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Title

Biosynthesis of long-chain polyunsaturated fatty acids in the African catfish *Clarias gariepinus*: Molecular cloning and functional characterisation of fatty acyl desaturase (*fads2*) and elongase (*elovl2*) cDNAs

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Abstract

Fish differ in their capacity for endogenous synthesis of long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA) from dietary C_{18} precursors (α-linolenic and linoleic acids). Understanding this capacity is of benefit to fish feed formulation. This, together with the importance of fish as the primary source of omega-3 LC-PUFA in the human diet has necessitated the rigorous study of the biochemical and molecular mechanisms involved in the LC-PUFA biosynthesis pathway in fish species. Studies have shown the potential of a species for LC-PUFA biosynthesis is associated with the complement and function of fatty acyl desaturase (*fads*) and elongase of very long chain fatty acid (*elovl*) gene it possesses. The present study therefore aimed to investigate these genes in the African catfish (*Clarias gariepinus*), the most commercially important farmed fish species in Sub-Saharan Africa. A *fads2* and an *elovl2* cDNA were cloned containing open reading frames (ORF) of 1338 base pair (bp) and 864 bp specifying proteins of 445 and 287 amino acids, respectively. Functional characterisation by heterologous expression in yeast showed that the Fads2 was bifunctional with Δ5Δ6 activities catalysing the desaturation of both 18:3n-3 and 20:4n-3 and their corresponding n-6 fatty acids, 18:2n-6 and 20:3n-6. The Elovl2 showed activity towards C_{18}, C_{20} and C_{22} PUFA with highest activity towards C_{20} and C_{22} PUFA. Tissue expression analysis showed a typical freshwater species expression pattern; higher expression in the liver compared to brain and all other tissues with the exception of *elovl5* which showed highest expression in the intestine. Consistent with feeding studies of typical freshwater fish species that show their essential fatty acid requirement can be satisfied by dietary C_{18} PUFA, the present study confirms that the LC-PUFA biosynthesis pathway is active in the African catfish *C. gariepinus*.

Keywords

Biosynthesis; *Clarias gariepinus*; elongase; essential fatty acids; desaturase; long-chain polyunsaturated fatty acids

Introduction

Fish, like all vertebrates, are dependent on dietary sources of polyunsaturated fatty acids (PUFA) such as α-linolenic (ALA, 18:3n-3) and linoleic (LA, 18:2n-6) acids as they lack the Δ12 and Δ15 desaturases required for the synthesis of LA and ALA from oleic acid (18:1n-9) (Tocher, 2010, 2015; Tocher and Glencross, 2015). However, whereas the C_{18} PUFA, ALA and LA, can satisfy essential fatty acid (EFA) requirements of some fish species, long-chain (C_{20-24}) polyunsaturated fatty acids (LC-PUFA) including eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6), which play physiologically important roles, are required in the diet to meet the EFA requirements of other species. This reflects the differing ability of fish species to endogenously synthesise LC-PUFA from C_{18} precursors, associated with the complement of fatty acyl desaturases (Fads) and elongation of very long-chain fatty acids (Elovl) enzymes they possess (Bell and Tocher, 2009; Castro et al., 2016; Tocher, 2010). This has important implications with regards to feed formulation for fish farming. Species with active and complete biosynthetic pathways can convert C_{18} PUFA contained in vegetable oils (VO) that are now common ingredients in aquafeeds, to LC-PUFA, and thus are less dependent on the inclusion of fish oil (FO) to supply LC-PUFA in their diets.

The LC-PUFA biosynthesis pathways involves successive desaturation and elongation of the C_{18} precursors catalysed by Fads and Elovl elongases (Castro et al., 2016; Monroig et al., 2011a; Tocher, 2003; Vagner and Santigosa, 2011). Fads enzymes introduce double bonds (unsaturations) at specific positions of the fatty acyl chain (Nakamura and Nara, 2004). It has been shown that all *fads* so far studied in teleost genomes are paralogues of *fads2*, a gene encoding an enzyme that typically acts as Δ6 Fads in vertebrates, while *fads1*, another vertebrate *fads* encoding an enzyme with Δ5 activity, appears to be absent in teleosts (Castro et al., 2012, 2016). While most fish Fads2 enzymes functionally characterised are typically

75 Δ6, others have been characterised as bifunctional Δ6Δ5 Fads2 (Fonseca-Madrigal et al.,
76 2014; Hastings et al., 2001; Li et al., 2010; Tanomman et al., 2013) or monofunctional Δ5
77 Fads (Abdul Hamid et al., 2016; Hastings et al., 2005). In recent years, Fads2 with Δ4
78 activities have been found in a variety of teleost species (Fonseca-Madrigal et al., 2014; Kuah
79 et al., 2015; Li et al., 2010; Morais et al., 2012). Furthermore, fish Fads2, as described in
80 mammals (Park et al., 2009), also display Δ8 activity, an activity that appeared to be
81 relatively higher in marine fish compared to freshwater fish species (Monroig et al., 2011a).

82 Elovl enzymes catalyse the condensation step in the elongation pathway resulting in the
83 addition of a two-carbon unit to the pre-existing fatty acid (Guillou et al., 2010). Functional
84 characterisation of fish Elovl2, Elovl4 and Elovl5, elongase enzymes that function in the LC-
85 PUFA biosynthesis pathway, show that they display somewhat overlapping activities (Castro
86 et al., 2016). Thus Elovl5 generally elongate C_{18} and C_{20} PUFA, whereas Elovl2 and Elovl4
87 are more efficient towards C_{20} and C_{22} PUFA (Gregory and James, 2014; Monroig et al.,
88 2011a, 2011b, 2009; Morais et al., 2009). While both *elovl5* and *elovl4* genes are present in
89 teleost genomes (Monroig et al., 2016), *elovl2* appears to be lost in Acanthopterygii, a
90 phylogenic group that, with the exception of salmonids, includes the vast majority of the most
91 important farmed fish species (Castro et al., 2016). To the best of our knowledge, Elovl2
92 cDNAs have been characterised only in Atlantic salmon (*Salmo salar*) (Morais et al., 2009),
93 zebrafish (*Danio rerio*) (Monroig et al., 2009) and rainbow trout (*Oncorhynchus mykiss*)
94 (Gregory and James, 2014).

95 Evidence indicates that the complement and functionalities of *fads* and *elovl* genes existing
96 in any teleost species has been shaped by evolutionary drivers leading to the retention,
97 subfunctionalisation or loss of these genes (Castro et al., 2016). Moreover, the habitat
98 (marine *vs* freshwater), and specifically the availability of LC-PUFA in food webs, has also
99 been implicated as influencing the LC-PUFA biosynthetic capability of fish (Bell and Tocher,

100 2009; Castro et al., 2016; Monroig et al., 2011b). Freshwater fish, having evolved on diets
101 low in LC-PUFA, are believed to have all the genes and/or enzymatic functionalities required
102 for endogenous LC-PUFA production (NRC, 2011; Tocher, 2015). Whereas, many marine
103 species have not retained all the genes and/or enzymatic functionalities required for
104 endogenous LC-PUFA production as a consequence of LC-PUFA being readily available in
105 their natural diets (NRC, 2011; Tocher, 2015). However, such dichotomy has been recently
106 seen as too simplistic and other factors including trophic level (Li et al., 2010) and trophic
107 ecology (Morais et al., 2015, 2012) also appear to influence LC-PUFA biosynthesis capacity
108 of fish species. Within an aquaculture nutrition context, investigations of the *fads* and *elovl*
109 gene repertoire involved in LC-PUFA biosynthesis, as well as the functions of the enzyme
110 they encode, are necessary to ascertain whether the EFA requirements of a species can be
111 satisfied by C_{18} PUFA or whether dietary LC-PUFA are required.

112 The African catfish *Clarias gariepinus*, a freshwater species belonging to the family
113 Clariidae and order Siluriformes, is the most important aquaculture species in Sub-Saharan
114 Africa (FAO, 2012). Yet, neither its LC-PUFA biosynthetic pathway nor EFA requirement is
115 fully understood. Studies on *C. gariepinus* and other African catfishes suggest they can
116 effectively utilise C_{18} PUFA contained in VO to cover their physiologically important LC-
117 PUFA requirements (Baker and Davies, 1996; Sotolu, 2010; Szabo et al., 2009). Intriguingly,
118 lower growth performance has been reported for *C. gariepinus* fed diets with FO compared to
119 those fed diets containing VO (Hoffman and Prinsloo, 1995; Ng et al., 2003) in contrast to
120 most fish species including those with full LC-PUFA biosynthetic capability like salmonids
121 (Sargent et al., 2002; Tocher and Glencross, 2015).

122 The aim of this study was to investigate the functions of the genes encoding putative Fads
123 and Elovl enzymes that account for the LC-PUFA biosynthetic capability of *C. gariepinus*
124 and thus understand the potential of this species to utilise diets containing VO and low

contents of LC-PUFA. Here, we report the cloning and functional characterisation of *fads2* and *elovl2* genes from *C. gariepinus*. We further investigated the mRNA tissue distribution of the newly cloned genes, as well as that of the previously cloned *elovl5* (Agaba et al., 2005).

Materials and Methods

Sample collection and RNA preparation

Tissue samples were obtained from adult *C. gariepinus* (~1.8 kg) raised in the tropical aquarium of the Institute of Aquaculture, University of Stirling, UK, on standard salmonid diets. Eight *C. gariepinus* individuals were sacrificed with an overdose of benzocaine before the collection of tissue samples including liver, intestine, pituitary, testis, ovary, skin, muscle, gills, kidney, and brain. The samples were immediately preserved in an RNA stabilisation buffer (3.6 M ammonium sulphate, 18 mM sodium citrate, 15 mM EDTA, pH 5.2) and stored at -80 °C prior to extraction of total RNA following homogenisation in TRI Reagent® (Sigma-Aldrich, USA). Purity and concentration of total RNA was assessed using the NanoDrop® (Labtech International ND-1000 spectrophotometer) and integrity was assessed on an agarose gel. First strand complementary DNA (cDNA) was synthesised using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, USA) following the manufacturer's instructions.

Molecular cloning of fads2 and elovl2 cDNAs

Amplification of partial fragments of the genes was achieved by polymerase chain reaction (PCR) using a mixture of cDNA from eye, liver, intestine and brain as template and primers FadCGF2F1 and FadCGF2R1 for *fads2*, and EloCGE2F1 and EloCGE2R1 for *elovl2* (Table 1). For clarity, it should be noted that the standard gene/protein nomenclature has been used in this study (Castro et al., 2016). Following conventions accepted for zebrafish (*Danio*

149 *rerio*), proteins are termed with regular fonts (e.g. Fads2) whereas genes are italicised (e.g.
150 *fads2*). Primers used for amplification of the first fragment of target genes were designed on
151 conserved regions of fish orthologues of *fads2* and *elovl2* according to the following strategy.
152 For *fads2*, sequences from the broadhead catfish (*Clarias microcephalus*) (gb|KF006248.1|),
153 spot pangasius (*Pangasius larnaudii*) (gb|KC994461.1|), striped catfish (*Pangasianodon*
154 *hypophthalamus*) (gb|JX035811.1|) and *Clarias* hybrid (*C. macrocephalus* and *C. gariepinus*)
155 (gb|KC994463.1|) were aligned with the ClustalW tool (BioEdit v7.0.9, Tom Hall,
156 Department of Microbiology, North Carolina State University, USA) for degenerate primer
157 design. For *elovl2*, homologous sequences from *D. rerio* (gb|NM_001040362.1|), *S. salar*
158 (gb|NM_001136553.1|) and Mexican tetra (*Astyanax mexicanus*) (gb|XM_007260074.2|)
159 were retrieved from NCBI (http://ncbi.nlm.nih.gov), aligned (BioEdit) and conserved regions
160 used to retrieve expressed sequence tags (ESTs) from catfish species. Six Channel catfish
161 (*Ictalurus punctatus*) ESTs (GenBank accession numbers GH651976.1, GH651977.1,
162 FD328544.1, FD284236.1, FD284235.1 and BM438219.1) were obtained and aligned with
163 BioEdit. Subsequently, the consensus *elovl2*-like sequences from *I. punctatus*, and those from
164 *D. rerio*, *S. salar* and *A. mexicanus*, were aligned for degenerate primer design. PCR
165 conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by 33 cycles
166 of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min 30
167 s, followed by a final extension at 72 °C for 7 min. The PCR fragments were purified using
168 the Illustra GFX PCR DNA/gel band purification kit (GE Healthcare, Little Chalfont, UK),
169 and sequenced (GATC Biotech Ltd., Konstanz, Germany). The primers used in this study and
170 their sequences are presented in Table 1.

171 In order to obtain full-length cDNA sequences, Rapid Amplification of cDNA Ends
172 (RACE) was performed with the FirstChoice® RLM-RACE RNA ligase mediated RACE kit
173 (Ambion®, Life Technologies™, USA). The 5' RACE outer primer and gene-specific primer

174 FadCGRF2R3 were used in a PCR using the 5' RACE cDNA as template (first round PCR)
175 for *fads2*. The resulting PCR product was then used as template for the second round PCR
176 with the 5' RACE inner primer and the gene-specific primer FadCGRF2R2. A similar
177 approach was followed to perform 3' RACE PCR, with primers FadCGRF2F1 and
178 FadCGRF2F2 used for first and second round PCR, respectively. For *elovl2*, the primers
179 CGRE2R3 and CGRE2R2 were designed and used for first and second round PCR,
180 respectively, for the 5' RACE PCR, while CGRE2F1 and CGRE2F2 were used for first and
181 second round PCR, respectively, for the 3' RACE PCR. The first fragments, 5' and 3' RACE
182 PCR fragments were then cloned into PCR 2.1 vector (TA cloning® kit, Invitrogen, Life
183 Technologies™, USA) and sequenced as above. The full nucleotide sequences of the *fads2*
184 and *elovl2* cDNAs were obtained by aligning sequences of the first fragments, together with
185 those of the 5' and 3' RACE PCR positive products (BioEdit).
186
187 *Sequence and phylogenetic analysis*
188 The deduced amino acid (aa) sequences of the *C. gariepinus fads2* and *elovl2* cDNAs were
189 compared to corresponding orthologues from other vertebrate species and sequence identity
190 scores were calculated using the EMBOSS Needle Pairwise Sequence Alignment tool
191 (http://www.ebi.ac.uk/Tools/psa/emboss_needle/). Phylogenetic analysis of the deduced aa
192 sequences of *fads2* and *elovl2* cDNAs from *C. gariepinus* and those from a variety of species
193 across vertebrate lineages were carried out by constructing trees using the neighbour-joining
194 method (Saitou and Nei, 1987), with the MEGA 4.0 software
195 (www.megasoftware.net/mega4/mega.html). Confidence in the resulting tree branch topology
196 was measured by bootstrapping through 1,000 iterations.
197

Functional characterisation of C. gariepinus fads2 and elovl2 by heterologous expression in Saccharomyces cerevisiae

PCR fragments corresponding to the open reading frame (ORF) of *C. gariepinus fads2* and *elovl2* were amplified from a mixture of cDNA synthesised from liver, intestine, eye and brain total RNA, using the high fidelity *Pfu* DNA polymerase (Promega, USA) with primers containing *Bam*HI (forward) and *Xho*I (reverse) restriction sites (Table 1). PCR conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 3 min 30 s followed by a final extension at 72 °C for 7 min. The DNA fragments obtained were purified as above, digested with the appropriate restriction enzymes, and ligated into similarly digested pYES2 yeast expression vector (Invitrogen).

Yeast competent cells InvSc1 (Invitrogen) were transformed with the plasmid constructs pYES2-*fads2* (desaturase) or pYES-*elovl2* (elongase) or with empty vector (control) using the S.c. EasyComp^{TM} Transformation Kit (Invitrogen). Selection of yeast containing the pYES2 constructs was performed on *S. cerevisiae* minimal medium minus uracil (SCMM^{-ura}) plates. One single yeast colony was grown in SCMM^{-ura} broth for 2 days at 30 ^{o}C, and subsequently subcultured in individual Erlenmeyer flasks until an optical density measured at a wavelength of 600nm (OD_{600}) reached 1, after which galactose (2 %, w/v) and a PUFA substrate were added. For both genes, final concentration of substrates were 0.5 mM (C_{18}), 0.75 mM (C_{20}) and 1.0 mM (C_{22}) to compensate for differential uptake related to fatty acyl chain (Zheng et al., 2009). For the *fads2*, Δ6 (18:3n-3 and 18:2n-6), Δ8 (20:3n-3 and 20:2n-6), Δ5 (20:4n-3 and 20:3n-6), and Δ4 (22:5n-3 and 22:4n-6) Fads substrates were used. For *elovl2*, substrates included C_{18}, (18:3n-3, 18:2n-6, 18:4n-3 and 18:3n-6), C_{20} (20:5n-3 and 20:4n-6) and C_{22} (22:5n-3 and 22:4n-6) PUFA. After 2 days, the yeasts were harvested, washed and homogenised in chloroform/methanol (2:1, v/v) containing 0.01 % butylated

hydroxytoluene (BHT) and stored at -20 °C until further use. All FA substrates (> 98-99 % pure) used for the functional characterisation assays, except for stearidonic acid (18:4n-3) and eicosatetraenoic acid (20:4n-3), were obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA). Stearidonic acid (> 99 % pure) and yeast culture reagents including galactose, nitrogen base, raffinose, tergitol NP-40 and uracil dropout medium were obtained from Sigma-Aldrich (UK). Eicosatetraenoic acid was purchased from Cayman Chemical Co. (Ann Arbor, USA).

Fatty acid analysis of yeast

Total lipids extracted according to Folch et al. (1957) from yeast samples were used to prepare fatty acid methyl esters (FAME). FAME extraction, purification and analysis were performed as described by Li et al. (2010). Substrate FA conversion was calculated as the proportion of exogenously added FA substrate desaturated or elongated [all product peak areas / (all product peak areas + substrate peak area)] x 100 (Monroig et al., 2016). GC-MS was used to confirm double bond positions when necessary (Li et al., 2010).

Gene expression analysis

Expression of the newly cloned *fads2* and *elovl2* genes, as well as that of the previously characterised elongase *elovl5* (Agaba et al., 2005), were determined by quantitative real-time PCR (qPCR). Extraction of RNA from tissues and cDNA synthesis were carried out as described above. qPCR amplifications were carried out in duplicate using Biometra Thermocycler (Analytik Jena company, Germany) and Luminaris Color Higreen qPCR master mix (Thermo Scientific, CA, USA) following the manufacturer's instruction. The qPCR was performed in a final volume of 20 µl containing 5 µl diluted (1/20) cDNA, 1 µl (10 µM) of each primer, 3 µl nuclease free water and 10 µl Luminaris Color Higreen qPCR master mix. The qPCR conditions were 50 °C for 2 min, 95 ^{o}C for 10 min followed by 35

cycles of denaturation at 95 °C for 15 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s. After the amplifications, a dissociation curve of 0.5 °C increments from 60 to 90 °C was performed, enabling confirmation of a single product in each reaction. Negative controls (no template control, NTC) containing no cDNA were systematically run. Absolute copy number of the target and reference gene in each sample was calculated from the linear standard curve constructed. Normalisation of each target gene was carried out by dividing the absolute copy number of the candidate gene by the absolute copy number of the reference gene 28S rRNA (gb|AF323692.1|). In order to prepare solutions of known copy numbers, DNA concentration linearised PCR 2.1 vectors containing a fragment of either candidate or reference genes was determined, and their molecular weights were estimated as 660 g bp x length (bp) of the plasmid constructs. Primers used for qPCR analysis are also presented in Table 1.

Statistical analysis

Tissue expression (qPCR) results were expressed as mean normalised ratios (±SE) corresponding to the ratio between the copy numbers of the target genes (*fads2*, *elovl2* and *elovl5*) and the copy numbers of the reference gene, 28S rRNA. Differences in gene expression among tissues were analysed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test at a significance level of $P \leq 0.05$ (IBM SPSS Statistics 21).

Results

Sequence and phylogenetic analysis

C. gariepinus Fads2 sequence was deposited in the GenBank database with the accession number KU925904. The full length of the *C. gariepinus* Fads2 was 1812 bp, comprising of a 5′ untranslated region (UTR) of 162 bp, an ORF of 1338 bp encoding a putative protein of 445 aa, and a 3′ UTR of 312 bp. The deduced *C. gariepinus* Fads2 enzyme showed distinctive structural features of fatty acyl desaturases including the three histidine boxes

HDFGH, HFQHH, and QIEHH (aa 181-185, 218-222 and 383-387, respectively) and cytochrome b5-domain (aa 26-77) containing the heme binding motif HPGG (aa 54-57). Pairwise aa sequence comparisons of *C. gariepinus* Fads2 with other Fads2-like proteins showed highest identities with Fads from members of the catfish family such as *C. macrocephalus* (97 %) and *P. hypophthalamus* (91.5 %). Comparisons with bifunctional Δ6Δ5 Fads2 of *D. rerio* (gb|AF309556.1|) and *C. estor* (gb|AHX39207.1|), bifunctional Δ5Δ4 Fads2 of *C. striata* (gb|ACD70298.1|) and *S. canaliculatus* (gb|ADJ29913.1|) and Δ4 Fads2 of *S. senegalensis* (gb|AEQ92868.1|) and *C. estor* (gb|AHX39206.1|) showed identities ranging from 65.2-70.2 %. Lowest identities were observed when *C. gariepinus* Fads was compared to Fads1-like sequences from different vertebrate lineages. Phylogenesis of *C. gariepinus* Fads with Fads from a variety of vertebrate species showed it clustered with all other Fads2 in one group that was separate from the Fads1 group confirming that the newly cloned *fads* was a *fads2* (Fig. 1). The *C. gariepinus* Fads2 clustered most closely with Fads2 from bony fish species (with the exception of the sarcopterygian, *L. chalumnae* which formed a separate cluster with Fads2 from chondrichthyes (*C. milli and S. canicula*), mammalian (*H. sapiens*, *M. musculus* and *B.* t*aurus*) and avian species (*G. gallus*) (Fig. 1).

C. gariepinus Elovl2 sequence was deposited in the GenBank database with the accession number KU902414. The full-length cDNA sequence of *C. gariepinus elovl2* was 1432 bp (5′ UTR 91 bp, ORF 864 bp, 3′ UTR 477 bp) encoding a protein of 287 aa. Analysis of the deduced aa sequence of *C. gariepinus* Elovl2 revealed characteristic features of fatty acyl elongases such as the highly conserved histidine box (HVYHH, aa 151-155) and the carboxyl-terminal region, but the aa residues at the carboxyl terminus were KHKLQ, more similar to the KXRXX found in Elovl5 than to the KKXX in *H. sapiens* and *S. salar* Elovl2 (Morais et al., 2009). Comparisons of *C. gariepinus* Elovl2 with homologues from *A.*

mexicanus (gb|XP_007260136.1|), *S. salar* (gb|ACI62500.1|), *D. rerio* (gb|XP_005162628.1|), *Clupea harengus* (gb|XP_012671565.1|), and *H. sapiens* (gb|NP_060240.3|) showed identities of 81.7, 72.9, 72.7, 69.1 and 64.8 %, respectively. *C. gariepinus* Elovl2 shared 52 % identity with *C. gariepinus* Elovl5. Phylogenetic analysis of the Elovl2 with members of the Elovl family confirmed that the newly cloned elongase was indeed an Elovl2 elongase. Thus, the *C. gariepinus* Elovl2 clustered together with all the Elovl2 and more distantly from Elovl5 sequences including that from *C. gariepinus* (Agaba et al., 2005) and even more distantly to Elovl4 enzymes (Fig. 2).

Functional characterisation of C. gariepinus Fads2 and Elovl2 in S. cerevisiae

Consistent with previous studies (Hastings et al., 2001), control yeast transformed with the empty pYES2 vector did not show any activity towards any of the PUFA substrates assayed (data not shown). Functional characterisation by heterologous expression in yeast revealed that the *C. gariepinus* Fads2 had the ability to introduce double bonds at Δ5, Δ6 and Δ8 positions in the appropriate PUFA substrates (Fig. 3; Table 2). The FA composition of the yeast transformed with pYES2-*fads2* showed peaks corresponding to the four main yeast endogenous FA, namely 16:0, 16:1n-7, 18:0 and 18:1n-9, the exogenously added PUFA and the corresponding PUFA product(s) (Fig. 3; Table 2). Thus, the C_{18} PUFA substrates 18:3n-3 and 18:2n-6 were desaturated to 18:4n-3 (42 % conversion) and 18:3n-6 (23 %), respectively, indicating the encoded protein had Δ6 Fads activity (Fig. 3A; Table 2). Moreover, the transgenic yeast was able to desaturate 20:4n-3 and 20:3n-6 to 20:5n-3 (19 %) and 20:4n-6 (14 %), respectively, indicating the *C. gariepinus* Fads2 also had Δ5 activity (Fig. 3C; Table 2), and thus these results confirm that this Fads2 from *C. gariepinus* is a bifunctional Δ6Δ5 Fads. Additionally, the *C. gariepinus* Fads2 showed Δ8 Fads activity as the yeast transformed with pYES2-*fads2* were able to desaturate 20:3n-3 and 20:2n-6 to 20:4n-3 and 20:3n-6,

respectively (Fig. 3B and Table 2). No additional peaks were observed when yeast expressing the *C. gariepinus fads2* were grown in the presence of 22:5n-3 and 22:4n-6 (Fig. 3D; Table 2).

The *C. gariepinus* Elovl2 showed the ability to elongate C_{18-22} PUFA substrates (Fig. 4; Table 3), with highest conversions towards the C_{20} substrates 20:5n-3 (73.4 %) (Fig. 4B) and 20:4n-6 (56 %). Conversion of the C_{22} substrate was 36.7 % for 22:5n-3 (Fig. 4C) and 9.7 % for 22:4n-6 (Table 3). Elongations of C_{18} PUFAs were generally lower compared to those for C_{20} and C_{22} substrates. Stepwise elongations derived from further activity of the *C. gariepinus* Elovl2 towards products of initial substrate elongation resulted in the production of several polyenes up to 24 carbons (Fig. 4; Table 3).

Tissue expression analysis of C. gariepinus fads2, elovl2 and elovl5

Tissue distribution analysis of *C. gariepinus fads2*, *elovl2* and *elovl5* transcripts confirmed that these genes were expressed in all tissues analysed (Fig. 5). Liver and brain were found to contain the highest transcript levels of the *C. gariepinus fads2*, followed by pituitary, intestine and kidney. Liver, brain and pituitary were also found to contain the highest transcript levels of the *C. gariepinus elovl2*. Generally, gonads including testis and ovary showed the lowest transcript levels for both *fads2* and *elovl2* (Fig. 5). Intestine and liver exhibited the highest level of *elovl5*, while the lowest expression levels were found in muscle.

Discussion

Elucidating the LC-PUFA biosynthesis pathway in farmed fish is crucial for formulating diets that satisfy physiological requirements and thus ensure normal growth and development. These studies are particularly relevant in the current scenario whereby FO are being replaced by VO in aquafeed, the latter naturally devoid of essential LC-PUFA and thus potentially

compromising both health of the fish and nutritional value for human consumers (Monroig et al., 2011b; Tocher and Glencross, 2015). Relevant to the present study, identification and production of fish that can efficiently utilise VO-based diets due to their high capacity for LC-PUFA biosynthesis is a valid strategy to expand aquaculture considering that marine ingredients (FO and fish meal) will be increasingly limited in the future (Tocher, 2015). *C. gariepinus* feed and grow well on a variety of feed ingredients and are, therefore, a good model for studying the endogenous capacity for LC-PUFA synthesis of freshwater fish.

Phylogenetic analysis of the *fads*-like desaturase cDNA isolated from *C. gariepinus,* together with the possession of all the main structural features common to the Fads2 protein family confirmed it to be a Fads2. Sequence and phylogenetic analyses also showed that the *C. gariepinus* Fads2 shared highest aa sequence similarities with other catfish species, with relatively low scores when compared with Fads from more distantly related fish lineages (Betancur-R et al., 2013). Nevertheless, recent advances in functional analyses of fish Fads have concluded that some Fads2 have acquired novel functions (subfunctionalisation) during evolution and thus phylogeny of fish Fads2 does not necessarily correlate with their functionalities (Castro et al., 2016). The herein reported functions of the *C. gariepinus* Fads2 further confirm such a conclusion.

Functional characterisation demonstrated that the *C. gariepinus* Fads2 is a bifunctional Δ6Δ5 desaturase able to operate towards a range of substrates including n-3 (18:3n-3 and 20:4n-3) and n-6 (18:2n-6 and 20:3n-6) PUFA. Similar substrate specificities were previously described in *D. rerio*, which represented the first ever report of dual Δ6Δ5 functionality in a vertebrate Fads (Hastings et al., 2001). More recent studies have now shown that bifunctionality appear to be a more common feature of fish Fads2 than originally thought. Thus dual Δ6Δ5 Fads have been described in *S. canaliculatus* (Li et al., 2010), Nile tilapia (*Oreochromis niloticus*) (Tanomman et al., 2013) and *C. estor* (Fonseca-Madrigal et al.,

374 2014). Interestingly, fish Fads2 with Δ4 capability reported in *S. canaliculatus* (Li et al.,
375 2010), *S. senegalensis* (Morais et al., 2012) and *C. striata* (Kuah et al., 2015) showed as well
376 some minor Δ5 activity and can thus be regarded as dual Δ5Δ4 Fads (Castro et al., 2016). In
377 contrast, other teleost Fads2 are single function Δ6 desaturases (González-Rovira et al., 2009;
378 Mohd-Yusof et al., 2010; Monroig et al., 2010; 2013; Zheng et al., 2009), in agreement with
379 Fads activities reported in mammalian FADS2 (Castro et al., 2016). Such substrate plasticity
380 exhibited amongst fish Fads2 is believed to be the result of a combination of multiple
381 evolutionary drivers including habitat, trophic level and ecology underlying the specific
382 phylogenetic position of each fish species (Castro et al., 2016, 2012; Li et al., 2010; Monroig
383 et al., 2011b). In contrast, Fads1, another "front-end" Fads encoding a Δ5 Fads in mammals
384 (Castro et al., 2012, 2016), appears to have been lost during evolution of teleost and is absent
385 in the vast majority of farmed fish species (Castro et al., 2016).

386 The *C. gariepinus* Fads2 also exhibited Δ8 desaturation capability, an intrinsic feature of
387 vertebrate Fads2 (Monroig et al., 2011a; Park et al., 2009). Although conversions in yeast
388 might quantitatively vary from those occurring *in vivo*, it appeared that the *C. gariepinus*
389 Fads2 had lower efficiency as Δ8 Fads than as Δ6 Fads, in agreement with the "Δ8 pathway"
390 being regarded as a minor pathway compared to the more prominent Δ6 desaturation pathway
391 (Monroig et al., 2011a; Park et al., 2009). Interestingly, the Δ8 desaturation capabilities of *C.*
392 *gariepinus* Fads2 towards 20:3n-3 (12.9 %) was relatively high leading to lower Δ6Δ8 ratio
393 (3.26), a parameter used to compare Δ8 desaturation capability among fish Fads2 enzymes
394 (Monroig et al., 2011a). Thus, the Δ6/Δ8 ratio of *C. gariepinus* Fads2 is more similar to that
395 of marine species like gilthead seabream (*Sparus aurata*) (2.7) and turbot (*Psetta maxima*)
396 (4.2). Whereas it is notably lower than those of freshwater or salmonid Fads2 including *D.*
397 *rerio* (22.4) and *S. salar* (12 and 14.7 for Fad_b and Fad_c, respectively) (Monroig et al.,
398 2011a). These results suggest that the Δ8 pathway, while possibly not to such an extent as the

399 Δ6 pathway, can still contribute to the initial steps of LC-PUFA biosynthesis in *C.*
400 *gariepinus*. Note that Δ8 activity introduces the same double bond as Δ6 activity, after
401 elongation rather than before, and so a Fads having Δ6Δ8 activity is not regarded as
402 "bifunctional".

403 The ability of the *C. gariepinus* Fads2 to desaturate a range of Δ5, Δ6 and Δ8 Fads
404 substrates from both n-3 and n-6 series clearly shows it is a multifunctional enzyme. This is
405 emphasised by the stepwise desaturation reactions that occurred when transgenic yeast
406 expressing the *C. gariepinus* Fads2 were grown in the presence of certain FA substrates such
407 as 20:3n-3 and 20:2n-6. *C. gariepinus* Fads2 enzyme activity toward 20:3n-3 led to the
408 production of either 20:4n-3 (Δ8 desaturation) that was subsequently desaturated to 20:5n-3
409 (Δ5 desaturation), or the non-methylene interrupted (NMI) FA products $^{\Delta5,11,14,17}$20:4 or
410 $^{\Delta6,11,14,17}$20:4 resulting from direct Δ5 or Δ6 desaturation, respectively. While the biological
411 significance of these pathways is difficult to determine, particularly for NMI FA biosynthesis,
412 the results further confirm that all the Fads capabilities (Δ5, Δ6 and Δ8) are present in the
413 characterised Fads2.

414 Moreover, we can further confirm that all the elongase activities required in the LC-PUFA
415 biosynthesis pathways also exist in *C. gariepinus.* Agaba et al. (2005) characterised an Elovl5
416 from *C. gariepinus* that, like the vast majority of fish Elovl5 investigated to date, showed C_{18}
417 and C_{20} PUFA as preferred substrates, with markedly lower affinity towards C_{22} substrates
418 (Castro et al., 2016). In contrast, the *C. gariepinus* Elovl2 showed higher elongation
419 efficiencies towards C_{20} and C_{22} PUFAs compared to C_{18} substrates. Generally, these results
420 are consistent with the activities shown by the only three fish Elovl2 enzymes characterised
421 to date, i.e. *S. salar*, *D. rerio* and *O. mykiss* (Gregory and James, 2014; Monroig et al., 2009;
422 Morais et al., 2009). Although, similar to the human orthologue, the latter did not show any
423 activity on C_{18} FA substrates (Leonard et al., 2002). The presence of Elovl2 and particularly

its ability to elongate C_{22} PUFA to a greater extent compared to Elovl5 elongases has been acknowledged as evidence supporting LC-PUFA biosynthetic capability in freshwater species and salmonids (Morais et al., 2009). The plethora of genomic and transcriptomic sequences currently available from a varied range of fish species and lineages strongly suggests that, rather than the habitat (freshwater versus marine) of fish, it is the phylogeny of each species that actually correlates with the presence or absence of Elovl2 within their genomes. Here we show that marine species such as the Atlantic herring *Clupea harengus* (Fig. 2) possess a putative Elovl2, whereas freshwater species including *O. niloticus* or medaka (*Oryzias latipes*) appear to have lost Elovl2 from their genomes.

The functions of the herein reported Fads2 and Elovl2, together with the previously characterised Elovl5 (Agaba et al., 2005), allow us to predict the biosynthetic pathways of LC-PUFA in *C. gariepinus* (Fig. 6). Thus, the dual Δ6Δ5 Fads2 catalyses the initial desaturation of 18:3n-3 and 18:2n-6 (Δ6 desaturation), as well as the desaturation of 20:4n-3 and 20:3n-6 (Δ5 desaturation). Although we cannot confirm whether the *C. gariepinus* Fads2 can desaturate 24:5n-3 and 24:4n-6 (Δ6 desaturation) required to synthesise 22:6n-3 and 22:5n-6, respectively, through the so-called Sprecher pathway (Sprecher et al., 2000). Such ability of vertebrate Fads2 has been demonstrated in *O. mykiss*, *S. salar* and *D. rerio* (Bell and Tocher, 2009; Buzzi *et al*., 1996; Tocher et al., 2003). Further studies will aim to elucidate whether the newly cloned Fads2 or other Fads potentially co-existing in the *C. gariepinus* genome, have the ability to desaturate C_{24} PUFA in position Δ6. The Elovl2 was able to catalyse the elongation of C_{18} (18:3n-3, 18:2n-6, 18:4n-3 and 18:3n-6), C_{20} (20:5n-3 and 20:4n-6) and C_{22} (22:5n-3 and 22:4n-6) PUFA. Its activity towards C_{18} PUFA was however very low compared to activity towards C_{20} and C_{22} PUFA. This, together with the activity of Elovl5, which is high towards C_{18} and C_{20} PUFA (Agaba et al., 2005), confirm that

448 the activities required to catalyse all the elongation steps required for LC-PUFA synthesis are
449 present in *C. gariepinus.*

450 Expression analysis showed *fads2*, *elovl2* and *elovl5* were expressed in all tissues analysed.
451 Consistent with the vast majority of freshwater species studied, the tissue distribution patterns
452 of *C. gariepinus fads2* and *elovl2* mRNAs showed liver as a major metabolic site for LC-
453 PUFA biosynthesis. In contrast, marine fish species typically have brain as the tissue with
454 highest expression levels of LC-PUFA biosynthesis genes, with production of DHA from
455 EPA in brain being hypothesised as driving the retention of at least part of the LC-PUFA
456 biosynthetic pathway in species with high inputs of dietary LC-PUFA (Monroig et al.,
457 2011b). An exception to this pattern is represented by the Nile tilapia *fads2*, with highest
458 expression in the brain (Tanomman et al., 2013). *C. gariepinus fads2* expression in liver was
459 approximately four-fold greater than in intestine, in contrast to salmonid *fads2* that have been
460 reported to be most highly expressed in intestine (Zheng et al., 2005). The expression of
461 *elovl5* was also high in liver but was highest in the intestine.

462 In conclusion, we have successfully cloned and characterised *fads2* and *elovl2* genes that
463 encode enzymes with a broad range of substrate specificities from *C. gariepinus*. These two
464 enzymes, and the previously reported Elovl5, enable the African catfish *C. gariepinus* to
465 carry out all the desaturation and elongation reactions required for endogenous LC-PUFA
466 synthesis from C_{18} precursors, namely ALA and LA. These results strongly suggest that *C.*
467 *gariepinus* has the ability to effectively utilise VO rich in C_{18} PUFA to satisfy essential LC-
468 PUFA requirements.

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Figures

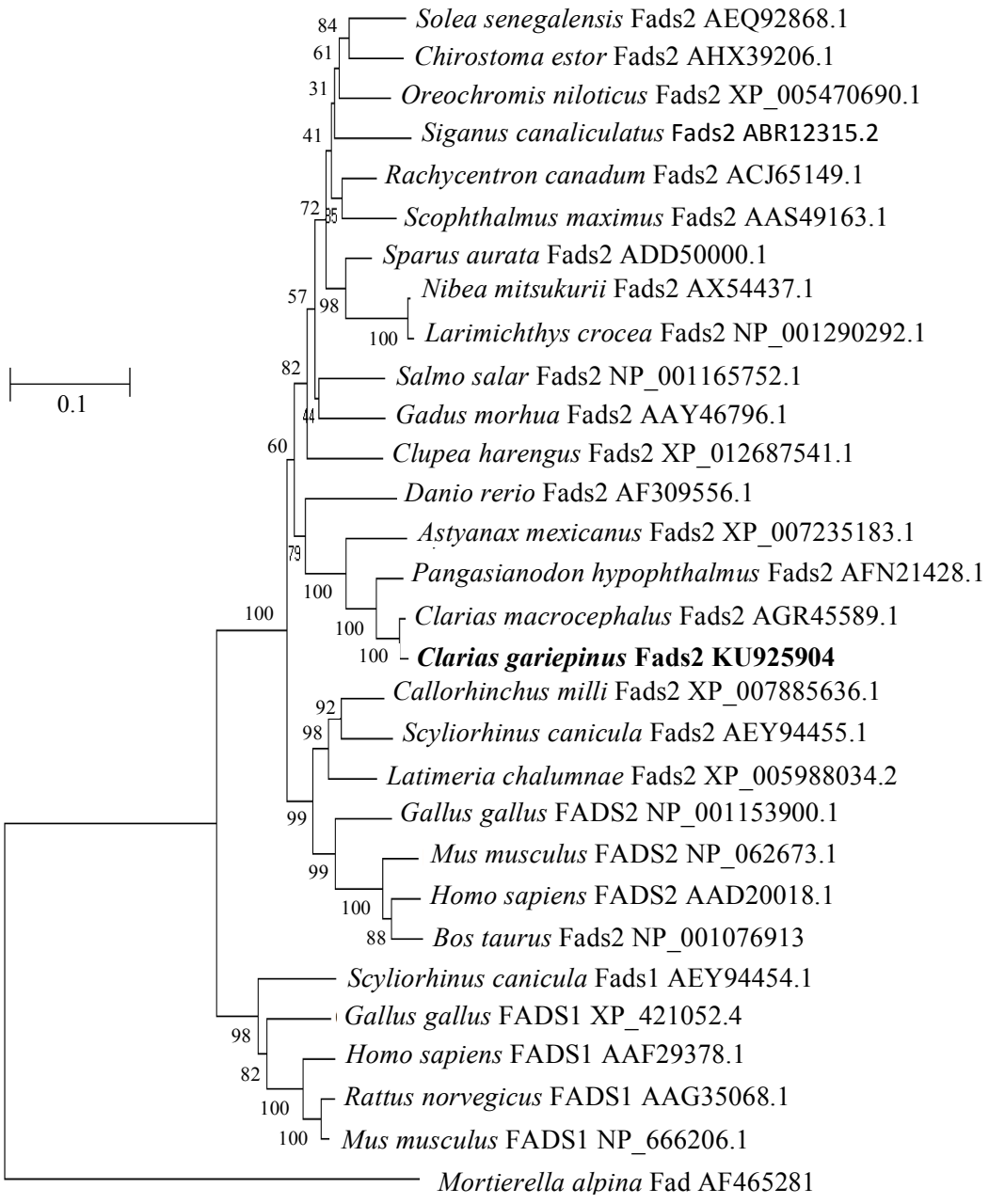


Fig. 1. Phylogenetic tree comparing the deduced amino acid sequence of *Clarias gariepinus fads2* with Fads from a range of vertebrates. The tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) with the MEGA 4.0 software. The numbers represent the frequency (%) with which the tree topology presented was replicated after 10,000 iterations.

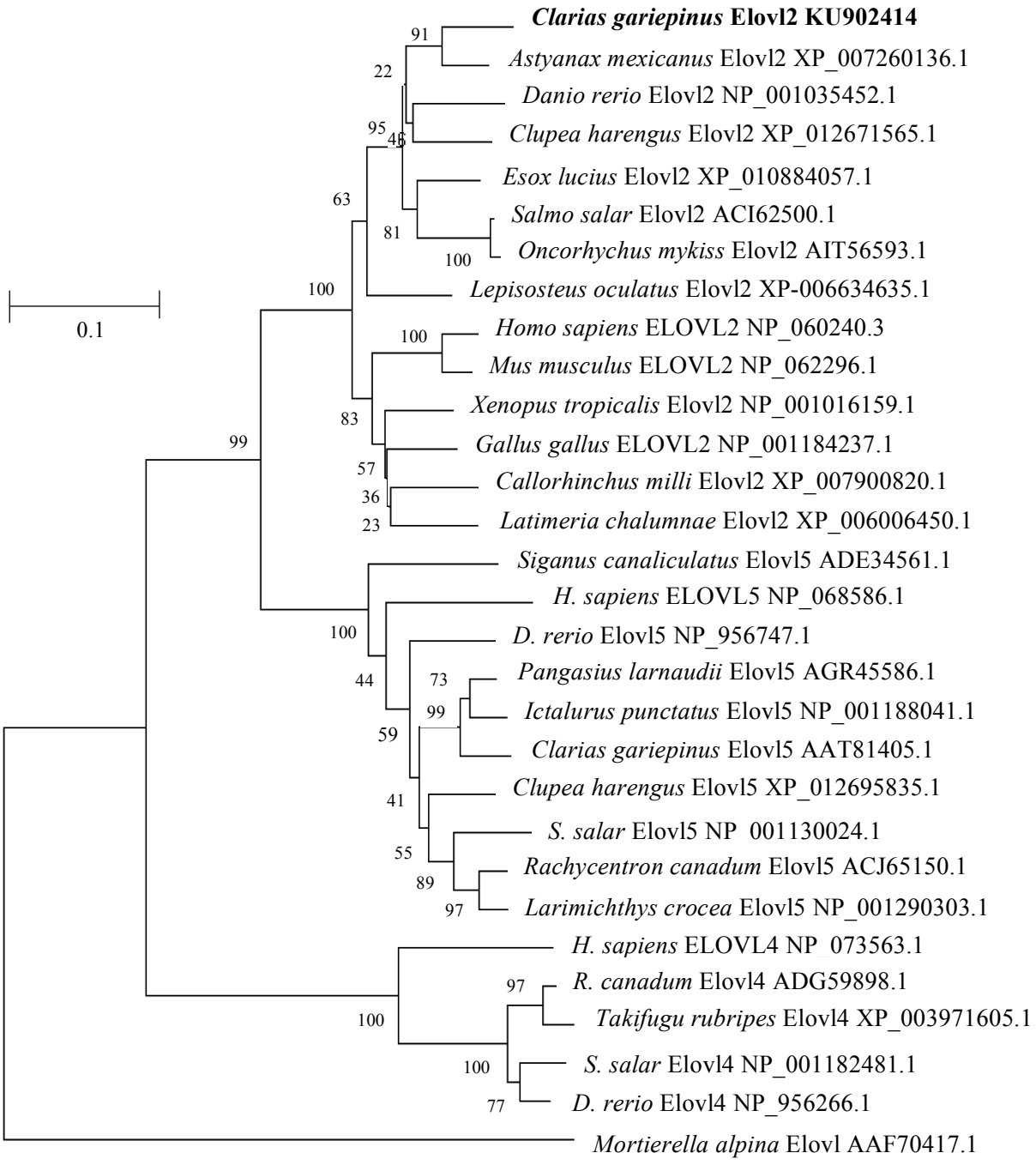


Fig. 2. Phylogenetic tree comparing the deduced amino acid (aa) sequence of *Clarias gariepinus elovl2* with Elovl2, Elovl4 and Elovl5 from a range of vertebrates. The tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) with the MEGA 4.0 software. The numbers represent the frequencies (%) with which the tree topology presented was replicated after 10,000 iterations.

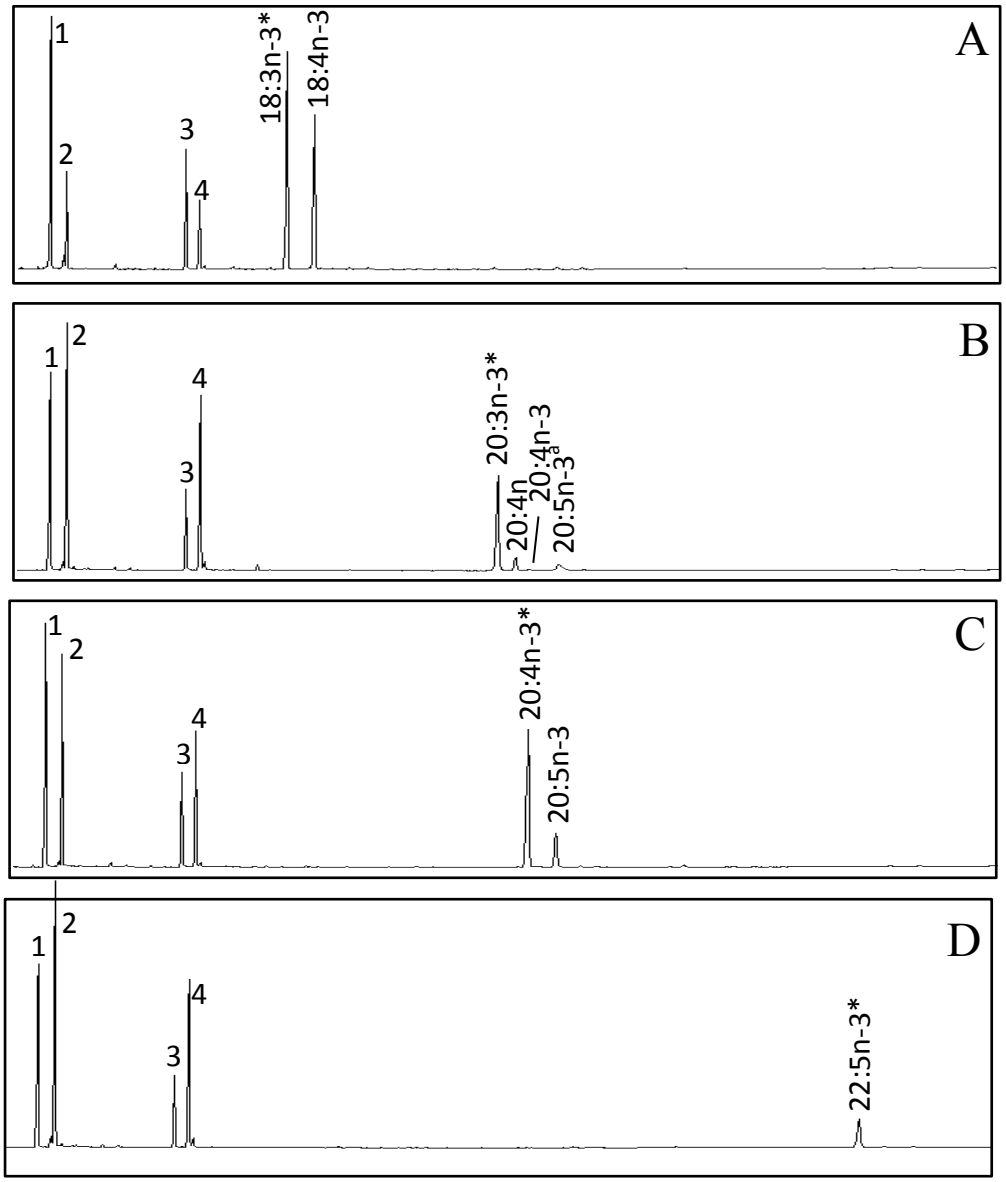


Fig. 3. Functional characterisation of the newly cloned *Clarias gariepinus* Fads2 in yeast (*Saccharomyces cerevisiae*). The fatty acid (FA) profiles of yeast transformed with pYES2 containing the coding sequence of *fads2* were determined after the yeast were grown in the presence of one of the exogenously added substrates 18:3n-3 (A), 20:3n-3 (B), 20:4n-3 (C) and 22:5n-3 (D). Peaks 1-4 represent the *S. cerevisiae* endogenous FA, namely 16:0 (1), 16:1 isomers (2), 18:0 (3) and 18:1n-9 (4). Additionally, peaks derived from exogenously added substrates (*) or desaturation products are indicated accordingly. The peak indicated as "20:4*" is a non-methylene interrupted FA ($^{\Delta 6,11,14,17}$ 20:4 or $^{\Delta 5,11,14,17}$ 20:4) (panel B).

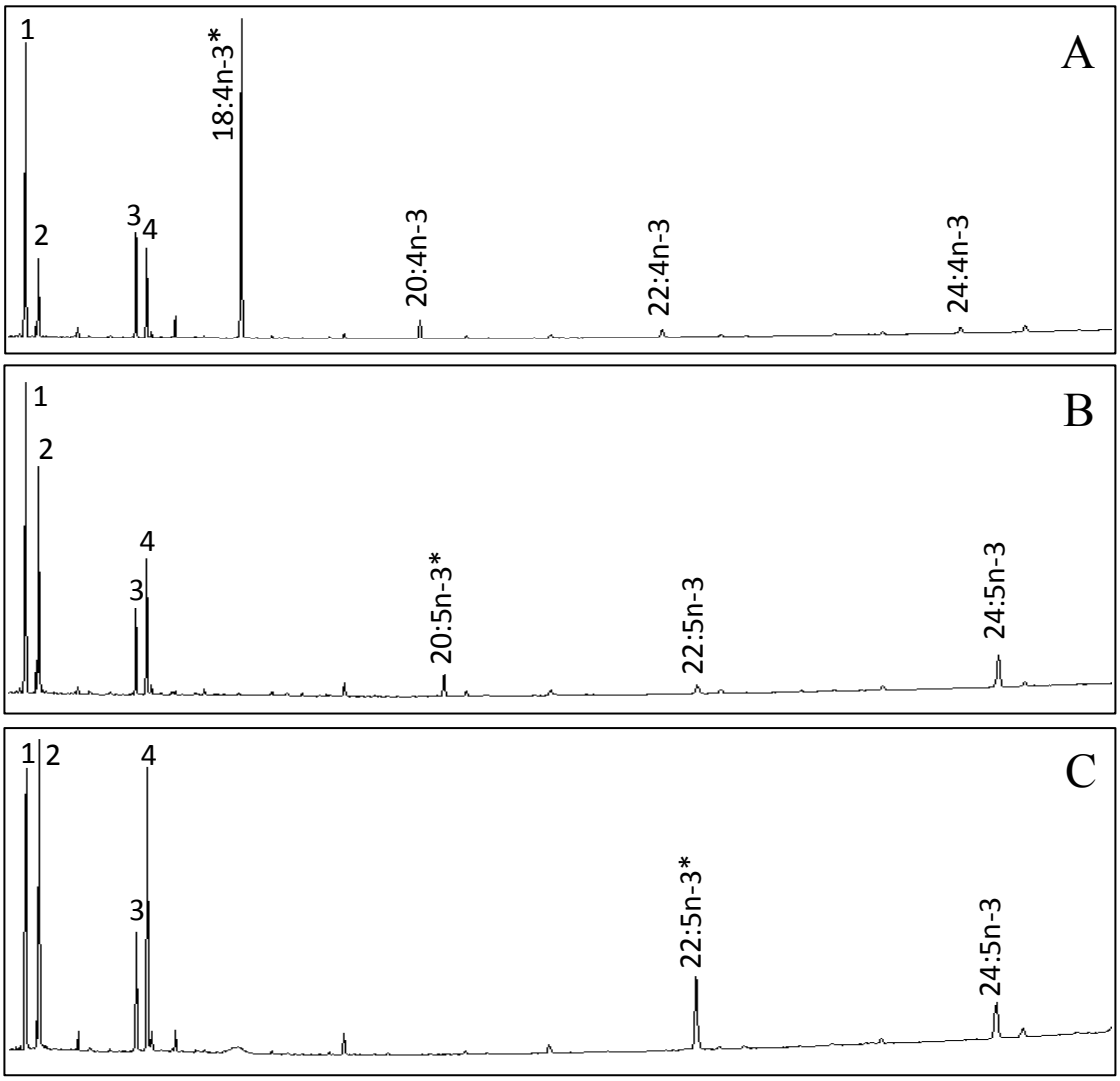
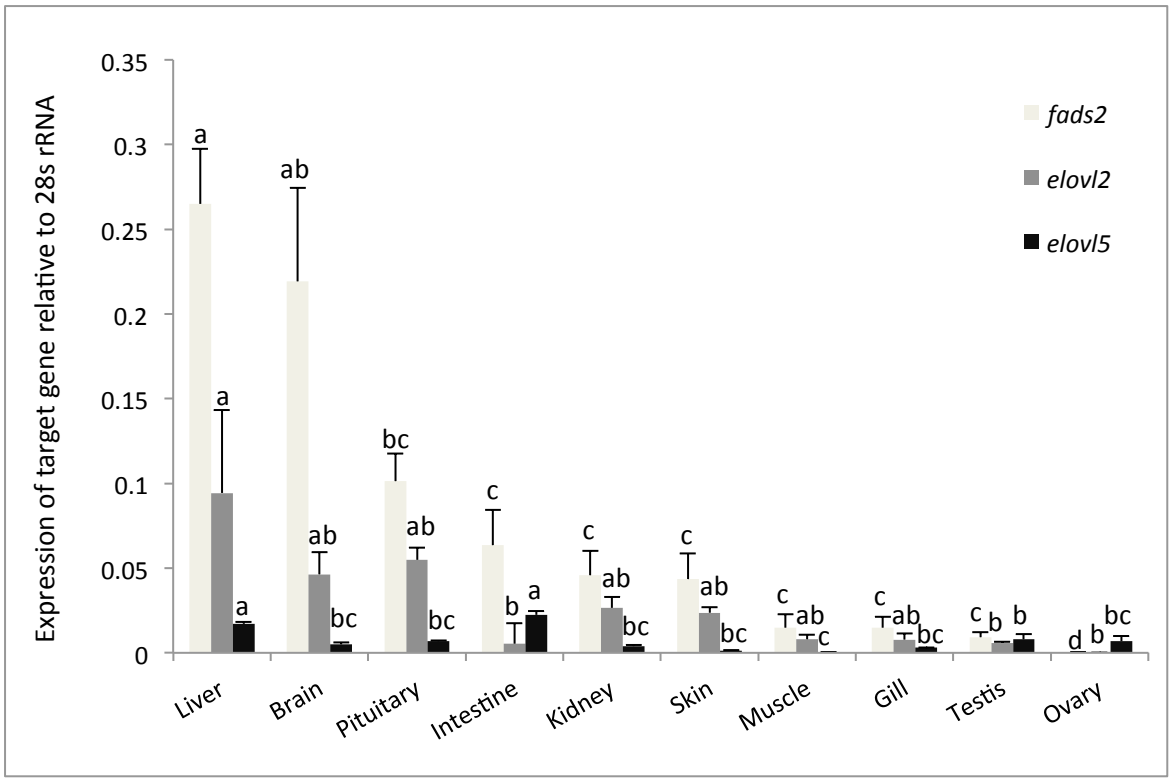


Fig. 4. Functional characterisation of the of the newly cloned *Clarias gariepinus* Elovl2 in yeast (*Saccharomyces cerevisiae*). The fatty acid (FA) profiles of yeast transformed with pYES2 containing the coding sequence of *elovl2* were determined after the yeast were grown in the presence of one of the exogenously added substrates 18:3n-3 (A), 18:4n-3 (B), 20:5n-3 (C) and 22:5n-3 (D). Peaks 1-4 represent *S. cerevisiae* endogenous FAs namely 16:0 (1), 16:1 (2), 18:0 (3) and 18:1n-9 (4). Additionally, peaks derived from exogenously added substrates (*) or elongation products are indicated accordingly.



720 Fig. 5. Tissue distribution of *fads2*, *elovl2* and *elovl5* transcripts in *Clarias gariepinus*.
721 Expression levels quantified for each transcript were normalised expression levels of the
722 reference gene (28s rRNA) of the same tissue. The data are reported as mean values with
723 their standard errors (n = 4). Within each target gene, different letters indicate statistically
724 significant differences between expression levels (ANOVA and Tukey's HSD *post hoc* tests).

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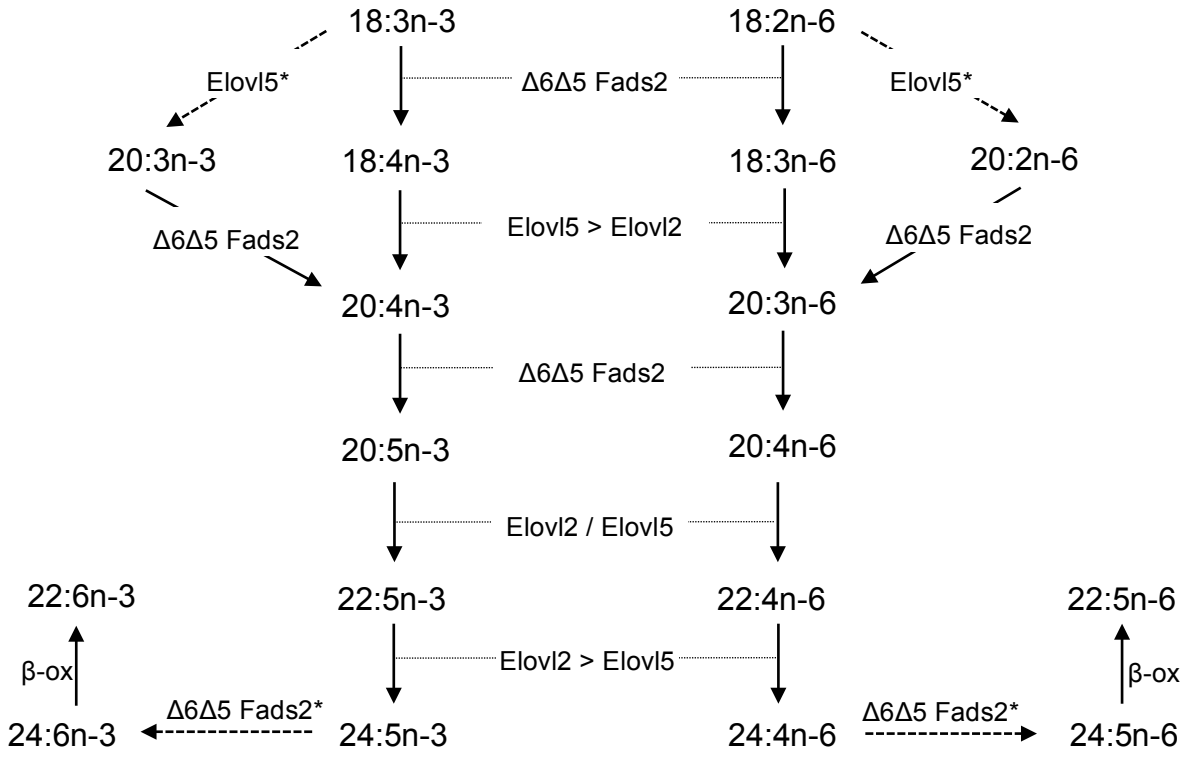

Fig. 6. The biosynthesis pathway of long-chain polyunsaturated fatty acids (≤ C_{24}) from α-linolenic (18:3n-3) and linoleic (18:2n-6) acids in *Clarias gariepinus*. Enzymatic activities shown in the scheme are predicted from heterologous expression in yeast of the herein investigated Δ6Δ5 fatty acyl desaturase 2 (Δ6Δ5 Fads2) and Elovl2 elongase, and the previously reported Elovl5 (Abaga et al., 2005). β-ox, partial β-oxidation; Elovl, fatty acyl elongase; Fads, fatty acyl desaturase.

* Enzymatic activities not yet demonstrated in *C. gariepinus*.

Tables

Table 1

Sequences of primers used for cDNA cloning and tissue expression analysis (qPCR) of *Clarias gariepinus fads2* and *elovl2*. Restriction sites *Bam*HI and *Xho*I are underlined.

Name	Direction	Sequence
Initial cDNA cloning		
FadCGF2F1	Forward	5'-ATGGGCGGCGGAGGACAC-3'
FadCGF2R1	Reverse	5'-GCATCTAGCCACAGCTCACC-3'
EloCGE2F1	Forward	5'-TACTTGGGACCAAAGTACATGA-3'
EloCGE2R1	Reverse	5'-AGATAGCGTTTCCACCACAG-3'
5′ RACE PCR		
FadCGRF2R2	Reverse	5'-CGATCACAACCCACTGATCA-3'
FadCGRF2R3	Reverse	5'-CGTCCTCCAGGATGTCTTTT-3'
EloCGRE2R3	Reverse	5'-AGCTTGCTGAAATAAGCTCCACT-3'
EloCGRE2R2	Reverse	5'-TGTAGAAGGACAGCATGGTGAC-3'
3′ RACE PCR		
FadCGRF2F1	Forward	5'-CAGTCGCCATTCAACGATT-3'
FadCGRF2F2	Forward	5'-GAACACCATCTCTTTCCCATG-3'
EloCGRE2F1	Forward	5'-TTGTCCACCATTCCTTCAATG-3'
EloCGRE2F2	Forward	5'-ACTGAACAGCTTCATCCATGTG-3'
ORF cloning		
FadCGF5UF1	Forward	5'-AGAGGAGCGCAGTGATGAG-3'
FadCGF3UR1	Reverse	5'-GTGGGAATTACAGAATTGTTATGG-3'
FadCGFVF	Forward	5'-CCCGGATCCAAGATGGGCGGCGGAGGAC-3'
FadCGFVR2	Reverse	5'-CCGCTCGAGTTATTTGTGGAGGTATGCATC-3'
EloCGE2VF	Forward	5'-CCCGGATCCAACATGGATTTTATTGTGAAGAA-3'
EloCGE2VR	Reverse	5'-CCGCTCGAGTCACTGCAGCTTATGTTTGGC-3'
EloCGE25UF	Forward	5'-CCAGTTACATTAAGAGGCACCG-3'
EloCGE23UR	Reverse	5'-AGATTAGTCAACATGAAAGGTGAA-3'
Quantitative PCR		
FadCGqF2F1	Forward	5'-TCCTATATGCTGGAACTAATGTGG-3'
FadCGqF2R1	Reverse	5'-AGGATGTAACCAACAGCATGG-3'
EloCGqE2F1	Forward	5'-GCAGTACTCTGGGCATTTGTC-3'
EloCGqE2R1	Reverse	5'-GGGACATTGGCGAAAAAGTA-3'
EloCGqE5F1	Forward	5'-ACTCACAGTGGAGGAGAGC-3'
EloCGqE5R1	Reverse	5'-GGAATGGTGGTAAACGTGCA-3'
28SrRNAF1	Forward	5'-GTCCTTCTGATGGAGGCTCA-3'
28SrRNAR1	Reverse	5'-CGTGCCGGTATTTAGCCTTA-3'

Table 2

Substrate conversions of *Saccharomyces cerevisiae* transformed with *Clarias gariepinus* *fads2* coding region and grown in the presence of exogenously added substrate (18:3n-3, 18:2n-6, 20:3n-3, 20:2n-6, 20:4n-3, 20:3n-6, 22:5n-3 or 22:4n-6). Conversions were calculated according to the formula [individual product peak area / (all products peak areas + substrate peak area)] × 100.

FA substrate	FA Product	Conversion (%)	Activity
18:3n-3	18:4n-3	42.0	Δ6
18:2n-6	18:3n-6	22.5	Δ6
20:3n-3	20:4n-3	12.9 [a]	Δ8
20:2n-6	20:3n-6	2.5 [a]	Δ8
20:4n-3	20:5n-3	18.7	Δ5
20:3n-6	20:4n-6	13.8	Δ5
22:5n-3	22:6n-3	Nd	Δ4
22:4n-6	22:5n-6	Nd	Δ4

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

FA, Fatty acid; Nd, not detected.

766 Table 3

767 Substrate conversions of *Saccharomyces cerevisiae* transformed with *Clarias gariepinus*
768 *elovl2* and grown in the presence of PUFA substrate exogenously added (18:3n-3, 18:2n-6,
769 18:4n-3, 18:3n-6, 20:5n-3, 20:4n-6, 22:5n-3 or 22:4n-6). Conversions were calculated for
770 each stepwise elongation according to the formula [peak areas of first products and longer
771 chain products / (peak areas of all products with longer chain than substrate + substrate peak
772 area)] x 100.

FA substrate	FA Product	Conversion (%)
18:3n-3	20:3n-3	7.5
18:2n-6	20:2n-6	3.0
18:4n-3	20:4n-3	15.2
18:3n-6	20:3n-6	20.5
20:5n-3	22:5n-3	73.4
20:4n-6	22:4n-6	56.0
22:5n-3	24:5n-3	36.7
22:4n-6	24:4n-6	9.7

773 FA, fatty acid

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