A complete enzymatic capacity for long-chain polyunsaturated fatty acid biosynthesis is present in the Amazonian teleost tambaqui, *Colossoma macropomum*

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Abstract

In vertebrates, the essential fatty acids (FA) that satisfy the dietary requirements for a given species depend upon its desaturation and elongation capabilities to convert the C_{18} polyunsaturated fatty acids (PUFA), namely linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n–3), into the biologically active long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA), including arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Recent studies have established that tambaqui (*Colossoma macropomum*), an important aquaculture-produced species in Brazil, is a herbivorous fish that can fulfil its essential FA requirements with dietary provision C_{18} PUFA LA and ALA, although the molecular mechanisms underpinning such ability remained unclear. The present study aimed at cloning and functionally characterizing genes encoding key desaturase and elongase enzymes, namely *fads2*, *elovl5* and *elovl2*, involved in the LC-PUFA biosynthetic pathways in tambaqui. First, a *fads2*-like desaturase was isolated from tambaqui. When expressed in yeast, the tambaqui Fads2 showed Δ6, Δ5 and Δ8 desaturase capacities within the same enzyme, enabling all desaturation reactions required for ARA, EPA and DHA biosynthesis. Moreover, tambaqui possesses two elongases that are *bona fide* orthologs of *elovl5* and *elovl2*. Their functional characterization confirmed that they can operate towards a variety of PUFA substrates with chain lengths ranging from 18 to 22 carbons. Overall our results provide compelling evidence that demonstrates that all the desaturase and elongase activities required to convert LA and ALA into ARA, EPA and DHA are present in tambaqui within the three genes studied herein, i.e. *fads2*, *elovl5* and *elovl2*.

Keywords

Biosynthesis; *Colossoma macropomum*; Desaturase; Elongase; Essential fatty acids.

1. Introduction

Long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (ARA, 20:4n–6), eicosapentaenoic acid (EPA, 20:5n–3) and docosahexaenoic acid (DHA, 22:6n–3), play many essential roles in growth, development and reproduction of vertebrates (Cook, 1996; Jaya-Ram et al., 2011; Innis, 2008; Vagner and Santigosa, 2011; Calder, 2014). In vertebrates, LC-PUFA can be obtained through the diet or endogenously synthesized from C_{18} polyunsaturated fatty acid (PUFA) precursors, namely linoleic acid (LA, 18:2n–6) and α-linolenic acid (ALA, 18:3n–3), themselves being dietary essential compounds since they cannot be biosynthesized *de novo* by vertebrates (Castro et al., 2016). Biosynthesis of the physiologically important LC-PUFA from the dietary essential C_{18} PUFA precursors requires the coordinated action of both fatty acid desaturases (Fads) and elongation of very long chain fatty acids (Elovl) proteins, membrane-bound enzymes localized in the endoplasmic reticulum (Guillou et al., 2010). The characterization of Fads and Elovl involved in LC-PUFA biosynthesis in fish has been extensively investigated (Castro et al., 2016; Kabeya et al., 2018; Monroig et al., 2018). This research has been mainly driven by the need to understand the endogenous ability that farmed species have to utilize commonly used ingredients such as vegetable oils (VO), devoid of LC-PUFA but often rich in their biosynthetic precursors LA and ALA (Turchini et al., 2009; Tocher, 2010). These studies revealed that the LC-PUFA biosynthetic capability varies remarkably among teleosts, depending upon the species-specific *fads* and *elovl* gene complement and function (Castro et al., 2016; Monroig et al., 2016, 2018).

Fads enzymes introduce a double bond (unsaturation) at a specific position of the fatty acid (FA) chain and are termed as "Δx desaturase", with "x" denoting the position of the carbon from the carboxylic group at which the new double bond is introduced (Leonard et al., 2004). Vertebrate Fads are characterized by a cytochrome b5-like domain

containing a heme-biding motif (HPGG), three histidine boxes consisting HXXXH, HXXHH and QXXHH, and several membrane-spanning domains (Sperling et al., 2003). Unlike most vertebrates that have Fads1 and Fads2 with Δ5 and Δ6 desaturase activities, respectively (Guillou et al., 2010), teleost fish appear to have lost the *fads1* gene, presenting exclusively *fads2* genes (Castro et al., 2012, 2016), with the exception of the Japanese eel *Anguilla japonica*, a basal teleostei possessing a Fads1 with Δ5 activity (Lopes-Marques et al., 2018). Interestingly, teleostei Fads2 are functionally more diverse than their non-teleost counterparts (Fonseca-Madrigal et al., 2014; Castro et al., 2016). While the majority of teleost Fads2 enzymes functionally characterized to date present Δ6 desaturase activity (Zheng et al., 2004, 2009; González-Rovira et al., 2009; Mohd-Yusof et al., 2010; Monroig et al., 2013, 2010a; Kabeya et al., 2018), an increasingly high number of teleost Fads2 have alternative desaturase substrate specificities including both Δ6 and Δ5 activities within the same enzyme (Hastings et al., 2001; Tanomman et al., 2013; Kuah et al., 2016; Oboh et al., 2016), Δ5 (Abdul Hamid et al., 2016) and Δ4, the latter often exhibiting some residual activity as Δ5 desaturase (Li et al., 2010; Morais et al., 2012; Fonseca-Madrigal et al., 2014; Oboh et al., 2017). Consistent with the mammalian FADS2 (Park et al., 2009), fish Fads2 typically possess Δ8 desaturation capacity (Monroig et al., 2011a), enabling the "Δ8 pathway", an alternative route to the most prominent "Δ6 pathway" leading to the biosynthesis of 20:3n–6 and 20:4n–3, immediate precursors of ARA and EPA, respectively (Castro et al., 2016).

Regarding Elovl, these enzymes catalyze the condensation of malonyl-CoA into an activated FA, a rate-limiting reaction in the elongation pathway resulting in the extension of the pre-existing FA in 2 carbons (Jakobsson et al., 2006). Several distinct Elovl (termed Elovl 1-7) have been described in vertebrates (Jakobsson et al., 2006; Guillou et al., 2010), each one having different substrate preferences. Interestingly, Elovl2, Elovl4 and

Elovl5 can elongate PUFA substrates and thus play major roles in LC-PUFA biosynthesis (Jakobsson et al., 2006; Guillou et al., 2010). In fish, the majority of Elovl5 orthologs investigated to date showed ability to efficiently elongate C_{18} and C_{20} PUFA substrates, with remarkably lower efficiency generally exhibited towards C_{22} PUFA (Castro et al., 2016). Vertebrate Elovl2 can elongate C_{20} and C_{22} PUFA, but it has little ability to elongate C_{18} PUFA. Loss of Elovl2 in recently emerged teleost lineages has been postulated to be partly compensated by Elovl4, an enzyme that, along its role in the biosynthesis of very long-chain (>C_{24}) PUFA (Monroig et al., 2010b), can operate towards C_{22} PUFA like Elovl2 thus playing a role in LC-PUFA biosynthesis (Monroig et al., 2011b; Kabeya et al., 2015).

Overall, it has become clear that the endogenous capacity for LC-PUFA production in fish species cannot be anticipated from their genetic repertoire of *fads* and *elovl*. Rather, functional analyses of the gene products (i.e. enzymes) is required to fully understand the physiological capacities that farmed fish species have to biosynthesize ARA, EPA and DHA from C_{18} precursors contained in VO. The *Colossoma macropomum*, popularly known as "tambaqui", is a freshwater fish and is currently the main native species farmed in Brazil, representing 28% of the total of the national production. Importantly, tambaqui displays high growth rates, adaptation to intensive culture systems and good fillet quality (Guimarães and Martins, 2015). The adult fish are predominantly herbivorous, feeding abundantly on fruits and seeds during flooding episodes (Almeida et al., 2008). Such natural adaptation to low LC-PUFA diets suggests that tambaqui possess an active LC-PUFA biosynthetic capacity that enable them to thrive with little input of these essential nutrients. In order to test this hypothesis, the present study aimed at cloning and functionally characterize the key desaturase and elongase genes, namely *fads2*, *elovl5* and *elovl2*, involved in the LC-PUFA biosynthetic pathways in this species.

2. Materials and Methods

2.1. RNA sampling and cDNA synthesis

Liver samples from an adult tambaqui were preserved in an RNA stabilization buffer (3.6 M ammonium sulphate, 18 mM sodium citrate, 15 mM EDTA, pH 5.2) and stored at −80 °C prior to RNA extraction. RNA extraction was performed using the Illustra RNA spin kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's guidelines including an on-column DNase treatment. Final RNA samples were run on a 1 % agarose TAE gel stained with GelRed™ nucleic acid stain (Biotium, Hayward, CA, USA) to evaluate integrity, while RNA quantification was performed using BiotTek® microplate reader to determine sample absorbance. Subsequently, cDNA synthesis was performed from 1 μg of total RNA and using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's recommendations. Additionally, 5' and 3' Rapid Amplification of cDNA Ends (RACE) cDNA was prepared using SMARTer® RACE 5'/3' Kit (Clontech, Mountain View, CA, USA).

2.2. Isolation of ORF fads and elovl genes from tambaqui

For amplification of the first fragments of the target genes *fads2, elovl5* and *elovl2*, polymerase chain reactions (PCR) were conducted using degenerated primers designed on conserved regions of teleosts orthologs of *fads2, elovl5* and *elovl2* using CODEHOP (http://blocks.fhcrc.org/codehop.html) (Rose et al., 2003). Amplifications of partial fragments of the target genes were carried out using a 1 μl of liver cDNA, 500 nM of sense and antisense primers, and Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), set for a final volume of 20 μl (see Supplementary Table 1 for primers, PCR conditions). Resulting PCR products were analyzed on a 1 % agarose gel, and fragments with the expected size were purified with NYZGelpure (NZYtech,

Lisbon, Portugal) and confirmed by DNA sequencing (GATC Biotech, Constance, Germany).

To obtain full-length cDNA sequences, RACE PCR were performed. Gene specific primers for RACE PCR were designed using the previously isolated first fragments, with adapter specific primers being provided with the kit (SMARTer® RACE 5'/3' Kit, Clontech, Mountain View, CA, USA). The RACE PCR were performed with Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) using as template 5' and 3' RACE cDNA prepared from liver RNA. Each RACE PCR contained 1 µl of gene specific primer combined with 2 µl Universal primer mix (Clontech, Mountain View, CA, USA) and corresponding RACE cDNA template (see Supplementary Table 1 for primers and PCR conditions). Resulting 5' and 3' RACE PCR products were confirmed by sequencing (GATC Biotech, Constance, Germany) and assembled with first fragments to obtain open reading frame (ORF) sequences for each target gene (Geneious V7.1.9).

2.3. Sequence collection and phylogenetic analysis

Blastp searches in the public database NCBI were performed using human Fads1, Fads2, Elovl2, Elovl5 and Elovl4, as queries. Fads and Elovl and amino acid sequences were collected from the major vertebrate lineages, namely Sarcopterygii (Mammalia, Reptilia, Aves, Amphibia, and Coelacanthiformes), Chondrichtyes (Elasmobranchii and Chimaera) and Actinopterygii (Elopomorpha, Osteoglossomorpha, Otomorpha, and Euteleosteomorpha), as well as from invertebrate groups including Hemichordata (*Saccoglossus kowalevskii*), Cephalochordata (*Branchiostoma lanceolatum*), Urochordata (*Ciona inestinalis*) and Cephalopod (*Octopus vulgaris*) (see Supplementary Table 2 for accession numbers). Next, two phylogenetic trees were constructed containing Fads and Elovl sequences, with alignments for both Fads and Elovl performed

independently in MAFFT (Katoh and Toh, 2008) with the L-INS-i method. The resulting sequence alignments were inspected and stripped of 90% columns containing gaps leaving a total of 59 sequences with 467 positions for phylogenetic analysis for Fads, and 53 sequences and 318 positions for Elovl. Final sequence alignments were submitted for phylogenetic analysis to PhyML V3.0 (Guindon et al., 2010). For each analysis evolutionary model was determined using the smart model selection (SMS) option resulting in a JTT+G+I for Fads and JTT+G+I for Elovl and branch support in both runs was calculated using aBayes. The resulting trees were visualized and analyzed in Fig. Tree V1.3.1 available at http://tree.bio.ed.ac.uk/software/figtree/.

2.4. Functional characterization of tambaqui fads2, elovl5 and elovl2

Functions of the tambaqui Fads2, Elovl5 and Elovl2 enzymes were characterized individually by heterologous expression in yeast, *Saccharomyces cerevisiae*. First, the ORF sequences of the tambaqui *fads2*, *elovl5* and *elovl2* were isolated and cloned into the yeast expression vector pYES2 (Thermo Fisher Scientific, Waltham, MA, USA) (Lopes-Marques et al., 2017). The ORF of the target genes were isolated by PCR (Flash High-Fidelity PCR Master Mix, Thermo Fisher Scientific, Waltham, MA, USA) using *C. macropomum* liver cDNA as template and primers containing appropriate restriction sites for further cloning into pYES2 (see Supplementary Table 1 for primers and PCR conditions). PCR products and pYES2 were subsequently digested with appropriate restriction enzymes (Promega, Madison, WI, USA) and ligated to produce the plasmid constructs pYES2-*fads2*, pYES2-*elovl5* and pYES2-*elovl2*. Sequences of inserts in each plasmid construct was confirmed (GATC Biotech, Constance, Germany) before being used to transform yeast competent cells.

One colony of transgenic yeast carrying either pYES2-*fads2*, pYES2-*elovl5* or pYES2-*elovl2* was grown in *S. cerevisiae* minimal (SCMM-uracil) medium lacking uracil

to produce a bulk culture and subsequently diluted to OD600 of 0.4 in all the Erlenmeyer flasks used to test each potential FA substrate assayed. For Fads2, transgenic yeast were grown in the presence of Δ6 (18:2n–6 and 18:3n–3), Δ8 (20:2n–6 and 20:3n–3), Δ5 (20:3n–6 and 20:4n–3) and Δ4 (22:4n–6 and 22:5n–3) desaturase substrates. In addition, to determine the desaturase activity towards 24:5n–3, the transgenic yeast co-expressing zebrafish *elovl2* and the tambaqui *fads2* were grown in the presence of 22:5n–3 as described previously by Oboh (Oboh et al., 2016). Here the conversion towards 18:3n-3 in the co-expression yeast system was also determined as positive control. With regards to tambaqui elongases, both Elovl5 and Elovl2 were functionally characterized by growing transgenic yeast in medium supplemented with one of the following PUFA substrates: 18:2n−6, 18:3n−3, 18:3n−6, 18:4n−3, 20:4n−6, 20:5n−3, 22:4n−6 and 22:5n−3. Given that PUFA uptake by yeast decreases with increasing chain length, PUFA substrates were added to the yeast cultures from both desaturase and elongase assays at final concentrations of 0.5 mM (C_{18}), 0.75 mM (C_{20}) and 1.0 mM (C_{22}) (Lopes-Marques et al., 2017). Additionally, yeast transformed with empty pYES2 were also grown in presence of PUFA substrates as control treatments. After 2 days of culture at 30 ℃, yeast were harvested, washed, and homogenized in chloroform/methanol (2:1, v/v) containing 0.01% (w/v) butylated hydroxytoluene (BHT) and kept -20 ºC until further analysis. All PUFA substrates, except stearidonic acid (18:4n-3), were from Nu-Chek Prep, Inc. (Elysian, MN, USA). Stearidonic acid and chemicals used to prepare SCMM-uracil were from Sigma-Aldrich (Darmstadt, Germany), except for the bacteriological agar obtained from Oxoid Ltd. (Hants, UK).

2.5. Fatty acid analysis of yeast

Total lipid extracted from yeast (Folch et al., 1957) was used to prepare fatty acyl methyl esters (FAME) that were further analyzed by gas chromatography as previously

described (Hastings et al., 2001; Li et al., 2010). FAME were identified based on retention times using an Fisons GC-8160 (Thermo Fisher Scientific, Waltham, MA, USA) gas chromatograph equipped with a 60 m x 0.32 mm i.d. x 0.25 μm ZB-wax column (Phenomenex, Macclesfield, UK) and flame ionization detector. The desaturation or elongation conversion efficiencies from exogenously added PUFA substrates were calculated by the proportion of substrate FA converted to desaturated or elongated products as [all product areas/(all products areas + substrate area)] × 100. For the tambaqui elongases, "all product areas" include those of the initial elongation products, as well as those from stepwise elongations occurring subsequently (Monroig et al., 2012). Similarly, the tambaqui Fads2 exhibited multifunctional abilities, and thus the conversions on Δ8 substrates (20:2n-6and 20:3n-3) include stepwise Δ5 desaturase reactions (Fonseca-Madrigal et al., 2014).

3. Results

3.1. Phylogenetic analysis of the tambaqui fads and elovl sequences

To determine orthology of the isolated sequences from tambaqui, two independent phylogenetic trees including Fads (Fig. 1) and Elovl (Fig. 2) were constructed. For Fads, the resulting phylogenetic tree showed two well supported vertebrate clades that contained either Fads1 or Fads2 sequences, and both being out-grouped by invertebrate Fads (Fig. 1). We found that the isolated Fads from tambaqui was placed within the Fads2 clade together with orthologs from other teleost species such as *Danio rerio* and *Ictalurus punctatus*. This positioning indicates that newly cloned tambaqui *fads* is a *bona fide* Fads2 desaturase, orthologous to previously characterized Fads2 from other vertebrate species (Fig. 1).

Regarding the Elovl phylogenetic analysis, the phylogenetic tree revealed that vertebrate Elovl4 sequences including the invertebrate elongase from *C. intestinalis* formed an independent monophyletic group positioned. The remaining vertebrate Elovl2 and Elovl5 sequences constituted two well supported independent clades with the invertebrate *B. lanceolatum* Elovl2/5 sequence as an outgroup (Monroig et al., 2016). Regarding the herein isolated elongases from tambaqui, one clustered within the Elovl2 clade and the other within the Elovl5 clade, in both cases including characterized Elovl sequences from other teleosts and vertebrate species. These results indicate that the isolated tambaqui elongases are orthologs of *elovl2* and *elovl5* (Fig. 2).

3.2. Functional characterization of C. macropomum fads2, elovl5 and elovl2 in S. cerevisiae

Control yeast transformed with the empty vector pYES2 did not show activity towards any of the substrates tested (data not shown). Transgenic yeast expressing the tambaqui *fads2* exhibited Δ6 desaturase activity since 18:2n–6 and 18:3n–3 were converted to 18:3n–6 (28.3 % conversion) and 18:4n–3 (63.5 % conversion), respectively (Table 1).

The tambaqui Fads2 also showed Δ5 desaturase activity since transgenic yeast converted 20:3n–6 and 20:4n–3 were converted to 20:4n–6 (14.8 % conversion) and 20:5n–3 (17.8 % conversion), respectively (Table 1). These results indicate that the tambaqui Fads2 is a dual Δ6Δ5 desaturase. Moreover, this enzyme exhibited Δ8 desaturase activity, with exogenously added 20:2n–6 and 20:3n–3 being desaturated to 20:3n–6 and 20:4n–3, respectively (Table 1). In addition, the tambaqui Fads2 showed Δ6 desaturation capacity towards 24:5n–3, which was desaturated to 24:6n–3.

Functional assays of tambaqui elongases showed that yeast expressing Elovl5 were able to elongate all C_{18} (18:2n-6, 18:3n-3, 18:3n-6 and 18:4n-3) and C_{20} (20:4n-6 and 20:5n-3) PUFA substrates, yet no elongation was detected for C_{22} (22:4n-6 and 22:5n-6) substrates (Table 2). Moreover, yeast expressing the tambaqui Elovl2 exhibited elongation ability for all PUFA including C_{18}, C_{20} and C_{22}, with C_{20} substrates appearing as preferred substrates for elongation (Table 2).

4. Discussion

The essential FA for a given species depends upon its desaturation and elongation capability to endogenously convert dietary C_{18} PUFA LA and ALA into the biologically active LC-PUFA, namely ARA, EPA and DHA (Tocher, 2015). Recent investigations have shown that tambaqui juveniles exhibited no difference in growth performance when fed diets containing mixtures of VO (corn and linseed oils) in comparison to fish fed a fish oil (control) diet (Pereira et al., 2017; Paulino et al., 2018). Consequently it was established that tambaqui can fulfil its essential FA requirements with dietary provision C_{18} PUFA (Paulino et al., 2018). The present study provides compelling molecular evidence that support previous findings and unequivocally demonstrates that all the desaturase and elongase activities required to convert C_{18} PUFA into ARA, EPA and DHA exist in tambaqui within the three genes studied herein, i.e. *fads2, elovl5* and *elovl2* (Fig. 3).

The phylogenetic analysis confirmed that the *fads*-like desaturase isolated from tambaqui is an ortholog of *fads2*. Additionally, sequence alignment of the tambaqui desaturase with Fads2 from other teleost species showed a high degree of conservation of the classic desaturase motifs, namely histidine boxes and heme binding region (Supplementary Fig. 1). Recently Lopes-Marques et al. (2018) demonstrated that, along the formerly characterized Fads2 (Wang et al., 2014), *Anguilla japonica* possess a Fads1 with Δ5 activity similarly to chondrichthyes and mammals (Marquardt et al., 2000; Castro et al., 2012), being the first report of a non-*fads2* desaturase described in teleosts. Nevertheless, the presence of *fads1* among teleosts appears to be anecdotic and restricted to certain post-3R lineages like Elopomorpha (Lopes-Marques *et al*., 2018) since the vast majority of teleosts possess only *fads2* as the sole *fads* desaturase found in their genomes (Castro et al., 2016). Indeed, loss of *fads1* has been often postulated as a major evolutionary driver partly explaining the unique functional plasticity of teleosts Fads2

(Fonseca-Madrigal et al., 2014; Castro et al., 2016). In agreement, the functional characterization of the tambaqui Fads2 demonstrated this is a functionalized desaturase that, in addition to the expected Δ6 activity, it also exhibited Δ5 desaturase capacity. Therefore, the tambaqui Fads2 can be categorized as a bifunctional or dual Δ6Δ5 desaturase, an enzyme type previously described in *D. rerio* (Hastings et al., 2001), *Siganus canaliculatus* (Li et al., 2010), *Oreochromis niloticus* (Tanomman et al., 2013), *Channa striata* (Kuah et al., 2016) and *Clarias gariepinus* (Oboh et al., 2016). Moreover, the tambaqui Fads2 also showed Δ8 desaturase activity as described in a wide range of teleosts (Monroig et al., 2011a; Oboh et al., 2016; Lopes-Marques et al., 2017; Kabeya et al., 2018). With the exception of the marine herbivore *S. canaliculatus*, all teleost species reported to have dual Δ6Δ5 desaturase inhabit freshwater ecosystems with limited abundance of LC-PUFA compared to marine environments (Colombo et al., 2016). Indeed, such dietary restriction of LC-PUFA has been hypothesized to account for the enhanced LC-PUFA biosynthesizing capacity of freshwater teleosts in comparison to their marine counterparts (Tocher et al., 2003; Leaver et al., 2008). Interestingly, the varied range of desaturase activities contained within the tambaqui Fads2 implies that this enzyme enables all the desaturation reactions required to biosynthesize ARA and EPA from the C_{18} PUFA precursors LA and ALA, respectively (Fig. 3). Also, the analysis of critical residues involved in determining desaturation position preference in mammalian FADS desaturases (residues marked with "*" in Supplementary Fig. 1) (Watanabe et al., 2016) revealed that tambaqui Fads2 has a similar profile to that found in the bifunctional *D. rerio* Fads2 (red boxes Supplementary Fig. 1), presenting no additional distinctive residue replacements. Similarly, the elongation capacities involved in the biosynthesis of ARA and EPA were also demonstrated within the two newly cloned *elovl* from tambaqui.

Phylogenetic analysis of the two tambaqui elongases showed that these were orthologs of Elovl2 and Elovl5 confirming the presence of both types of elongases as anticipated by the phylogenetic position of tambaqui (Characiformes) (Ravi and Venkatesh, 2018). Certainly, whereas Elovl5 is present virtually in all teleosts (Castro et al., 2016), distribution of Elovl2 is more restricted, with absence in recently emerged teleosts (Morais et al., 2009; Monroig et al., 2016) and presence reported only in relatively ancient lineages such as Cypriniformes (Monroig et al., 2009), Siluriformes (Oboh et al., 2016) and Salmoniformes (Morais et al., 2009; Gregory and James, 2014). Importantly, functional characterization of the tambaqui elongases further demonstrated their role in LC-PUFA biosynthesis, with both enabling the elongation capacity required to produce ARA and EPA from their C_{18} precursors (Fig. 3). Thus, the tambaqui Elovl5 showed elongation capacity towards C_{18} and C_{20} PUFA, while no activity detected towards C_{22} substrates. Such elongation capacities exhibited by the tambaqui Elovl5 are generally consistent with those of other teleost species (Castro et al., 2016), with some interesting particularities. Thus, the tambaqui Elovl5 showed no activity towards the C_{22} PUFA substrates 22:4n-6 and 22:5n-3, in contrast to Elovl5 from other species such as *S. canaliculatus* and *Thunnus thynnus*, with remarkably high C_{22} elongation capacity when expressed in yeast (Morais et al., 2011; Monroig et al., 2012). On the other hand, the tambaqui Elovl2 showed activity towards all C_{18}, C_{20} and C_{22} PUFA substrates tested. Yet, certain C_{18} substrates, namely LA (18:2n-6) and ALA (18:3n-3), were elongated at a comparatively lesser extent, suggesting that Elovl2 has a minor role in the Δ6 desaturase – elongase - Δ5 desaturase pathway leading to ARA and EPA biosynthesis from LA and ALA, respectively (Fig. 3). Unlike Elovl5 though, the tambaqui Elovl2 had efficiency to operate towards C_{22} substrates. For instance, 22:5n–3 was efficiently elongated to 24:5n–3, a key intermediate component of the so-called "Sprecher pathway", a metabolic route

that accounts for DHA biosynthesis in vertebrates (Buzzi et al., 1996, 1997; Sprecher, 2000) and recently demonstrated to be widespread among teleosts (Oboh et al., 2017). Certainly, our results show that tambaqui can also biosynthesize DHA through the Sprecher pathway since, in addition to the capacity of Elovl2 to produce 24:5n-3 mentioned above, its Fads2 has the ability to desaturate 24:5n-3 to 24:6n-3, a key desaturation reaction preceding a final β-oxidation step required to produce DHA. Similarly, to the enzymatic reactions required for ARA and EPA, our results confirm that both the elongation and desaturase capacities involved to biosynthesize DHA from EPA (elongase – elongase - Δ6 desaturase) are present in tambaqui.

In conclusion, the combination of the enzymatic capacities demonstrated by the tambaqui Fads2, Elovl2 and Elovl5 allows this species to convert the dietary essential C_{18} PUFA LA (18:2n–6) and ALA (18:3n–3) into the biologically active ARA, EPA, and DHA. These results confirm that tambaqui can fulfil its essential FA requirements with an adequate dietary provision of C_{18} PUFA. Therefore, tambaqui emerges as a valuable species for the sustainable development of Brazilian aquaculture, given its ability to efficiently utilize alternative sources of essential FA such as VO as confirmed at molecular level by the enzymatic capabilities shown in this study.

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Figure Captions

Figure 1. Maximum likelihood phylogenetic analysis of Fads amino acid sequences rooted with the invertebrate clade. Numbers at nodes indicate branch support in posterior probabilities calculated using aBayes. *C. macropomum* Fads2 studied herein is highlighted. Accession numbers for all Fads sequences are available in Supplementary Table 2.

Figure 2. Maximum likelihood phylogenetic analysis of Elovl amino acid sequences rooted with the Elovl4 sequences. Numbers at nodes indicate branch support in posterior probabilities calculated using aBayes. *C. macropomum* sequences (Elovl2 and Elovl5) studied herein are highlighted. Accession numbers for all Elovl sequences are available in Supplementary Table 3.

Figure 3. The pathways of biosynthesis long-chain (C_{20-24}) polyunsaturated fatty acids in *C. macropomum* predicted from activity of Fads2, Elovl2 and Elovl5 measured in yeast. Desaturation reactions are indicated as "Δx", whereas elongation reactions are indicated as "Elo". LA, linoleic acid (18:2n-6); ALA, α-linolenic acid (18:3n-3); ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. β-ox, beta-oxidation.

Figures

Figure 1

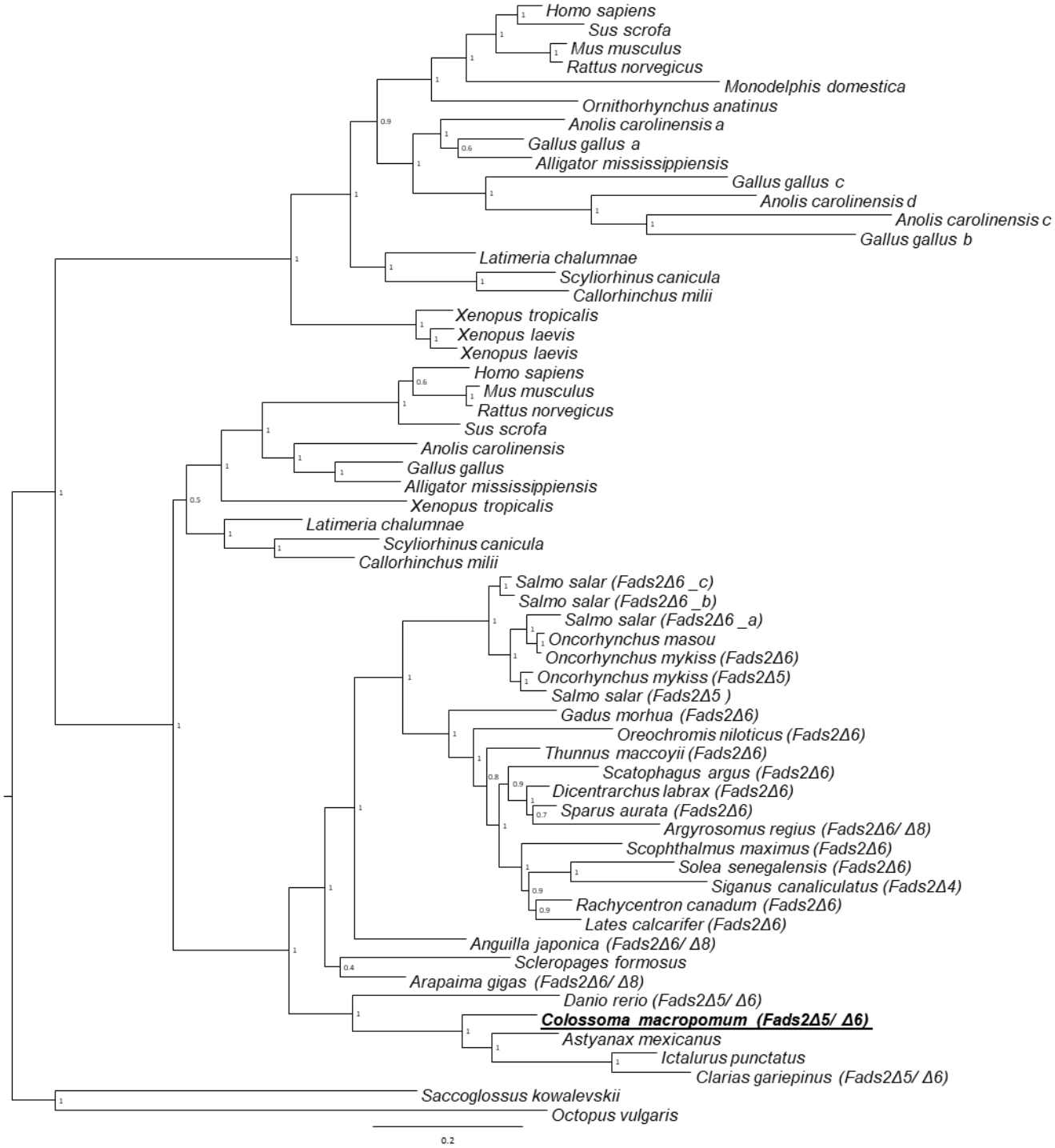


Figure 2

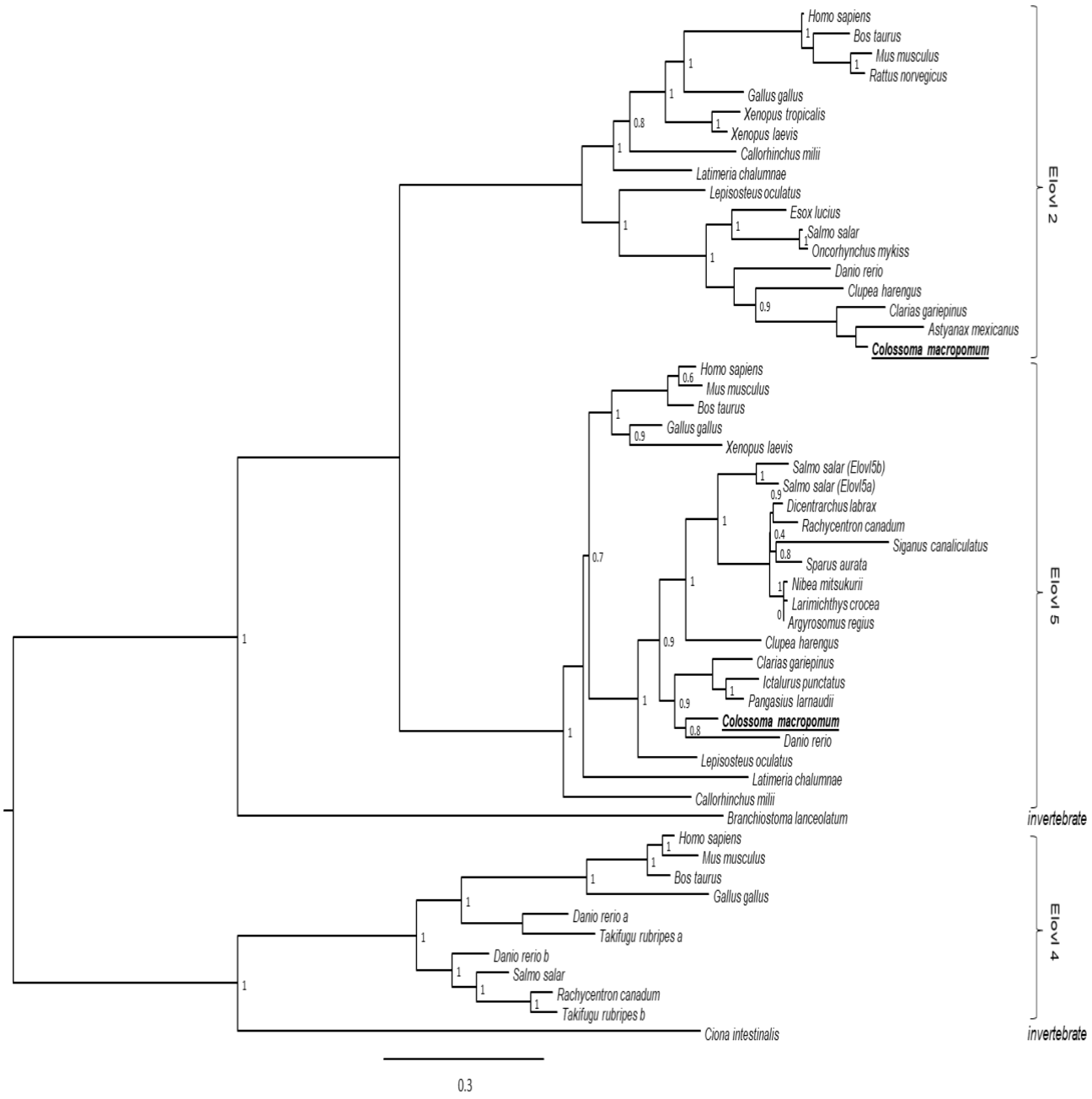
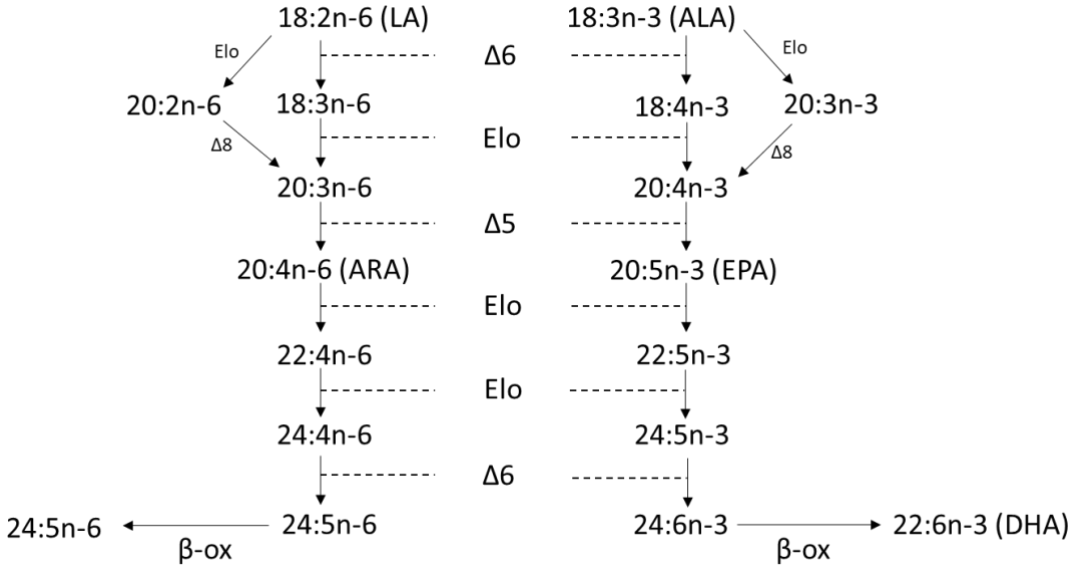

Homo sapiens
Bos taurus
Mus musculus
Rattus norvegicus
Gallus gallus
Xenopus tropicalis
Xenopus laevis
Callorhinchus milii
Latimeria chalumnae
Lepisosteus oculatus
Esox lucius
Salmo salar
Oncorhynchus mykiss
Danio rerio
Clupea harengus
Clarias gariepinus
Astyanax mexicanus
Colossoma macropomum
Elovl 2
Homo sapiens
Mus musculus
Bos taurus
Gallus gallus
Xenopus laevis
Salmo salar (Elovl5b)
Salmo salar (Elovl5a)
Dicentrarchus labrax
Rachycentron canadum
Siganus canaliculatus
Sparus aurata
Nibea mitsukurii
Larimichthys crocea
Argyrosomus regius
Clupea harengus
Clarias gariepinus
Ictalurus punctatus
Pangasius larnaudii
Colossoma macropomum
Danio rerio
Lepisosteus oculatus
Latimeria chalumnae
Callorhinchus milii
Elovl 5
Branchiostoma lanceolatum
invertebrate
Homo sapiens
Mus musculus
Bos taurus
Gallus gallus
Danio rerio a
Takifugu rubripes a
Danio rerio b
Salmo salar
Rachycentron canadum
Takifugu rubripes b
Elovl 4
Ciona intestinalis
invertebrate
0.3

Figure 3


18:2n-6 (LA)
18:3n-3 (ALA)
Elo
Δ6
Elo
20:2n-6
18:3n-6
18:4n-3
20:3n-3
Δ8
Elo
Δ8
20:3n-6
20:4n-3
Δ5
20:4n-6 (ARA)
20:5n-3 (EPA)
Elo
22:4n-6
22:5n-3
Elo
24:4n-6
24:5n-3
Δ6
24:5n-6
24:5n-6
24:6n-3
22:6n-3 (DHA)
β-ox
β-ox

Tables

Table 1. Functional characterization of the tambaqui (*C. macropomum*) Fads2 in yeast *S. cerevisiae*. Yeast were transformed with the *C. macropomum fads2* ORF and grown in the presence of Δ6 (18:2n-6 and 18:3n-3), Δ8 (20:2n-6 and 20:3n-3), Δ5 (20:3n-6 and 20:4n-3), and Δ4 (22:4n-6 and 22:5n-3) fatty acid (FA) substrates. Desaturation of 24:5n-3 was tested by co-expressing both *D. rerio elovl2* and *C. macropomum fads2* in *S. cerevisiae* (Oboh et al., 2017). Conversions in both expression systems were calculated according to the formula [all product areas/(all product areas + substrate area)] × 100.

FA substrate	FA product	Conversion (%)	Activity
18:2n–6	18:3n–6	28.3	Δ6
18:3n–3	18:4n–3	63.5	Δ6
20:2n–6	20:3n–6	2.8[a]	Δ8
20:3n–3	20:4n–3	4.5[a]	Δ8
20:3n–6	20:4n–6	14.8	Δ5
20:4n–3	20:5n–3	17.8	Δ5
22:4n–6	22:5n–6	Nd	Δ4
22:5n–3	22:6n–3	Nd	Δ4
Expressed with *D. rerio elovl2*			
18:3n–3	18:4n–3	20.5	Δ6
24:5n–3	24:6n–3	21.9	Δ6

[a] Conversions of Δ8 substrates (20:2n-6 and 20:3n-3) include stepwise reactions due to multifunctional desaturation abilities. Thus, the conversions of the *C. macropomum* Fads2 on 20:2n-6 and 20:3n-3 include the Δ8 desaturation toward 20:3n-6 and 20:4n-3, respectively, and their subsequent Δ5 desaturations to 20:4n-6 and 20:5n-3, respectively.

Nd, not detected.

Table 2. Functional characterization of the tambaqui (*C. macropomum*) Elovl5 and Elovl2 elongases in yeast *S. cerevisiae*. Yeast transformed with the *C. macropomum elovl5* and *elovl2* ORF sequences and grown in the presence of exogenously added fatty acid (FA) substrates. Conversions by Elovl5 and Elovl2 were calculated according to the formula [all product areas/(all product areas + substrate area)] × 100.

		Conversion (%)		
FA substrate	**FA product**	**Elovl5**	**Elovl2**	**Activity**
18:2n–6	20:2n–6	29.8	1.5	$C_{18}\rightarrow C_{20}$
18:3n–3	20:3n–3	47.7	7.6	$C_{18}\rightarrow C_{20}$
18:3n–6	20:3n–6	79.8	13.5	$C_{18}\rightarrow C_{20}$
18:4n–3	20:4n–3	79.0	26.3	$C_{18}\rightarrow C_{20}$
20:4n–6	22:4n–6	33.4	30.9	$C_{20}\rightarrow C_{22}$
20:5n–3	22:5n–3	53.1	66.5	$C_{20}\rightarrow C_{22}$
22:4n–6	24:4n–6	Nd	16.4	$C_{22}\rightarrow C_{24}$
22:5n–3	24:5n–3	Nd	21.8	$C_{22}\rightarrow C_{24}$

Nd, not detected.

Supplementary Material

Supplementary Table 1. Primer sets and corresponding PCR conditions used in the cloning of the *C. macropomum fads2*, *elovl5* and *elovl2* ORF sequences.

Supplementary Table 2. Accession numbers of all sequences used in phylogenetic analysis of Fads amino acid sequences.

Supplementary Table 3. Accession numbers of all sequences used in phylogenetic analysis of Elovl amino acid sequences.

Supplementary Figure 1. Sequence alignment and conservation analysis of destaturase sequence motifs of *C. macropomum* Fads2. *- indicates residues involved in the determination of the destaturation activity in mammalian desaturases (Watanabe et al., 2016). Red boxes highlight conserved residue replacements with *Danio rerio* Δ5Δ6 Fads2.

Supplementary Table 1. Primer sets and corresponding PCR conditions used in the cloning of the *C. macropomum fads2*, *elovl5* and *elovl2* ORF sequences.

	Primer set function	Primer name	Primer sequence	Cycles	TM	Extension (size bp)
FADS2	Degenerate primers	FADS2_degen_F	GCGCCTCCGCCAAytggtggaayc	40	54 °C	72 °C/10 s
		FADS2_degen_R	TGGCCGGAGAACcartcrttraa			
	Gene specific race	Cma_FADS2_3RACE_F	GGAGTCTTCGGATCATTTGCGCTTC	45	65 °C	72 °C/15 s
		Cma_FADS2_5RACE_R	GCAGAGGAGGACCAATCAGGAAGAA			
	Full ORF	Cma_FADS2_ORF_F	TCATCAGAGAGAGCAGCGAG	35	59 °C	72 °C/24 s
		CmA_FADS2_ORF_R	CCAGCATAGATGGCAGAGGA			
	Restriction site	Cma_FADS2_Pyes_KPNI_F	CCCGGTACCATAATGGGTGGGGGCACTCAT	35	68 °C	72 °C/21 s
		Cma_FADS2_Pyes_XBAL_R	CCCTCTAGATTATTTGTGGAGGTATGCGTCC			
ELOVL5	Degenerate primers	ELOVEL5_degen_F	TGAACGTCCTGTgtggtaytayt	45	52 °C	72 °C/5 s
		ELOVEL5_degen_R	GCTGCACCTGGGTGATGTACykyttccacca			
	Gene specific race	Cma_ELOVEL5_3RACE_F	TGGACACGTTCTTCTTCATCCTGCG	20	65 °C	72 °C/8 s
	nested	Cma_ELOVEL5_3RACE_F2	GTCCTGTGCTGTAGTCTGGCCCTGC	25	65 °C	72 °C/6 s
		Cma_ELOVEL5_5RC_R	CGCAGGATGAAGAAGAACGTGTCCA	45	65 °C	72 °C/5 s
	Restriction site	Cma_ELOVEL5_Pyes_KPNI_F	CCCGGTACCAAGATGGAGGCCTTTAATCACA	35	58 °C	72 °C/15 s
		Cma_ELOVEL5_Pyes_XBAI_R	CCCTCTAGATCAATCTGCCCGCGGCTT			
ELOVL2	Degenerate primers	ELOVL2_degen_F	TACTTGGGACCAAAGTACATGA	45	60 °C	72 °C/7 s
		ELOVL2_degen_R	AGATAGCGTTTCCACCACAG			
	Gene specific race	Cma_ELOVL2_3RACE_F	ACCACGCCTCCATGTTCAATATCTGGTG	45	72 °C	72 °C/7 s
		Cma_ELOVL2_5RACE_R	CCCGCTGACCAGGTTGCTGAAATTA	45	69 °C	73 °C/7 s
	Full ORF	Cma_ELOVL2_ORF_F	GAGAGGCGCGGCGAGGAAACAAC	35	68°C	73 °C/19s
		Cma_ELOVL2_ORF_R	GGCTGTGGTTGTGCATATGTCTCAG			
	Restriction site	Cma_ELOVL2_Pyes_KPNI_F	CCCGGTACCACCATGGAGCTCTTTAGCATGAA	35	62°C	73 °C/14 s
		Cma_ELOVL2_Pyes_XBAL_R	CCCTCTAGATTACTGTAGCTTATGTTTGGCTCC			

Supplementary Table 2. Accession numbers of all sequences used in phylogenetic analysis of Fads amino acid sequences.

	Accession numbers
Fads 1	
Homo sapiens	NP_037534.3
Sus scrofa	NP_001106512.1
Mus musculus	AAH26848.1
Rattus norvegicus	NP_445897.2
Monodelphis domestica	H9H609
Ornithorhynchus anatinus	XP_016084018.1
Latimeria chalumnae	XP_005988035.1
Scyliorhinus canicula	AEY94454.1
Callorhinchus milii	XP_007885635.1
Xenopus tropicalis	XP_002943012.2
Xenopus laevis	XP_018112981.1
Xenopus laevis	XP_018115603.1
Anolis carolinensis a	XP_003224167.1
Anolis carolinensis c	XP_003224188.1
Anolis carolinensis d	XP_003224187.1
Gallus gallus a	XP_421052.4
Gallus gallus b	XP_426408.2
Gallus gallus c	XP_421051.3
Alligator mississippiensis	XP_006274989.1
Fads 2	
Homo sapiens	AAG23121.1
Mus musculus	NP_062673.1
Rattus norvegicus	NP_112634.1
Sus scrofa	NP_001165221.1
Anolis carolinensis	XP_003224168.1
Gallus gallus	NP_001153900.1
Alligator mississippiensis	XP_006274951.1
Xenopus tropicalis	NP_001120262.1
Salmo salar (Fads2Δ6 _c)	NP_001165752.1
Salmo salar (Fads2Δ6 _b)	NP_001165251.1
Salmo salar (Fads2Δ6 _a)	NP_001117047.1
Salmo salar (Fads2Δ5)	NP_001117014.1
Oncorhynchus masou	ABU87822.1
Oncorhynchus mykiss (Fads2Δ6)	NP_001117759.1
Oncorhynchus mykiss (Fads2Δ5)	AFM77867.1
Gadus morhua (Fads2Δ6)	AAY46796.1
Scatophagus argus (Fads2Δ6)	AHA62794.1
Oreochromis niloticus (Fads2Δ6)	AGV52807.1
Dicentrarchus labrax (Fads2Δ6)	ACD10793.1

Argyrosomus regius (Fads2Δ6/ Δ8)	AGG69480.1
Sparus aurata (Fads2Δ6)	AAL17639.1
Thunnus maccoyii (Fads2Δ6)	ADG62353.1
Scophthalmus maximus (Fads2Δ6)	AAS49163.1
Solea senegalensis (Fads2Δ6)	AEQ92868.1
Siganus canaliculatus (Fads2Δ4)	ADJ29913.1
Lates calcarifer (Fads2Δ6)	ACS91458.1
Rachycentron canadum (Fads2Δ6)	ACJ65149.1
Anguilla japonica (Fads2Δ6/ Δ8)	AHY22375.1
Arapaima gigas (Fads2Δ6/ Δ8)	AOO19789.1
Scleropages formosus	XP_018585703.1
Danio rerio (Fads2Δ5/ Δ6)	NP_571720.2
Colossoma macropomum	MH734335
Astyanax mexicanus	XP_007235183.1
Ictalurus punctatus	XP_017341187.1
Clarias gariepinus (Fads2Δ5/ Δ6)	AMR43366.1
Latimeria chalumnae	XP_005988034.2
Scyliorhinus canicula	AEY94455.1
Callorhinchus milii	XP_007885636.1
invertebrates	Fads
Saccoglossus kowalevskii	XP_006822674.1
Octopus vulgaris	AEK20864.1

Supplementary Table 3. Accession numbers of all sequences used in phylogenetic analysis of Elovl amino acid sequences.

Accession numbers Elovl	
Elovl2	
Homo sapiens	NP_060240.3
Bos taurus	NP_001076986.1
Mus musculus	NP_062296.1
Rattus norvegicus	NP_001102588.1
Gallus gallus	NP_001184237.1
Xenopus tropicalis	NP_001016159.1
Xenopus laevis	NP_001087564.1
Callorhinchus milii	XP_007900820.1
Latimeria chalumnae	XP_006006450.1
Esox lucius	XP_010884057.1
Salmo salar	NP_001130025.1
Oncorhynchus mykiss	AIT56593.1
Clupea harengus	XP_012671565.1
Danio rerio	NP_001035452.1
Colossoma macropomum	MH734337
Astyanax mexicanus	XP_007260136.1
Clarias gariepinus	AOY10780.1
Elovl5	
Homo sapiens	NP_068586.1
Mus musculus	NP_599016.2
Bos taurus	NP_001040062.1
Gallus gallus	NP_001186126.1
Xenopus laevis	NP_001089883.1
Sparus aurata	AAT81404.1
Dicentrarchus labrax	CBX53576.1
Rachycentron canadum	ACJ65150.1
Salmo salar (Elovl5b)	NP_001130024.1
Salmo salar (Elovl5a)	NP_001117039.1
Larimichthys crocea	AFB81415.1
Nibea mitsukurii	ACR47973.1
Argyrosomus regius	AGG69479.1
Siganus canaliculatus	ADE34561.1
Clupea harengus	XP_012695835.1
Clarias gariepinus	AAT81405.1
Ictalurus punctatus	NP_001188041.1
Pangasius larnaudii	AGR45586.1
Danio rerio	NP_956747.1
Colossoma macropomum	MH734336
Branchiostoma lanceolatum	ALZ50284.1

	Elovl4
Homo sapiens	NP_073563.1
Mus musculus	NP_683743.2
Bos taurus	NP_001092520.1
Gallus gallus	NP_001184238.1
Danio rerio a	NP_957090.1
Danio rerio b	NP_956266.1
Takifugu rubripes a	XP_003966009.1
Takifugu rubripes b	XP_003971605.1
Salmo salar	NP_001182481.1
Rachycentron canadum	ADG59898.1
Ciona intestinalis	NP_0010290

Supplementary Figure 1. Sequence alignment and conservation analysis of destaturase sequence motifs of *C. macropomum* Fads2. *- indicates residues involved in the determination of the destaturation activity in mammalian desaturases (Watanabe et al., 2016). Red boxes highlight conserved residue replacements with *Danio rerio* Δ5Δ6 Fads2.