., Dick, J. R., Davie, A. & Tocher, D. R. Biosynthesis of very long-chain fatty acids (C>24) in Atlantic salmon: Cloning, functional characterisation, and tissue distribution of an Elovl4 elongase. *Comp. Biochem. Physiol. B** **159**, 122–129 (2011).
32. Monroig, Ó. et al. Elongation of long-chain fatty acids in rhabitfish *Siganus canaliculatus*: Cloning, functional characterisation and tissue distribution of Elovl5- and Elovl4-like elongases. *Aquaculture* **350**, 63–70 (2012).
33. Fonseca-Madrigal, J. et al. Diversification of substrate specificities in teleostei Fads2: characterization of Δ6-Δ5 desaturases of *Chirostoma estor*. *J Lipid Res.* **55**, 1408–1419 (2014).
34. Agbaga, M. P., Mandal, M. N. A. & Anderson, R. E. Retinal very long-chain PUFAs: new insights from studies on ELOVL4 protein. *J Lipid Res.* **51**, 1624–1642 (2010).
35. Zadravec, D. et al. ELOVL2 controls the level of n-6 28:5 and 30:5 fatty acids in testis, a prerequisite for male fertility and sperm maturation in mice. *J Lipid Res.* **52**, 245–25 (2011).
36. Sprecher, H. Metabolism of highly unsaturated n-3 and n-6 fatty acids. *BBA-Mol. Cell Biol. L.* **1486**, 219–231 (2000).
37. Kabeya, N. et al. Polyunsaturated fatty acid metabolism in a marine teleost, Nibe croaker *Nibea mitsuakura*: Functional characterization of Fads2 desaturase and Elovl5 and Elovl4 elongases. *Comp. Biochem. Physiol. B** **188**, 37–45 (2015).
38. Monroig, Ó., Webb, K., Ibarra-Castro, L., Holt, G. J. & Tocher, D. R. Biosynthesis of long-chain polyunsaturated fatty acids in marine fish: Characterization of an Elovl4-like elongase from cobia *Rachycentron canadum* and activation of the pathway during early life stages. *Aquaculture** **312**, 145–153 (2011).
39. Gregory, M. K., See, V. H. L., Gibson, R. A. & Schuller, K. A. Cloning and functional characterisation of fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*). *Comp. Biochem. Physiol. B** **155**, 178–185 (2010).
40. Wang, S. et al. Investigating long-chain polyunsaturated fatty acid biosynthesis in teleost fish: Functional characterization of fatty acyl desaturase (Fads2) and Elovl5 elongase in the catadromous species, Japanese eel (*Anguilla japonica*). *Aquaculture* **454**, 57–65 (2014).
41. Lewis, M. J. et al. Targeted dietary micronutrient fortification modulates n-3 LC-PUFA pathway activity in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture** **412**, 215–222 (2013).
42. Vagner, M. & Santigosa, E. Characterization and modulation of gene expression and enzymatic activity of delta-6 desaturase in teleosts: a review. *Aquaculture** **315**, 131–143 (2011).
43. Postic, C., Dentin, R., Denechaud, P. D. & Giraud, J. Chrebp, a transcriptional regulator of glucose and lipid metabolism. *Annu. Rev. Nutr.* **27**, 179–92 (2007).
44. Minghetti, M., Leaver, M. J. & Tocher, D. R. Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (*Salmo salar*) established cell line, skh-1. *BBA-Mol. Cell Biol. L.* **1811**, 194–202 (2011).
45. Saitou, N. & Nei, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425 (1987).
46. Agaba, M. K. et al. Cloning and functional characterisation of polyunsaturated fatty acid elongases from marine and freshwater teleost fish. *Comp. Biochem. Physiol. B** **142**, 342–352 (2005).
47. Li, M. et al. Characterization of two Δ5 fatty acyl desaturases in abalone (*Haliotis discus hannai* Ino). *Aquaculture* **416**, 48–56 (2013).
48. Monroig, Ó., Tocher, D. R., Hontoria, F. & Navarro, J. C. Functional characterisation of a Fads2 fatty acyl desaturase with Δ6/Δ4 activity and an Elovl5 with C16, C18 and C20 elongase activity in the anadromous teleost meagre (*Argyrosomus regius*). *Comp. Biochem. Physiol. B** **155**, 63–70 (2012).
49. Zhang, Q. et al. miR-17 is involved in the regulation of LC-PUFA biosynthesis in vertebrates: Effects on liver expression of a fatty acyl desaturase in the marine teleost *Siganus canaliculatus*. *BBA-Mol. Cell Biol. L.* **1841**, 934–943 (2014).
50. Zhuo, M. Q. et al. Regulation of insulin on lipid metabolism in the sterol regulatory element-binding protein-1c (SREBP-1c) gene by antagonizing ligand-dependent activation of the LXR. *P. Natl. Acad. Sci. USA* **108**, 6027–6032 (2011).
51. Zheng, J. J. et al. Antioxidant defences at transcriptional and enzymatic levels and gene expression of Nr72-Keap1 signaling molecules in response to acute zinc exposure in the spleen of the large yellow croaker *Pseudosciaena crocea*. *Fish Shellfish Immun.* **52**, 1–8 (2016).
52. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2^-ΔΔCT method. *Methods* **25**, 402–408 (2001).

**Acknowledgements**

This work was funded by the National Science Fund for Distinguished Young Scholars of China (31525024) and Spanish Government through the LONGFAQUA project (AGL2013–40986–R). We are grateful to prof. Qicun Zhou for their selfless assistance in vitro study. Additionally, we thank Xiaojing Dong for her help in promoter study, and Jianlong Du and Hanlin Xu for their help in fish rearing.

**Author Contributions**

S.L.L., Q.H.A., K.S.M., WX. and D.R.T. designed the research; S.L.L. conducted most of the research with the help of T.J.W., Y.H.Y. and K.L.; M.O. determine the function of fatty acid elongase with the help of C.N. and E.H.; S.L.L. analyzed the data and wrote the paper. All authors read and approved the final manuscript.

**Additional Information**

Supplementary information accompanies this paper at doi:10.1038/s41598-017-02646-8

**Competing Interests:** The authors declare that they have no competing interests.

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
