Long-Chain Polyunsaturated Fatty Acids in Fish: Recent Advances on Desaturases and Elongases Involved in Their Biosynthesis

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Summary

The pathways of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis have been extensively investigated in farmed fish both for determining the specific dietary essential fatty acids that guarantee optimal growth and development in captivity, and also for maintaining high levels of the healthy n-3 LC-PUFA for human consumers. It is now well established that the capacity for LC-PUFA biosynthesis of a particular species depends upon the complement of the key enzymes required, namely the fatty acyl desaturases (Fad) and elongases of very long-chain fatty acids (Elovl). This paper reviews the recent progress made on the molecular aspects of Fad- and Elovl-encoding genes isolated so far from fish species. More specifically, we discuss the potential implications that Fad and Elovl functions and expression patterns have on the ability of a particular fish species to thrive on sustainable vegetable-based aquafeeds.

Keywords: biosynthesis, desaturase, elongase, essential fatty acid, fish nutrition, long-chain polyunsaturated fatty acids

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Introduction

Lipid and fatty acid nutrition in fish has attracted the interest of researchers over the last two decades such that some aspects of lipid metabolism, in particular, the pathways of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis, are arguably better understood in fish than in other vertebrates including mammals (Bell and Tocher, 2009a). This has in part been driven by the crucial role that fish play in the human food chain as the primary source of n-3 (the so-called "omega-3") LC-PUFA, which have proven health benefits in neural development and a range of human diseases and pathologies such as cardiovascular, inflammatory neurological disorders (Brouwer *et al.*, 2006; Eilander *et al.*, 2007; Torrejon *et al.*, 2007; Calder and Yaqoob, 2009).

With declining fisheries worldwide (Worms *et al.*, 2006), aquaculture produces an ever-increasing proportion of fish/seafood in the human food basket amounting to around 50 % in 2008 (FAO, 2009). Until recently, high n-3 LC-PUFA levels in flesh of farmed fish were obtained by dietary fish oils, paradoxically themselves derived from marine reduction fisheries that are also at their sustainable limit (Tacon and Metian, 2008; Naylor *et al.*, 2009). In consequence, the continued use of fish oil in feeds is not sustainable and will constrain continuing growth of aquaculture activities and so replacement dietary oils are urgently required (Bell and Waagbø, 2008). The main sustainable alternatives presently available are vegetable oils, which are rich in short chain (C_{18}) polyunsaturated fatty acids (PUFA), but devoid of the n-3 C_{20-22} LC-PUFA abundant in fish oil (Turchini *et al.*, 2010). Feeding fish on vegetable oil can have important consequences for the human consumer as it lowers the n-3 LC-PUFA content of the flesh compromising the nutritional value (Bell and Tocher, 2009b: Turchini *et al.*, 2010).

As with all vertebrates, PUFA are essential in the diet of all fish, but requirements vary with species (Tocher, 2010). With some species, the C_{18} PUFA found in vegetable oil such as α-linolenic acd (ALA; 18:3n-3) and linoleic acid (LOA; 18:2n-6) can satisfy essential fatty acid (EFA) requirements but others have a dietary requirement for preformed LC-

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PUFA such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (Tocher, 2010). Therefore, understanding the LC-PUFA biosynthesis pathway in fish has been important for aquaculture, both for determining the specific dietary requirements of a particular species for PUFA to ensure optimal growth and development in captivity, and also for maintaining levels of n-3 LC-PUFA in the flesh as high as possible to ensure that farmed fish continue to be key components of a healthy human diet.

LC-PUFA biosynthetic pathways in fish were originally investigated by analysing the effects that experimental diets with suboptimal levels of potential LC-PUFA metabolic precursors had on fish physiology. More direct evidence was later obtained through the use of biochemical assays with radiolabeled fatty acids substrates (Tocher *et al.*, 2003). The application and expansion of molecular methodologies to aquaculture research has facilitated the rapid advancement of our knowledge of LC-PUFA biosynthesis in fish. Thus, it is now well established that EFA requirements of fish depend upon the ability of the species to biosynthesise LC-PUFA, which itself is dependent upon their complement of the critical enzymes required for endogenous LC-PUFA biosynthesis (Tocher, 2010). Below we aim to review the recent progress made on the molecular aspects of key enzymes that participate in the LC-PUFA pathway, namely the fatty acyl desaturases (Fad) and elongases of very long-chain fatty acids (Elovl). Briefly, desaturases catalyse the introduction of a double bond (or unsaturation) into the acyl chain, whereas elongases, as their name indicates, elongate a pre-existing fatty acyl chain by 2 carbon atoms.

Fatty acid nomenclature

Fatty acids can be simply defined as aliphatic chains containing a carboxylic (-COOH) and a methyl group (-CH_3) at their two ends (Fig. 1). Fatty acids are designated by their chain lengths, degree of unsaturation (number of double bonds) and the position of the double bonds in the aliphatic chain. Thus, 16:0 (palmitic acid or hexadecanoic acid) is a saturated fatty acid with 16 carbons. For unsaturated fatty acids, the position of the double bonds can be designated in two alternative ways. In the n- or ω nomenclature, in general use in fish

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nutrition research, the position of the double bond is counted from the methyl end of the molecule (Fig. 1). Thus, 16:1n-7 (palmitoleic acid) is a C_{16} fatty acid with one double bond (monounsaturated) positioned at the seventh carbon from the methyl group (Fig. 1A). Alternatively, the older Δ nomenclature, traditionally used for characterising Fad enzyme activities involved in the production of PUFA and LC-PUFA, designates the position of double bonds from the carboxyl group, and thus 16:1n-7 can be designated as $16{:}1\Delta^{9}$, which is the Δ9 desaturation product of 16:0 (Fig. 1A). PUFA are those fatty acids containing two or more double bonds. A common example of PUFA is EPA, 20:5n-3 or $20{:}5\Delta^{5,8,11,14,17}$, a fatty acid with five double bonds with the first being at the third carbon from the methyl end group or at carbons 5, 8, 11, 14 and 17 from the carboxylic group carbon in the Δ nomenclature (Fig. 1B). In this paper, we define long-chain PUFA (LC-PUFA) as fatty acids with two or more double bonds and having 20 or more carbons.

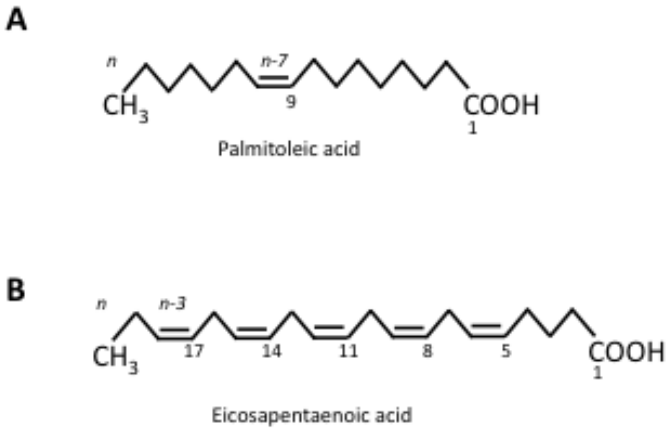

Figure 1. Fatty acid nomenclature. A) Example of a monounsaturated fatty acid, palmitoelic acid, termed either 16:1n-7 or $16{:}1\Delta^{9}$. B) Example of a polyunsaturated fatty acid, eicosapentaenoic acid (EPA), termed either 20:5n-3 or $20{:}5\Delta^{5,8,11,14,17}$.

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Biosynthesis pathways of long-chain polyunsaturated fatty acids in fish

In fish, like virtually all living organisms, fatty acid synthase (FAS), a cytosolic multifunctional enzymatic complex, is responsible for *de novo* biosynthesis of saturated fatty acids up to C_{16} (palmitic acid, 16:0). The fatty acids synthesised by FAS, as well as dietary FA, can be further converted by desaturase and elongases enzymes located in the endoplasmic reticulum (ER). Thus, in fish saturated fatty acids can be desaturated by the action of an enzyme termed stearoyl coA desaturase (SCD), which has Δ9-desaturation activity and can therefore convert 16:0 and 18:0 to 16:1n-7 and 18:1n-9, respectively (Sargent *et al.*, 2002). However, vertebrates including fish, cannot produce PUFA *de novo* as they lack the Δ12 and Δ15 (ω3) desaturases required to desaturate 18:1n-9 to 18:2n-6 (LOA) and then to 18:3n-3 (ALA) (Fig. 2). Consequently, PUFA are considered as EFA that vertebrates must obtain through their diet. However, in some species, the C_{18} PUFA, LOA and ALA, with relatively low biological activity, can be bioconverted to C_{20} and C_{22} LC-PUFA, including arachidonic acid (ARA; 20:4n-6), EPA (20:5n-3) and DHA (22:6n-3) that have physiologically essential roles (Fig. 2). For example, ARA and EPA are precursors of eicosanoids, biologically active compounds that regulate and modulate physiological processes including inflammation, reproduction and hemostasis (Funk, 2001), and DHA is a critical component of cell membrane lipids of neuronal tissues (Salem *et al.*, 2001).

The LC-PUFA biosynthetic pathway in fish was traditionally accepted to proceed through consecutive enzymatic reactions that convert the C_{18} PUFA 18:3n-3 and 18:2n-6 to $C_{20\text{-}22}$ LC-PUFA (Fig. 2). For synthesis of ARA, 18:2n-6 is desaturated by Δ6 Fad to 18:3n-6, which is elongated to 20:3n-6 and then desaturated by Δ5 Fad to ARA. The same enzymes participate in the desaturation and elongation of 18:3n-3 to EPA. The synthesis of DHA, however, was generally thought to occur solely through two further elongations of EPA to 24:5n-6, which was further Δ6 desaturated and chain-shortened, this latter reaction occurring in the peroxisomes, in a pathway often referred to as the "Sprecher shunt" (Sprecher, 2000). Nevertheless, an alternative more direct way for DHA biosynthesis via

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Δ4-desaturation of 22:5n-3 has been recently described for the first time in vertebrates in the herbivorous marine fish, *Siganus canaliculatus* (Li *et al.*, 2010).

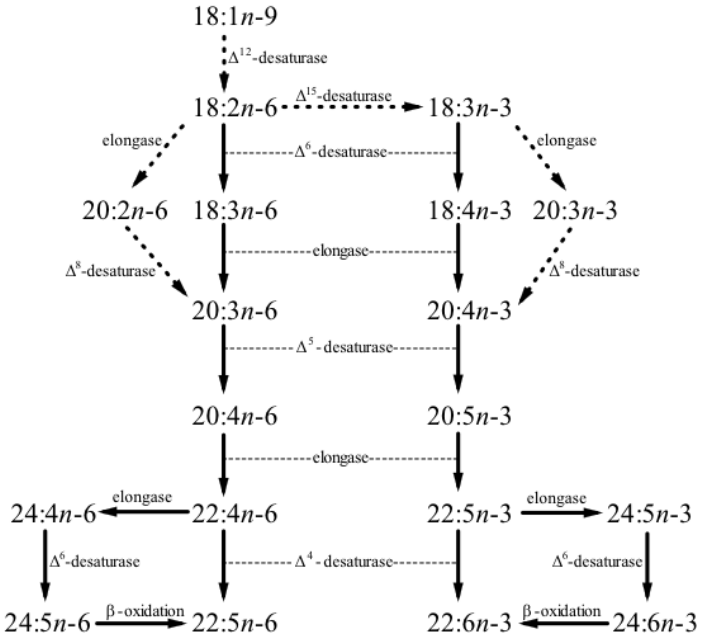

Figure 2. Potential pathways in fish for the biosynthesis of long-chain polyunsaturated fatty acids from the C_{18} precursors, 18:2n-6 and 18:3n-3.

Fatty acyl desaturases

Fatty acyl-coenzyme A (CoA) desaturases are enzymes responsible for the introduction of a double bond at a specific position of the acyl chain of a long-chain fatty acid (Guillou *et al.*, 2010). The CoA desaturases can be divided into two broad families including the stearoyl-CoA desaturases (SCD) and the fatty acyl desaturases (Fad) (Marquardt *et al.*, 2000). SCD exhibits Δ9-desaturation activity and is the enzyme alluded to above that is responsible for the desaturation of 16:0 and 18:0 to produce 16:1n-7 and 18:1n-9, respectively. On the other hand, Fad enzymes constitute a family of genes in vertebrates with three members

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termed *FADS1*, *FADS2* and *FADS3*[1] in mammals. The *FADS1* gene product has been demonstrated to have Δ5 desaturation activity, whereas *FADS2* encodes a protein with prominent Δ6 activity. Despite sequence similarities with other *FADS* family members, no functional activity has been yet attributed to *FADS3* (Guillou *et al.*, 2010).

Several Fad cDNAs have been isolated and functionally characterised from a relatively wide range of fish species (Table 1). Two studies published in 2001 investigated for the first time Fad-encoding cDNAs from rainbow trout (Seiliez *et al.*, 2001) and zebrafish (Hastings *et al.*, 2001). Whereas amino acid sequence homology suggested a putative Δ6-desaturation function for the rainbow trout Fad cDNA, functional characterisation of the zebrafish Fad cDNA by heterologous expression in yeast demonstrated it encoded a protein that possessed interesting and, at the time, unique bifunctional Δ6/Δ5 desaturation activity (Hastings *et al.*, 2001). The conversion rates of n-3 substrates were consistently higher than those of n-6 substrates, a feature that has been later confirmed as common for most fish Fad enzymes that have been functionally characterised. Thus, several Fad cDNAs have been subsequently isolated from a variety of freshwater, marine and diadromous fish species including gilthead sea bream (Seiliez *et al.*, 2003; Zheng *et al.*, 2004a), Atlantic salmon (Hastings *et al.*, 2005; Zheng *et al.*, 2005a; Monroig *et al.*, 2010a), Nile tilapia (Zheng *et al.*, 2004a), carp (Zheng *et al.*, 2004a), turbot (Zheng *et al.*, 2004a), Atlantic cod (Tocher *et al.*, 2006), cherry salmon (Alimuddin *et al.*, 2005, 2007), rabbitfish (Li *et al.*, 2008, 2010), cobia (Zheng *et al.*, 2009a), European sea bass (Gonzalez-Rovira *et al.*, 2009; Santigosa *et al.*, 2011), barramundi (Mohd-Yusof *et al.*, 2010), nibe croaker (Yamamoto *et al.*, 2010), striped snakehead fish (Jaya-Ram *et al.*, 2011), Northern bluefin tuna (Morais *et al.*, 2011) and red sea bream (Sarker *et al.*, 2011).

Phylogenetic analyses performed on the deduced amino acid sequences of the Fad genes revealed that all the desaturases so far isolated from fish are *FADS2* orthologues (Monroig

[1] The terms Fad and FADS are not equivalent and need to be distinguished. Whereas we use Fad to abbreviate "fatty acyl desaturase", FADS is the approved name given to the mammalian gene (*FADS*) or protein (FADS) (www.genenames.org).

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et al., 2011b). Whereas mammalian *FADS2* has predominantly Δ6-desaturase activity, the fish Fad enzymes show a large variety of functions (Table 1). Thus, fish Fad with Δ6-desaturation functionality have been isolated from a variety of both marine (gilthead sea bream, turbot, Atlantic cod, cobia, European sea bass, barramundi, and Northern bluefin tuna) and freshwater fish (common carp and rainbow trout). Interestingly, the diadromous species Atlantic salmon, probably the fish species whose LC-PUFA metabolism has been more investigated, possesses four distinct genes encoding Fad proteins with high homology to mammalian FADS2. Whereas three desaturases, termed Δ6Fad_a, Δ6Fad_b, and Δ6Fad_c were found to be strict Δ6-like desaturases (Zheng *et al.*, 2005a; Monroig *et al.*, 2010a), a fourth desaturase gene was determined to have a Δ5-desaturase capability despite being phylogenetically closer to FADS2 (mammalian Δ6) than FADS1 (mammalian Δ5) (Hastings *et al.*, 2005). Salmon Δ5Fad is the only monofunctional Δ5-desaturase so far discovered, and enables this species to perform all desaturation reactions required in the LC-PUFA biosynthesis pathways, i.e. Δ6 and Δ5 desaturations. It is therefore important to emphasise that Δ5 desaturases are apparently missing in marine teleosts, and this has been hypothesised to be a limiting step explaining the low ability of marine fish to biosynthesise DHA.

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Table 1. Fatty acyl desaturases (Fad) investigated in fish. The desaturation activities determined for each characterised Fad enzyme are indicated.

Species*	Common name	Reported activities	References
Danio rerio	Zebrafish	Δ6, Δ5, Δ8	Hastings *et al.* (2001)
Oncorhynchus mykiss	Rainbow trout	Δ6, Δ8	Seiliez *et al.* (2001); Zheng *et al.* (2004)
Salmo salar (Δ5Fad)	Atlantic salmon	Δ5	Hastings *et al.* (2005)
S. salar (Δ6Fad_a)	Atlantic salmon	Δ6	Zheng *et al.* (2005a)
S. salar (Δ6Fad_b)	Atlantic salmon	Δ6, Δ8	Monroig *et al.* (2010a)
S. salar (Δ6Fad_c)	Atlantic salmon	Δ6, Δ8	Monroig *et al.* (2010a)
Cyprinus carpio	Common carp	Δ6	Zheng *et al.* (2004a)
Sparus aurata	Gilthead sea bream	Δ6, Δ8	Seiliez *et al.* (2003); Zheng *et al.* (2004a)
Psetta maxima	Turbot	Δ6, Δ8	Zheng *et al.* (2004a)
*Oncorhynchus masou (*Δ6-like)	Masu salmon	Not determined	Alimuddin *et al.* (2005)
*O. masou (*Δ5-like)	Masu salmon	Not determined	Alimuddin *et al.* (2007)
Gadus morhua	Atlantic cod	Δ6, Δ8	Tocher *et al.* (2006)
Rachycentron canadum	Cobia	Δ6, Δ8	Zheng *et al.* (2009a)
Siganus canaliculatus (Fad1)	Rabbitfish	Δ6, Δ5, Δ8	Li *et al.* (2008); Li *et al.* (2010)
S. canaliculatus (Fad2)	Rabbitfish	Δ4, Δ8	Li *et al.* (2010)
Dicentrarchus labrax	European sea bass	Δ6	González-Rovira *et al.* (2009); Santigosa *et al.* (2011)
Lates calcarifer	Asian sea bass/Barramundi	Δ6	Mohd-Yusof *et al.* (2010)
Nibea mitsukurii	Nibe croaker	Not determined	Yamamoto *et al.* (2010)
Channa striata	Striped snakehead fish	Not determined	Jaya-Ram *et al.* (2011)
Thunnus thynnus	Northern bluefin tuna	Δ6	Morais *et al.* (2011)
Pagrus major	Red sea bream	Not determined	Sarker *et al.* (2011)

*In species in which more than one desaturase has been isolated (*Salmo salar* and *Siganus canaliculatus*), the name of the specific gene is indicated according to published information.

A unique vertebrate Fad was recently discovered in the rabbitfish (*Siganus canaliculatus*), a herbivorous marine teleost fish (Li *et al.*, 2010). Production of DHA from EPA in animals

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was originally thought to occur via an elongation to 22:5n-3 followed by direct Δ4-desaturation (Cook and McMaster, 2004). However, studies in rats allowed Sprecher and co-workers to demonstrate an alternative pathway that involved sequential elongations of EPA or ARA to C_{24} fatty acids (24:5n-3 and 24:4n-6, respectively) followed by a Δ6-desaturation (Sprecher, 2000). Biochemical studies indicated that this pathway also likely operated in rainbow trout (Buzzi *et al.*, 1996, 1997). Furthermore, mammalian FADS2 (De Antueno *et al.*, 2001; D'Andreas *et al.*, 2002) and the zebrafish Δ6/Δ5 Fad (Tocher *et al.*, 2003) were demonstrated to be capable of Δ6 desaturation of both C_{18} and C_{24} fatty acid substrates, suggesting that the same enzyme operated at two steps in the pathway. Moreover, none of the previously cloned and functionally characterised Fad of fish had shown any measurable Δ4 desaturation activity (Hastings *et al.*, 2001, 2005; Zheng *et al.*, 2004a, 2005; Monroig *et al.*, 2010a). Thus, it became the paradigm that vertebrates in general produced DHA from EPA via the alternative "Sprecher" pathway and did not possess a Δ4 Fad (Sprecher, 2000). Therefore, it was unexpected when the study on rabbitfish desaturases revealed the presence of a Fad with Δ4 activity and demonstrated that an alternative pathway via direct Δ4-desaturation of 22:5n-3 was possible for the production of DHA from EPA in some vertebrates. Furthermore, the demonstration of a gene encoding an enzyme with Δ4 Fad activity in the marine species *S. canaliculatus* suggested that the generalization that marine fish were characterised by low LC-PUFA biosynthetic ability should be revised. Possibly non-carnivorous marine fish, or at least fish such as *S. canaliculatus* that feed exclusively on macroalgae, have greater LC-PUFA biosynthetic capability than carnivorous species on which the above generalization was based. Furthermore, these species arise as potential candidates for diversification of marine aquaculture with species capable of effectively utilising more sustainable vegetable-based diets.

Plasticity of fish desaturases is not only limited to the existence of monofunctional Δ6, Δ5 or Δ4 enzymes, but also some of the enzymes characterised from fish have demonstrated multifunctional enzymatic capabilities. As mentioned previously, the zebrafish Fad was the first bifunctional Δ6/Δ5 desaturase described (Hastings *et al.*, 2001), but subsequently other

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bifunctional desaturases were described in fungi (Damude *et al.*, 2006, Zhang *et al.*, 2007), protozoans (Sayanova *et al.*, 2006) and moths (Serra *et al.*, 2006). Very recently, Li *et al.* (2010) discovered that, in addition to the Δ4 Fad, rabbitfish also possess a bifunctional Δ6/Δ5 Fad, which was the first report of a marine fish species having the capability for Δ5-desaturation, an enzymatic activity previously believed to be generally absent in marine fish (Tocher, 2010).

Overall results obtained by the cloning and functional analyses of fish Fad suggest that multifunctionality might be an extended feature among teleost Fads. We have recently undertaken a retrospective study to assess the ability of fish Fad to desaturate fatty acids at position Δ8 in addition to their previously determined activities (Monroig *et al.*, 2011b). Dual Δ6/Δ8 activity had been demonstrated for the baboon FADS2 (Park *et al.*, 2009), but the presence of Δ8 activity had not been investigated previously in fish Fad. Thus the capability for Δ8 activity was tested in Fad from a range of freshwater, diadromous and marine species. We demonstrated that Δ8 desaturation activity was a characteristic of fish FADS2-like enzymes and so Δ8 substrates including 20:3n-3 and 20:2n-6 were converted to 20:4n-3 and 20:3n-6, respectively, the products being suitable substrates for Δ5 desaturation (Fig. 2). Interestingly, the Δ8 activity varied notably between freshwater/diadromous and marine fish species, with marine fish Fad having higher Δ8 capability than Fad from freshwater/diadromous species. It is not clear why Fad from marine fish have diverged to have greater Δ8 activity than freshwater/diadromous species, but possibly there may be increased dietary intake of Δ8 LC-PUFA substrate, 20:3n-3, in marine environments, which could have partly driven the bifunctionalisation of marine teleost desaturases towards Δ6/Δ8 enzymes. However, it is unclear what benefit this evolutionary adaptation process would have in species deficient in Δ5-desaturation that limits LC-PUFA biosynthesis or, indeed, that are receiving adequate dietary EPA and DHA (Bell and Tocher, 2009a; Leaver *et al.*, 2008a). Clearly though, these findings indicate that the general concept regarding 20:3n-3 and 20:2n-6 as 'dead-end' metabolic products needs to be revised, at least for some marine teleosts (Oxley *et al.*, 2010; Pratoomyot *et al.*, 2008; Ruyter *et al.*, 2003).

Monroig, O. *et al*. 2011. Long-Chain Polyunsaturated Fatty Acids in Fish: Recent Advances on Desaturases and Elongases Involved in Their Biosynthesis. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Hernández-Hernández, L. (Eds), Avances en Nutrición Acuícola XI – Memorias del Décimo Primer Simposio Internacional de Nutrición Acuícola, 23-25 de Noviembre, San Nicolás de los Garza, N. L., México. ISBN 978-607-433-775-4. Universidad Autónoma de Nuevo León, Monterrey, México, pp. 257-283.

Elongases of very long-chain fatty acids

Elongases of very long-chain fatty acids (Elovl) are the initial and rate-limiting enzymes responsible for the condensation of activated fatty acids with malonyl-CoA required for biosynthesis of long-chain fatty acids (Nugteren, 1965). Seven members of the Elovl family, termed ELOVL 1-7, have been identified in mammals that differ from each other in their substrate specificity (Jakobsson *et al.*, 2006). Generally, ELOVL1, ELOVL3, ELOVL6 and ELOVL7 have been determined to participate in elongation of saturated and monounsaturated fatty acids and, to the best of our knowledge, no studies have been conducted on the characterisation of these enzymes in fish. Contrarily, Elovl family members involved in the biosynthesis of LC-PUFA, namely ELOVL2, ELOVL4 and ELOVL5, have been extensively investigated in a range of fish, especially farmed species (Table 2).

Elovl5-encoding cDNAs have been isolated from many species, with zebrafish Elovl5 being the first cloned and functionally characterised (Agaba *et al.*, 2004). Similar to mammalian ELOVL5, zebrafish Elovl5 efficiently elongated PUFA including C_{18} (18:4n-3 and 18:3n-6) and C_{20} (20:5n-3 and 20:4n-6), with only low conversion shown towards C_{22} substrates (22:5n-3 and 22:4n-6). Later, Elovl5s were cloned and characterised in Atlantic salmon (Hastings *et al.*, 2005) and in catfish, tilapia, turbot, gilthead sea bream and Atlantic cod (Agaba *et al.*, 2005). Further publications on cobia (Zheng *et al.*, 2009a), barramundi (Mohd-Yusof *et al.*, 2010), Southern (Gregory *et al.*, 2010) and Northern (Morais *et al.*, 2011) bluefin tuna, confirmed that fish Elovl5s all demonstrated the ability to elongate C_{18} and C_{20} PUFA with n-3 substrates generally being preferred as substrates over n-6 series fatty acids. Other potential substrates for Elovl5 are 18:3n-3 and 18:2n-6, which can be converted to 20:3n-3 and 20:2n-6, respectively, suitable substrates for Δ8-desaturation (Fig. 2) (Guillou *et al.*, 2010). However, elongation of 18:3n-3 and 18:2n-6 by fish Elovl5 has not been investigated, and future functional characterisation analyses of fish Elovl5, particularly in marine species, should be explored as it may be a key enzyme in the recently suggested potential "elongation → Δ8 desaturation → Δ5 desaturation" pathway, an

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alternative to the 'classical' "Δ6 desaturation → elongation → Δ5 desaturation" pathway (Monroig *et al.*, 2011b).

The understanding of fatty acid elongation pathways in fish has considerably advanced over the last few years with the investigation of other Elovl family members involved in the biosynthesis of LC-PUFA. Thus, Elovl2 enzymes with the ability to elongate C_{20} (20:5n-3 and 20:4n-6) and C_{22} (22:5n-3 and 22:4n-6) LC-PUFA substrates have been cloned and functionally characterised in Atlantic salmon (Morais *et al.*, 2009) and zebrafish (Monroig *et al.*, 2009). Although 20:5n-3 and 20:4n-6 are also elongation substrates for Elovl5, 22:5n-3 and 22:4n-6 are only efficiently converted by Elovl2. Importantly, the ability of Elovl2 to elongate 22:5n-3 to 24:5n-3 has been regarded as key for the production of DHA via the 'Sprecher pathway'. To date, no Elovl2 cDNA has been isolated from a marine fish species, and this has been hypothesised as another possible factor underlying their very limited ability for DHA biosynthesis (Morais *et al.*, 2009). Recent studies, however, have revealed that other enzymes may effectively elongate C_{22} PUFA in marine fish, which could compensate for the lack of Elovl2.

Monroig, O. *et al*. 2011. Long-Chain Polyunsaturated Fatty Acids in Fish: Recent Advances on Desaturases and Elongases Involved in Their Biosynthesis. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Hernández-Hernández, L. (Eds), Avances en Nutrición Acuícola XI – Memorias del Décimo Primer Simposio Internacional de Nutrición Acuícola, 23-25 de Noviembre, San Nicolás de los Garza, N. L., México. ISBN 978-607-433-775-4. Universidad Autónoma de Nuevo León, Monterrey, México, pp. 257-283.

Table 2. Elongases of very long-chain fatty acids (Elovl) investigated in fish. The type of Elovl is determined by amino acid similarities with mammalian orthologues and, in most cases, by functional characterisation analyses.

* Not functionally characterised; Elovl5 annotation is based on amino acid sequence homology.

Species	Common name	Elongase type	Reference
Danio rerio	Zebrafish	Elovl5	Agaba *et al.* (2004)
D. rerio	Zebrafish	Elovl2	Monroig *et al.* (2009)
D. rerio	Zebrafish	Elovl4 (isoforms a and b)	Monroig *et al.* (2010b)
Salmo salar	Atlantic salmon	Elovl5 (isoforms a and b)	Agaba *et al.* (2005); Morais *et al.* (2009)
S. salar	Atlantic salmon	Elovl2	Morais *et al.* (2009)
S. salar	Atlantic salmon	Elovl4	Carmona-Antoñanzas *et al.* (2011)
Cyprinus carpio	Nile tilapia	Elovl5	Agaba *et al.* (2005)
Clarius gariepinus	African catfish	Elovl5	Agaba *et al.* (2005)
Sparus aurata	Gilthead sea bream	Elovl5	Agaba *et al.* (2005)
Psetta maxima	Turbot	Elovl5	Agaba *et al.* (2005)
Gadus morhua	Atlantic cod	Elovl5	Agaba *et al.* (2005)
Oncorhynchus masou	Masu salmon	Elovl5*	Alimuddin *et al.* (2008)
Rachycentron canadum	Cobia	Elovl5	Zheng *et al.* (2009a);
R. canadum	Cobia	Elovl4	Monroig *et al.* (2011a)
Lates calcarifer	Asian sea bass/Barramundi	Elovl5	Mohd-Yusof *et al.* (2010)
Thunnus maccoyii	Southern bluefin tuna	Elovl5	Gregory *et al.* (2010)
Nibea mitsukurii	Nibe croaker	Elovl5*	Yamamoto *et al.* (2010)
Thunnus thynnus	Northern bluefin tuna	Elovl5	Morais *et al.* (2011)
Pagrus major	Red sea bream	Elovl5*	Sarker *et al.* (2011)

Elovl4 is the most recent member of the Elovl family that has been investigated in fish. Elovl4 elongases are responsible for the biosynthesis of so-called "very long-chain fatty acids" (VLC-FA), saturated or polyunsaturated fatty acids (VLC-PUFA) with chain-lengths greater than C_{24}. In mammals, saturated VLC-FA have been identified as components of sphingolipids and ceramides (Cameron *et al.*, 2007; Li *et al.*, 2007a, b), whereas VLC-

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PUFA are relatively abundant in particular lipid classes of specific tissues such as retina (Aveldaño, 1987, 1988; Suh *et al.*, 2002; McMahon *et al.*, 2007), brain (Robinson *et al.*, 1990; Poulos, 1995) and testis (Aveldaño *et al.*, 1993; Furland *et al.*, 2003, 2007a,b). Two Elovl4 isoforms present in zebrafish (Elovl4a and Elovl4b) were functionally characterised showing that both proteins efficiently elongated saturated fatty acids up to C_{36}. Interestingly, activity for the elongation of PUFA substrates was only shown by the b isoform, which effectively converted 20:5n-3 and 20:4n-6 to corresponding elongated polyenoic products up to C_{36} of the n-3 and n-6 series, respectively. Similar to zebrafish Elovl4b, cobia and Atlantic salmon Elovl4 enzymes have been shown to participate in the biosynthesis of both saturated and polyunsaturated VLC-FA (Carmona-Antoñanzas *et al.*, 2011; Monroig *et al.*, 2011a). The ability of fish Elovl4 for elongation of 22:5n-3 to 24:5n-3 clearly showed that these enzymes have the potential to participate in the production of DHA similar to Elovl2. As described above, such functionalisation of teleost Elovl4, may be critical in marine species such as cobia apparently lacking Elovl2 in their genomes (Morais *et al.*, 2009).

Expression of desaturase and elongase genes

Tissue distribution

Analysis of gene expression by quantitative real-time-PCR (qPCR) analysis of ten tissues showed expression of Δ6 Fad, Δ5 Fad, Elovl5 and Elovl2 were all highest in intestine, liver and brain in Atlantic salmon (Zheng *et al.*, 2005a; Morais *et al.*, 2009). Similarly, Northern analysis of six tissues showed expression of Δ6 Fad gene in rainbow trout was also highest in intestine, liver and brain (Seiliez *et al.*, 2001). In contrast, expression of Δ6 Fad in the marine fish, Atlantic cod, cobia and Asian sea bass, was considerably higher in brain than in any other of the ten tissues examined by qPCR (Tocher *et al.*, 2006; Zheng *et al.*, 2009a; Mohd-Yusof *et al.*, 2010). This finding has prompted speculation that retention of Δ6 Fad in marine fish may be to maintain membrane DHA levels in neural tissues at times of high demand such as embryonic and larval development despite fluctuations in dietary EPA:DHA.

Monroig, O. *et al*. 2011. Long-Chain Polyunsaturated Fatty Acids in Fish: Recent Advances on Desaturases and Elongases Involved in Their Biosynthesis. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Hernández-Hernández, L. (Eds), Avances en Nutrición Acuícola XI – Memorias del Décimo Primer Simposio Internacional de Nutrición Acuícola, 23-25 de Noviembre, San Nicolás de los Garza, N. L., México. ISBN 978-607-433-775-4. Universidad Autónoma de Nuevo León, Monterrey, México, pp. 257-283.

Nutritional regulation of desaturase and elongase expression

Nutritional regulation of gene expression and the activity of the LC-PUFA biosynthesis pathway has also been demonstrated in freshwater and salmonid fish species. For example, LC-PUFA biosynthesis activity was increased by EFA-deficiency and modulated by different C_{18} PUFA in carp cells (Tocher and Dick, 1999, 2000). Furthermore, in feeding trials with salmonids, LC-PUFA biosynthesis was increased (Tocher *et al.* 1997, 2001), and expression of LC-PUFA biosynthesis genes, especially Δ6 Fad, was induced in salmon liver and intestine in fish fed diets containing vegetable oils lacking LC-PUFA compared to fish fed diets containing fish oil rich in EPA and DHA (Zheng *et al.*, 2004b, 2005a,b; Leaver *et al.*, 2008b; Taggart *et al.*, 2008). In contrast, liver and intestinal Δ6 Fad expression and activity was generally not significantly affected in Atlantic cod fed diets containing either vegetable or fish oil (Tocher *et al.*, 2006), possibly reflecting differences in the Fad gene promoters in cod and salmon (Zheng *et al.*, 2009b). Regulation of Fad and Elovl genes was also investigated in marine fish larvae, a developmental stage particularly sensitive to suboptimal LC-PUFA supply in the diet (Navarro et al., 1997; Sargent *et al.*, 1997, 1999; Izquierdo, 2000). Therefore, Δ6 Fad appeared to be up-regulated in larval gilthead sea bream fed on microdiets formulated on rapeseed and soybean oil with low EPA and DHA compared to larvae fed on diet formulated with fish oil (Izquierdo *et al.*, 2008). A recent study on nibe croaker, a marine sciaenid teleost, reported increased expression of a putative Δ6 Fad in response to *Artemia* diets containing low C_{20-22} LC-PUFA (Yamamoto *et al.*, 2010). The expression of an Elovl5-like elongase, however, did not show nutritional regulation in this species. Nutritional regulation of Fad expression or activity in other tissues such as brain has not been studied in fish.

Developmental regulation of desaturase and elongase expression

LC-PUFA are critical compounds for early life stages of fish as emphasised by their selective accumulation in specific lipid classes of neuronal tissues during embryogenesis (Tocher, 2010). Investigations have also demonstrated that deposition of LC-PUFA in

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embryos (here referring to life stages from zygote to the oesophagus opening) is greatly influenced by the diet of broodstock fish (Rodríguez *et al.*, 1998; Mazorra *et al.*, 2003; Izquierdo *et al.*, 2001) and genetic factors (Pickova *et al.*, 1997). Moreover, Ishak *et al.* (2008) studied the regulation of the Δ6/Δ5 Fad and Elovl5 elongase during follicule maturation of zebrafish, suggesting that the LC-PUFA pathway was activated to promote oocyte maturation and ovulation processes. However, the endogenous capability of fish embryos for biosynthesis of LC-PUFA, a mechanism proven to be very active in other oviparous organisms such as avians (Noble and Shand, 1985; Cherian and Sim, 2001), was largely unexplored. Thus, we recently investigated the expression of three genes, namely the dual Δ6/Δ5 Fad and the Elovl5 and Elovl2 elongases, during zebrafish embryogenesis (Monroig *et al.*, 2009). According to the LC-PUFA biosynthesis pathways (Fig. 2), these three genes could enable zebrafish to perform all conversions required to transform dietary C_{18} PUFA (18:3n-3 and 18:2n-6) to biologically active C_{20-22} LC-PUFA. Our results revealed that transcripts (mRNA) of Δ6/Δ5 Fad, Elovl5 and Elovl2 were found during zebrafish embryogenesis, suggesting that the endogenous biosynthetic pathway was indeed active. Although slightly delayed in comparison, the marine teleost cobia also showed expression of key biosynthetic enzymes (Δ6 Fad, Elovl5 and Elovl4) during embryogenesis (Monroig *et al.*, 2011a). In both species, the genes investigated were expressed from the zygote stage (0 hours post-fertilisation), suggesting maternal mRNA transfer to the embryo. This highlights that the maternal role is not limited to the transfer of critical preformed LC-PUFA in the yolk, but also includes the transfer of key enzyme mRNA transcripts to the embryo.

Using whole-mount *in situ* hybridisation techniques, our investigations also determined the metabolic sites showing endogenous production of LC-PUFA in embryonic tissues. Thus, the yolk syncytial layer, a structure that forms a boundary between the embryo and the yolk mass, expressed Δ6/Δ5Fad, Elovl5 and Elovl2 in zebrafish, revealing an important role in remodelling of yolk fatty acids during early embryogenesis in fish (Monroig *et al.*, 2009). Interestingly, Elovl5 elongase expression was specifically located in the pronephric ducts, and thus suggested an as yet unknown role of embryonic kidney in fatty acid metabolism

Monroig, O. *et al*. 2011. Long-Chain Polyunsaturated Fatty Acids in Fish: Recent Advances on Desaturases and Elongases Involved in Their Biosynthesis. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Hernández-Hernández, L. (Eds), Avances en Nutrición Acuícola XI – Memorias del Décimo Primer Simposio Internacional de Nutrición Acuícola, 23-25 de Noviembre, San Nicolás de los Garza, N. L., México. ISBN 978-607-433-775-4. Universidad Autónoma de Nuevo León, Monterrey, México, pp. 257-283.

during early embryonic development of zebrafish and possibly other fish species. Additionally, high expression signals of Δ6/Δ5Fad and Elovl2 in the head area suggested accretion of newly biosynthesised LC-PUFA in developing neuronal tissues. A subsequent study revealed that similar expression patterns were observed for zebrafish Elovl4a (Monroig *et al.*, 2010b). Additionally, zebrafish Elovl4b, an enzyme involved in the production of VLC-PUFA, was specifically expressed in pineal gland and photoreceptor cells in the retina. These findings were consistent with the fact that fish retina and pineal gland have a common evolutionary origin, with both tissues possessing photoreceptor cells.

Application of molecular knowledge of LC-PUFA biosynthesis to aquaculture nutrition

The above studies have shown options for alternative strategies for the optimisation of the LC-PUFA biosynthesis pathway in farmed fish. Knowledge of the precise complement of genes for enzymes in the LC-PUFA biosynthesis pathway will enable the design of diets and feeds to specifically match the abilities of different fish species for endogenous biosynthesis. Furthermore, knowledge of the developmental regulation of the pathway will enable diet formulations to be tailored to particular life stages. Knowledge of the nutritional regulation of the genes of the LC-PUFA biosynthesis pathway opens the door for the activation/optimisation of the endogenous pathway through careful diet formulation.

In contrast to strategies aimed at designing feeds to better suit the fish, an alternative approach would be to design the fish to better suit the feeds that are likely to predominate in the near future. Therefore, the genes of LC-PUFA biosynthesis may be appropriate targets for marker-assisted genetic selection to develop strains of fish with enhanced ability to thrive on more sustainable vegetable-based feed formulations. Another novel variation of this strategy involving transgenic technology was explored by Takeuchi and coworkers in Japan. Using zebrafish, a series of studies investigated the effects that the overexpression of genes encoding enzymes of LC-PUFA biosynthetic pathway from masu salmon (*Oncorhynchus masou*) had on n-3 LC-PUFA endogenous production. Firstly, zebrafish

Monroig, O. *et al*. 2011. Long-Chain Polyunsaturated Fatty Acids in Fish: Recent Advances on Desaturases and Elongases Involved in Their Biosynthesis. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Hernández-Hernández, L. (Eds), Avances en Nutrición Acuícola XI – Memorias del Décimo Primer Simposio Internacional de Nutrición Acuícola, 23-25 de Noviembre, San Nicolás de los Garza, N. L., México. ISBN 978-607-433-775-4. Universidad Autónoma de Nuevo León, Monterrey, México, pp. 257-283.

embryos were transfected with a masu salmon Δ6-like Fad resulting in increased production of EPA (1.4-fold) and DHA (2.1-fold) compared to non-transgenic fish (Alimuddin *et al.*, 2005). In a second experiment, zebrafish were transformed with Δ5-like Fad and moderate increases of EPA and DHA (both ~ 1.2-fold) were observed in transgenic compared to non-transgenic fish (Alimuddin *et al.*, 2007). Finally, a masu salmon *elovl5*-like gene was overexpressed in zebrafish with enhanced production of EPA and DHA (both ~ 1.3-fold) (Alimuddin *et al.*, 2008). The authors suggested that transgenesis was a potential strategy to alleviate and possibly eliminate the need of supplying preformed C_{20-22} LC-PUFA in the diet of on-growing and larval stages of farmed fish. These studies have obvious interest to understand the molecular and physiological processes related to a LC-PUFA enhancement. Their applicability, however, to fish farming in many parts of the world, not least Europe, is still far from possible due in part to safety food regulations and also to the fact that methodological difficulties might arise when applying transgenic technologies into fish other than model species.

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