MOLECULAR CLONING AND FUNCTIONAL CHARACTERIZATION OF BLUE-SPOTTED MUDSKIPPER (*Boleophthalmus boddarti*) FATTY ACYL DESATURASE AND ELONGASE

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by

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LIST OF ABBREVIATIONS

aa	amino acid
ACP	acyl carrier protein
ALA	α-linolenic acid
ANOVA	analysis of variance
ARA	arachidonic acid
bHLH-Zip	basic helix-loop-helix leucine zipper
BLAST	basic local alignment search tool
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FA	fatty acid
ef1α	elongation factor 1 alpha
EFA	essential fatty acid
Elovl	elongase of very long chain of fatty acid
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
Fads	fatty acyl desaturase
FAME	fatty acid methyl ester
GAL1	galactokinase
GC	gas chromatography
HDL	high-density lipoprotein
IPTG	isopropyl β-D-thiogalactopyranoside
KCR	β-ketoacyl-CoA reductase
KCS	β-ketoacyl-CoA synthase
LIN	linoleic acid
LB	Luria-Bertani
LCFA	long chain (saturated) fatty acid

LC-PUFA	long-chain polyunsaturated fatty acid
LDL	low-density lipoprotein
MEGA	Molecular Evolutionary Genetic Analysis
M-MLV	Moloney murine leukemia virus
mRNA	messenger RNA
MUFA	monounsaturated fatty acid
NCBI	National Center for Biotechnology Information
NMI	Non-Methylene-Interrupted
OA	oleic acid
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PUFA	polyunsaturated fatty acid
qPCR	quantitative real-time PCR
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RT-PCR	reverse transcription-polymerase chain reaction
SCMM-U	*Saccharomyces cerevisiae* minimal medium without uracil
SEM	standard error of the mean
SFA	saturated fatty acid
TBE	Tris-borate-EDTA
UTR	untranslated region
VLC-FA	very long chain (saturated) fatty acid
VLC-PUFA	very long chain polyunsaturated fatty acid
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPD	yeast extract peptone dextrose

PENGKLONAN MOLEKULAR DAN PENCIRIAN

ENZIM ELONGASE DAN DESATURASE DALAM

IKAN BELACAK (*Boleophthalmus boddarti*)

ABSTRAK

Asid lemak tak tepu yang berantai panjang (LC-PUFA) membawa banyak manfaat kepada manusia dan amat penting untuk fungsi tubuh dari aspek fisiologi dan pertumbuhan. Berbanding dengan desaturase (Fads) dan elongase (Elovl) mammalia berfungsi terhadap substrat yang terhad, Fads dan Elovl dalam ikan teleost mempamerkan aktiviti terhadap pelbagai substrat yang diakibatkan perbezaan sumber LC-PUFA di dalam habitat ikan. Kajian ini menyumbangkan kepada sains untuk memahami proses biosintesis LC-PUFA di dalam ikan belacak, *Boleophthalmus boddarti*. Dua gen yang berperanan dalam melakukan sintesis LC-PUFA telah diklonkan, dinamakan *BbFads2* dan *BbElovl5*. Gen *BbFads2* mempunyai rangka bacaan terbuka (ORF) sepanjang 1590bp menghasilkan protein memiliki 436 asid amino. Jujukan protein mendedahkan kesemua ciri yang menyifatkan sesuatu desaturase asil lemak mikrosom, termasuk domain N-terminal mirip-b_5, tiga kotak histidina HXXXH, HXXHH, QXXHH yang penting untuk pemangkinan, dan satu "heme-binding motif", HPGG. Pencirian fungsi BbFads2 dalam yis menunjukkan protein tersebut memiliki aktiviti Δ6 dan Δ8. Sama dengan Δ6 Fads2 daripada kebanyakan ikan marin, qPCR mendedahkan bahwa otak ikan belacak mempunyai ekspresi gen *BbFads2* yang paling tinggi dan ini adalah untuk memastikan tahap DHA yang stabil di dalam otak. Aktiviti Δ6 dari Fads2 tersebut dianggapkan terlibat dalam penghasilan DHA di dalam otak melalui Sprecher Pathway. Sebagai tambahan, rangka baca terbuka *BbElovl5* yang sepanjang 1387bp

mengekod protein memiliki 290 asid amino. BbElovl5 tersebut mempunyai kesemua ciri yang sama dengan enzim elongase PUFA dari haiwan mammalia dan ikan lain, seperti kotak histidina (HXXHH), lisina (K) and arginina (R) pada hujung kumpulan karboksil (C-terminal) dalam retikulum endoplasma (KXRXX). qPCR menunjukkan bahwa usus mempunyai ekpresi gen *BbElovl5* yang tertinggi. Hasil ini adalah normal bagi *B.boddarti* sebagai ikan herbivore untuk mengubahsuai asid lemak C_{18} dari makanan kepada PUFA yang diperlukan. Pencirian fungsi dalam yis menunjukkan protein BbElovl5 mempamerkan aktiviti pemanjangan terhadap kesemua substrat yang telah diuji, dan keutama substrat terhadap C_{20}>C_{18}>C_{22}. Namun begitu, enzim Elovl5 ikan belacak mempunyai aktiviti yang agak tinggi terhadap substrat C_{22} berbanding dengan Elovl5 dalam ikan lain. Dengan demikian, penelitian ini dapat disimpulkan bahwa dua LC-PUFA gen telah dipencilkan dari ikan belacak dan dicirikan.

MOLECULAR CLONING AND FUNCTIONAL CHARACTERIZATION OF MUDSKIPPER (*Boleophthalmus boddarti*) FATTY ACYL DESATURASE AND ELONGASE

ABSTRACT

Long chain polyunsaturated fatty acids (LC-PUFAs) serve many important roles in human are important for physiological and developmental well being. Unlike mammalian desaturases (Fads) and elongases (Elovl) that have tight selections of fatty acid substrates, the desaturase and elongase in teleost displayed much diversified functionality and this was hypothesized to be driven mainly by PUFA abundance in diet and their respective habitat. This study present the first evidence to the understanding of LC-PUFA biosynthetic pathway in blue-spotted mudskipper, *Boleophthalmus boddarti*. Two genes involved in the LC-PUFA biosynthesis were isolated. The *BbFads2* carried an open reading frame of 1590bp specifying a protein of 436 amino acids. The protein includes all the characteristics of microsomal fatty acid desaturase, including N-terminal b_5-like domain and three catalytically important histidine boxes HXXXH, HXXHH, QXXHH, and a heme-binding motif, HPGG. Functional analysis in yeast showed that the BbFads2 protein has Δ6 activity and Δ8 activity. In agreement with Δ6 *Fads2* of most other marine species, qPCR revealed the *BbFads2* gene was highly expressed in brain to maintain constant DHA level in the nervous system. The Δ6 activity found in the BbFads2 protein was speculated to involve in the DHA production in brain via classical Sprecher Pathway. In addition, *BbElovl5* carried an open reading frame of 1387bp specifying a protein of 290 amino acids was isolated. The protein has all the common features of mammalian and fish PUFA elongases, such as histidine box (HXXHH), lysine (K)

and arginine (R) residues at the canonical C-terminal ER retrieval signal (KXRXX). qPCR showed that the *BbElovl5* gene was highly expressed in intestine, which is normal to a herbivorous species to enable the endogenous formation of LC-PUFA from dietary C_{18} precursors. Functional analysis in yeast showed that the BbElovl5 protein was active towards all tested PUFA substrates, with the rank order being C_{20}>C_{18}>C_{22}. Interestingly, unlike any other teleostei Elovl5, the BbElovl5 was shown to have high activity towards C_{22} conversion that was typically seen in Elovl2. The unusual high C_{22} conversion was speculated to aid in the DHA production via Specher Pathway. Here, I concluded that two genes involved in LC-PUFAs bioconversions were successfully isolated from the blue-spotted mudskipper and functionally characterized.

CHAPTER ONE

INTRODUCTION

1.1 Background

PUFAs are fatty acids that carry two or more double bonds in the carbon backbone. PUFAs that have more than 18 carbons, such as arachidonic acid (ARA, 22:4n-6), eicosapentaenoic acid (EPA, 22:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are known as long-chain polyunsaturated fatty acids (LC-PUFAs). LC-PUFAs play vital roles in the development (Koletzko et al., 2008) and physiological well being, especially important to the neural and visual system (Belkind-Gerson et al., 2008) (Innis et al., 2008). ARA, EPA and DHA are synthesized from their C_{18} precursors, namely linoleic acid (LIN, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3). As vertebrates lack of key enzymes synthesizing LIN and ALA, these essential fatty acids (EFAs) must be obtained from dietary route (Simopoulos, 2008).

In this context, I studied two groups of enzymes that are critical for LC-PUFA biosynthesis: fatty acyl desaturase and elongase. Desaturases add double bond(s) to the carbon chain. Elongases elongate the carbon chain by two carbons in each cycle of reaction. The level of LC-PUFAs being synthesized or converted in a particular species ultimately relies on the efficiency of these enzymes involved in the bioconversion pathway. In human, EPA and ARA can form DHA and docosapentaenoic acid (Osbond acid, 22:5n-6) respectively. However, the conversion rates by the corresponding enzymes are very slow (Xie and Innis, 2008) (Rzehak et al., 2009).

Nearly all PUFAs are produced in primary producers inhabiting aquatic environment such as microalgae, bacteria and heterotrophic protists. Compared with

terrestrial oil, the oils from the marine ecosystem contain much higher levels of n-3 LC-PUFAs. Therefore fishes and seafood form an important source of these vital nutrients in the human diet (Tur et al., 2012).

Fish, like other vertebrates, cannot synthesize LIN and ALA from saturated fatty acids or MUFA (Torcher, 2003). However, they serve as a "transit station" where the LC-PUFAs get bio-accumulated along the food chain, which is then consumed by human. LC-PUFA biosynthesis pathways in fish has drawn more attention compared to other species due to the increasing need of sustainable LC-PUFA source for aquaculture, which could potentially be overcome by replacing the traditional feed ingredients containing fish oil with more sustainable vegetable oils (Sargent et al., 2002). Vegetable oils are rich in C_{18} PUFA, but do not contain LC-PUFAs. Therefore, it is worth looking into the ability of LC-PUFA biosynthesis from C_{18} precursors in particular farmed species.

Metabolism of LC-PUFAs has been extensively studied in fish (Torcher, 2003). It has been widely perceived that marine fish experience far less evolutionary pressure to retain LC-PUFA biosynthesis machineries due to high level of EPA and DHA produced by phytoplankton in the marine environment (Sargent et al., 1995). In contrast, fresh water species such as zebrafish have diversified their gene and catalytic complement to compensate with the reduced levels of LC-PUFA in their natural diets (Leaver et al., 2008). The lack of LC-PUFA can also be seen in terrestrial habitats that are mainly fuelled by green plants (Sargent et al., 1989) (Sargent et al., 1995). As a result, both Δ5 and Δ6 desaturase genes are retained in terrestrial tetrapods. However, it is inappropriate to assume the LC-PUFA biosynthesis capabilities of any fish based on freshwater or marine environment.

It was subsequently shown that an extreme herbivore marine species, the rabbitfish (*Siganus canaliculatus*) possesses all the genes enabling LC-PUFA biosynthesis from C_{18} precursors (Li et al., 2008). Extreme carnivorous fish can obtain abundant preformed 20:4n-6, 20:5n-3, and 22:6n-3 and appear to have low expression of Δ6 and Δ5 desaturases (Li et al., 2010). These reports suggested that trophic level / feeding preferences can also be a factor accounting for the LC-PUFA conversion capabilities.

LC-PUFA biosynthesis in mudskippers has not been explored before. These unique teleost fishes under the family of Gobiidae; subfamily of Oxudercinae are distinguished by their unique "out of water" lifestyle. The degree of terrestrial life varies amongst species. After diverging from other teleosts, mudskippers have acquired some characteristic adaptations that put them into a unique ecological niche. There are some herbivorous species, and some are carnivorous. Adaptations such as locomotion on land using their pectoral fins, air breathing, and improved aerial vision to escape from predator make the mudskipper a good model to understand evolutionary aspect of sea-land transition.

In the present study, I focus on studying the LC-PUFA genes of the herbivorous blue-spotted mudskipper, *Boleopthalmus boddarti*.

1.2 Problem statement

Despite fat content of mudskipper tissues have been reported, and the full genome of several mudskippers species have been sequenced, molecular aspect of de novo LC-PUFA biosynthesis in *Boleophthalmus boddarti* and other mudskipper species has not been explored. Current literatures categorize desaturases and elongases of fishes based on their habitat and trophic level. It would be interesting to look into the molecular machineries and LC-PUFA biosynthesis pathway of this relatively unexplored herbivorous marine teleost. For instance, does *B.boddarti* have *Fads* and *Elovl* genes? Did the unique semiterrestrial life-style of *B.boddarti* affect the functionality of its Fads and Elovl genes compared to other teleost? Do these genes actively participate in the LC-PUFA biosysnthesis in terms of gene expression? How well do these enzymes convert the FA substrate to corresponding products?

1.3 Research objectives

In order to understand the molecular mechanism of LC-PUFA biosynthesis in the blue-spotted mudskipper, tissue specific gene expression, functional role of the key enzymes involved in the sequential desaturation and elongation steps have to be first elicidated. In this study, efforts were made to achieve the objectives of:

1 To isolate and clone the full length cDNA of blue-spotted mudskipper *Fads* and Elovl genes.

2 To determine the gene expression profile of Fads and Elovl in various tissues of blue-spotted mudskipper.

3 To functionally characterize the blue-spotted mudskipper Fads and Elovl proteins.

CHAPTER TWO

LITERATURE REVIEW

2.1 Fatty acid

As a broad group of naturally occurring macromolecules, lipids such as fats, wax, phospholipids, and fat-soluble vitamins play a wide variety of biological roles in organisms; such as energy storage, structure component of cell membrane, and signal transduction (e.g. steroid hormone) (Fofana et al., 2010). Fatty acid is a group of long chain carboxylic acid molecules (**Figure 2.1**). They are the main component of various classes of lipids, which gives the long carbon chain acyl group that is joined to glycerol via ester linkage in triglycerides.

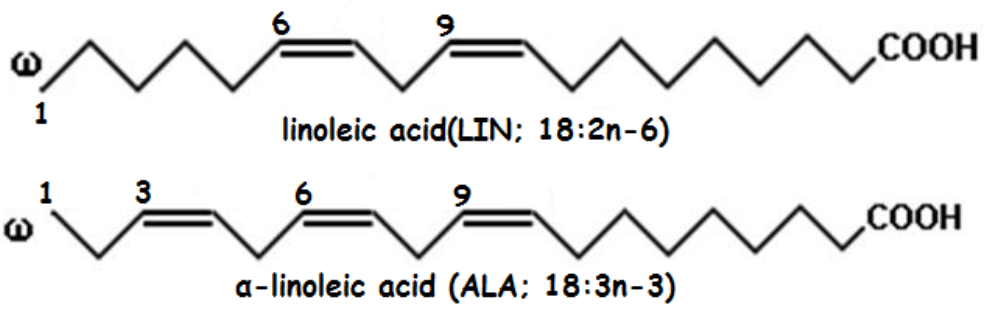
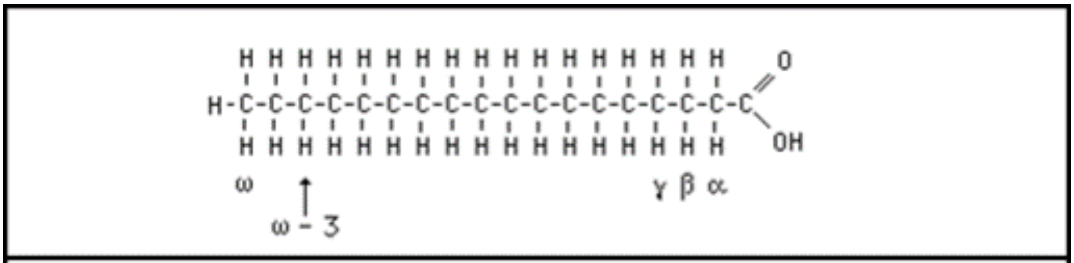


Figure 2.1 Molecular structures of LIN and ALA.

Figure 2.2 illustrates the nomenclature system of fatty acids. Standard that is internationally accepted that is defined by the International Union of Pure and Applied Chemistry (IUPAC) (McNaught & Wilkinson, 1997). However, many literatures use the n-x (or "omega x") system of nomenclature, for example, Linoleic acid (LIN; 18:2n-6), indicating the common name of the fatty acid in short form, followed by total number of carbon versus number of double bond and the position

of the first double bond from the methyl end (also called ω end) of the chain comes last.



Nomenclature of Fatty Acids

Names		Abbreviations	
trivial	IUPAC	carboxyl-reference	ω - reference
palmitic acid	hexadecanoic acid	16:0	16:0
stearic acid	octadecanoic acid	18:0	18:0
oleic acid	9-octadecenoic acid	18:1 Δ^{9}	18:1 (ω-9)
linoleic acid	9,12-octadecenoic acid	18:2 $\Delta^{9,12}$	18:2 (ω-6)
linolenic acid	9. 12. 15-octadecenoic acid	18:3 $\Delta^{9,12,15}$	18:3 (ω-3)

Figure 2.2 Four common ways of designating fatty acids. Figure on top shows the use of Greek letters to designate carbons. The carbon next to the –COOH group is designated α; the next one is β and so forth. The most distant carbon is designated ω. Sometimes carbon atoms close to the ω carbon are designated in relation to it. E.g., the third from the end is ω-3 (omega minus 3)

2.2 Formation of fatty acids

Intensive work has been done in various species in order to understand PUFA biosynthesis better. Carbon chain of the fatty acid is formed by combining eight two-carbon (2C) units derived from acetyl-CoA. These acetyl-CoA are derived from pyruvate-CoA that comes from the breakdown of glucose. The malonyl-CoA is an activated unit formed from the acetyl-CoA at the expense of ATP, which are then added to the growing chain by malonyl-CoA decarboxylation. The cycle continues until a 16C saturated fatty acids is formed, called palmitic acid, or palmitate (C16:0). (**Figure 2.3**)

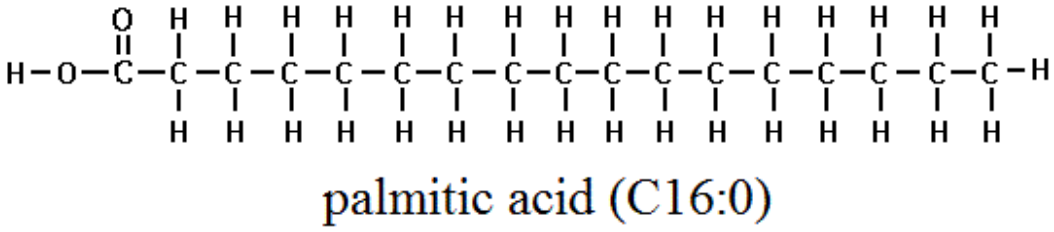


Figure 2.3 Molecular structure of palmitic acid.

This whole reaction is catalyzed by acetyl-CoA decarboxylase and fatty acid synthases (FAS). Acetyle-CoA decarboxylase converts acetyle-CoA to Malonyl-CoA. Then FAS use one acetyl-coA and seven malonyl-CoA molecules to synthesize the 16C palmitate (Lomakin et al., 2007). Eukaryotes FAS comprises of several functional domains, which includes acetyl transferase (AT) , acyl carrier protein (ACP), β-enoyl reductase (ER), β-ketoacyl reductase (KR), β-ketoacyl synthase (KS), dehydrase (DH), malonyl-CoA-acetyl-CoA-ACP (MAT), malonyl/palmitoyl transferase (MPT), phosphopantetheinyl transferase (PPT) and thioesterase (TE). Each domain carries out its own unique function and role in fatty acids formation (Lomakin et al., 2007). However, this elongation by FAS usually ends at palmitate (C16:0), a point where the fatty acid is detached from the enzyme. The elongation of fatty acids longer than this may require another class of enzyme, called the Elongase of Very Long chain fatty acids (Elovl).

2.3 Polyunsaturated fatty acids (PUFAs)

Fatty acids that carry two or more double bonds along their carbon backbone are known as PUFAs. They can be categorized as either ω3 or ω6. ω3 (also called n-3) PUFAs have the first C=C double bond between 3rd and 4th carbon counting from the methyl terminus, while ω6 (n-6) PUFAs have the first C=C double between 6th and 7th carbon. They play important physiological roles in organism at many levels.

Many aquatic algae can form LC-PUFAs from LIN and ALA. While many higher plants and terrestrial animals have virtually lost the ability to make LC-PUFAs or cannot synthesize LC-PUFAs at levels sufficient to meet their developmental needs.

2.3.1 Essential fatty acids

While Δ6 desaturase catalyzes the double bond formation between 6th and 7th carbon counting from the carboxyl end, the ω6 desaturase catalyzes desaturation at carbon counting from the methyl end (**Figure 2.2**). Stearoyl-ACP desaturase found only in the stroma of plant plastids enable for desaturation at Δ9 position the saturated stearic acid (18:0), forming monounsaturated oleic acid (18:1n-9) (Shanklin & Cahoon, 1998). Following this, an ω6 desaturase in higher plants) (Martin et al., 2002) or an Δ12 desaturase in cyanobacteria (Chi et al., 2008) is required to desaturate the oleic acid at Δ12 position, forming LIN (18:2n-6). Some yeast strains have Δ12 desaturases and therefore are capable of producing their own PUFAs (Pereira et al., 2003). From 18:2n-6, for formation of ALA (18:3n-3) involve the ω3 or Δ15 desaturases (Harwood, 1996) (Kotajima et al., 2014). Some protozoans (Jones et al., 1993) (Sayanova et al., 2006) and fungi (Zhang et al., 2007) were reported to have bifunctional desaturases showing both Δ12 and Δ15 activities. However, all vertebrates cannot form their own LIN and ALA as they lack the necessary Δ12/ω6

and Δ15/ω3 desaturases (Torcher et al., 1998). These are known as essential fatty acids (EFAs) and must be obtained from food (Anderson et al., 2009). Once consumed, the LIN and ALA can be further processed to LC-PUFAs through a series of desaturation and elongation steps (Anderson et al., 2009) (Sealls et al., 2008).

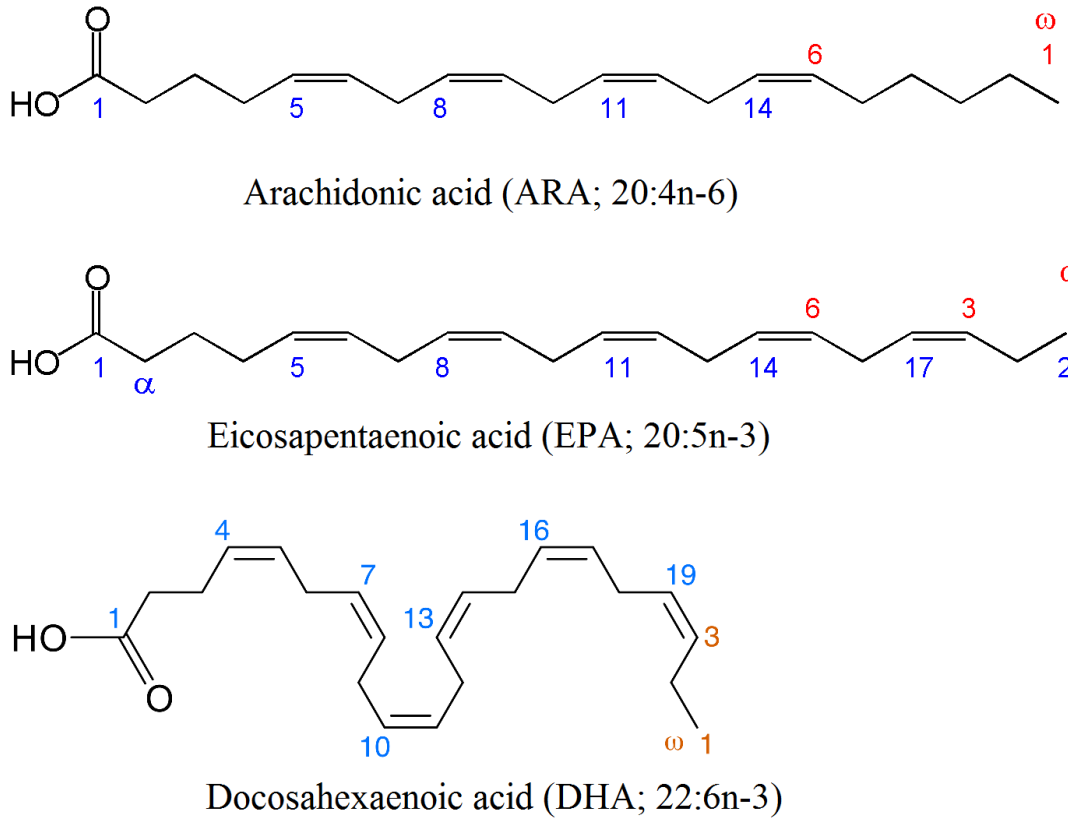


Figure 2.4 Structures of LC-PUFAs.

2.4 Importance of LC-PUFAs to human

Long chain polyunsaturated fatty acids (LC-PUFAs) have more than 20 carbons and more than 3 double bonds (Arts et al., 2009), such as the arachidonic acid (ARA; 20:4n-6), eicosa-pentanoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22-6n-3) (**Figure 2.4**). The functions of LC-PUFAs in human are diverse and complex, from modulation of dynamic properties of membranes, to regulation of gene expression, and production of active mediators. These LC-PUFAs have been

reported to have beneficial effect to many diseases such as cancer (Serini et al., 2011), heart diseases (Kris-Etherton et al., 2002), rheumatoid arthritis (Horrocks and Yeo, 1999), asthma (Bilal et al., 2011), lupus, visual acuity, kidney disease, respiratory disease, peroxisomal disorder, dermatitis, psoriasis, cystic fibrosis, schizophrenia, dyslexia, malaria, multiple schlerosis and even migraine headaches. Studies showed that a lower n-3 LC-PUFA level was correlated with alcoholism, depression or both (Nieminen et al., 2006).

All these years, DHA have received much attention due to its essential functions in the neural and visual system development. Deficiency in DHA and EPA at critical level of human neurodevelopment result in suboptimal neurotransmitter systems, which in turns, limit the regulation of the limbic system, which is the center for emotion and memory (Hibbeln et al., 2006). ARA and its metabolites are crucial for growth and maturation of multiple organs, including the immune system and gastrointestinal tract (Innis, 1991) (Hadders--Algra et al., 2007) (Field et al., 2001) (Stenson, 2007). For such a simple molecule to affect so many seemingly unrelated processes, LC-PUFAs must function at a level fundamental to most cells.

2.4.1 Fish consumption and medical significance of LC-PUFAs

In vivo studies in human showed that only less than 0.5% of ALA is converted to DHA (Plourde & Cunnane, 2007). This suggested that human must have adequate LC-PUFAs in the diet for optimal physiological development. Since the first AHA (American Heart Association) Science Advisory "Fish Consumption, Fish Oil, Lipids, and Coronary Heart Disease," the beneficial effect of ω3 long-chain polyunsaturated fatty acids (LC-PUFAs), especially EPA and DHA, are important for human health has been widely accepted. (Kris-Etherton et al., 2003) These LC-PUFAs have been suggested to prevent heart diseases and associated precursor

conditions such as metabolic syndrome and obesity (Swanson et al., 2012). Another study showed that fish consumption help reducing the risk of getting ischemic heart disease and stroke mortality across 36 countries (Zhang et al., 1999). A study in Japan reported a corelation of fish intake and reduced cardiovascular disease risk factor such as obesity, hypertension, glycohemoglobin in a dose dependent fashion (Mizushima et al., 1997). As aquatic plankton and algae are primary producers of PUFAs, and many fish species are able to convert the PUFA to LC-PUFAs, fishery represents a relatively easy way to obtain LC-PUFAs (Ruiz-Lopez et al., 2015).

2.5 Fish Oil and LC-PUFAs

In comparison to terrestrial oil, fish oil and other marine oils (seal and whales) are well known for their much lower saturated oils and high proportions of omega-3 LC-PUFAs (Dyerberg et al., 1975). Oily fish such as sardines, sprat, salmon, and mackerel deposit their fat reserves throughout their flesh and tissues and these lipids are commonly referred to as “fish body oil”. Triacylglycerols (TAG) is the major component of fish body oils, contributing in excess of 90% of the total fatty acid composition. In comparison, fish liver oils are extracted from fish liver, usually from cod, halibut and shark. Fish liver oil is a rich source for vitamin A (strong oxidant) and D (maintaining healthy joint).

Fish oils contain an abundance of EPA and DHA. EPA content can range from 7.6% to 22.0% in Peruvian anchovy oil, 6.1% to 8.0% in capelin oil, 3.9% to 15.2% in herring oil. For DHA, the percentage can be anywhere between 9.0% to 12.7% in Peruvian anchovy oil, 3.7% to 6.0% in capelin oil, and 2.0% to 7.8% in herring oil. The oil of the Peruvian anchovy also contains some unsaturated and monosaturated fatty acids: 17% to 19.4% of palmitic acid (16:0), 9.0% to 13.0% of

16:1n-7 and 10.0% to 22.0% of oleic acid (18:1n-9). On the other hand, the content of 20:1n-9 and 22:1n-11 can be as high as 17.0% and 15.4% respectively in capelin oil. Similarly, herring (*Clupea harengus*) oil also contains high levels 20:1n-9 (7.3% to 19.9%) and 22:1n-11 (6.9% to 30.6%) (De Silva et al., 2011). Despite the huge variation of EPA and DHA content within and between species, MUFA are generally present in low concentrations and EPA and DHA come in greater abundance in fish oil.

A lot of LC-PUFA research have focused on fish compared to other terrestrial species mainly because the fish constitutes a big part of human diet that form the major source of n-3 LC-PUFAs such as EPA and DHA. It has become a more important topic due to global decline marine fisheries (Sargent & Tacon, 1999). Not only that, a big portion of animal feed relies on the marine fisheries as the major source of LC-PUFAs (Simopoulos, 1999). Perhaps this overwhelming demand of marine fish resources can be resolved by fish farming. However, the farmed species do require adequate level of LC-PUFAs in their diet for physiological development. These EPA and DHA in the feed are mainly extracted from wild captured marine fish, such as sand eels, capelin, and anchovies (Sargent & Tacon, 1999), which is not really sustainable in long run as these marine fish are finite resources themselves. Vegetable contains quite an amount of LIN and ALA. For example, corn oil and sunflower oil contain high amount (between 60~70%) of 18:2n-6. Linseed oil can contain up to 54.24% 18:3n-3 (Zambiazi et al., 2007). Thus, as one might ask, can the fish make use of these C_{18} PUFAs from vegetable oil and make their own LC-PUFAs? If so, how efficient is the conversion rate in particular farmed fish species? Does that meet the developmental need? With the aim to replace fish oil with

vegetable oil in aquaculture feed, the understanding of LC-PUFAs biosynthesis from the C_{18} precursors at molecular basis becomes very important.

Moreover, a wide variation in PUFA biosynthesis ability is observed among fish species (Sargent et al., 1989) (Sargent et al., 1995) (Sargent & Tacon, 1999). Freshwater species seem to be more capable of producing LC-PUFAs from their C_{18} precursor fatty acids, whereas most piscivorous and carnivorous marine species have virtually lost the ability (Owen et al., 1975) (Henderson & Torcher, 1987). Knowing the genetic difference in these enzymes provides invaluable knowledge about the mode of actions in the LC-PUFA biosynthesis pathway(s), and provides new insights of how these genes could have evolved since the ancient time.

2.6 Overview of LC-PUFA biosynthesis

Figure 2.5 illustrates the pathway of PUFA biosynthesis that was demonstrated in rats by Sprecher's and coworkers in late 1990s (Sprecher et al., 2000). In 2010, Li and colleagues found the Δ4 desaturase in rabbitfish and indicated an alternative pathway of DHA synthesis from EPA (Li et al., 2010). Generally, the synthesis of LC-PUFAs requires the delta 6 desaturase (Δ6D) to insert a double bond between 6^{th} and 7^{th} carbon counting from the carboxyl end of the fatty acid molecule, converting linoleic acid (LIN, 18:3n-3) and α-linolenic acid (ALA, 18:2n-6) to steridonic acid (18:4n-3) and gamma-linolenic acid (18:3n-6), respectively. These intermediates will then go through an elongation step and followed by a second desaturation step by delta 5 desaturase (Δ5D), forming 20:4n-6 (ARA) and 20:5n-3 (EPA). The Δ5D introduces double bond at the 5^{th} carbon counting from the carboxyl end of the carbon chain. The ARA and EPA are further elongated to 24-carbon via two elongation steps, and inserted a double bond into the Δ6 position via Δ6D activity, which are then shortened to docosapentaenoic acid (Osbond acid, 22:5n-6) and docosahexaenoic acid (DHA, 22:6n-3) via β-oxidation by acyl-coA oxidase (Schmitz & Ecker, 2008). First and second Δ6 desaturation steps were reported to be catalyzed by the same desaturase enzyme (D' Andrea et al., 2002) (Dreesen et al., 2006). Morever, as both n-3 and n-6 fatty acids competes for the same reaction sites of the participating elongases and desaturases, excess of n-3 fatty acids could cause a significant decrease in the conversion of n-6 PUFA or vice versa (Simopoulos, 1999).

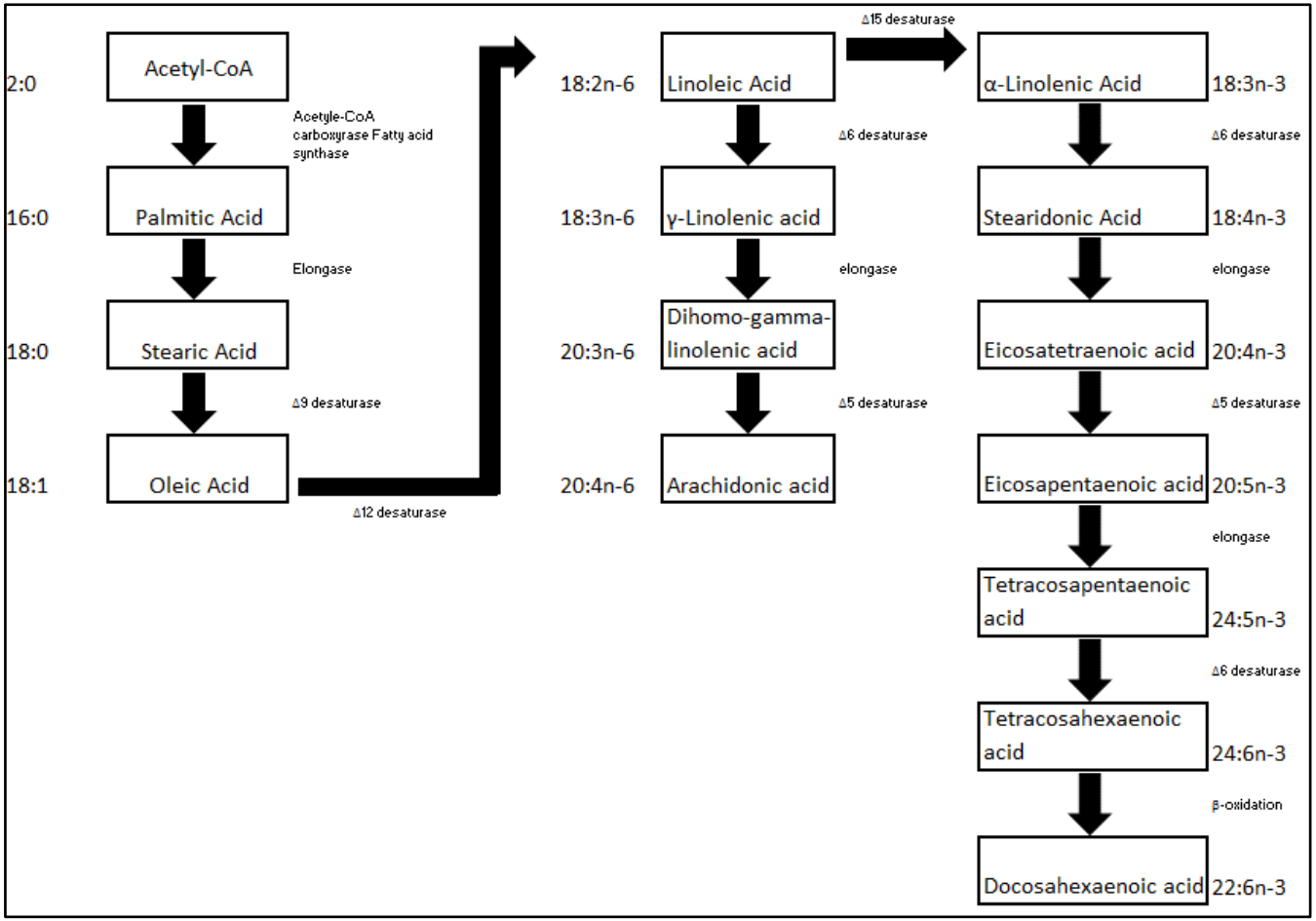


Figure 2.5 Schematic illustration of the n-3 and n-6 LC-PUFA formation from palmitic acid as described by Sprecher and coworkers in late 1990s.

2.7 Fatty acyl desaturases and elongases

Although a lot of effort has been done in the past decades, yet, the complete pathway of LC-PUFA biosynthesis is far from understood. Functional studies on desaturases and elongases have been feeding new information that gives clearer image of the overall pathway and the mode of action of these enzymes. It is important to understand these genes, their products and look into those factors that influence their expression and functions at molecular level. (Tocher et al., 1998)

2.7.1 Desaturase

As described in section 2.6, the formation of ARA (20:4n-6) and EPA (20:5n-3) from their C_{18} precursors by sequential route of Δ6-desaturation → elongation → Δ5-desaturation (Cook and McMaster, 2004). Subsequent studies showed that some Δ6 desaturases also possess Δ8 activity, which allows an alternative pathway for the formation of ARA & EPA (Park et al., 2009). The formation of DHA from EPA requires two further elongations, one Δ6 desaturation and one peroxisomal chain shortening step (Sprecher et al., 2000). These Δ5 and Δ6/Δ8 desaturase are encoded by *Fads* genes. They belong to the family of "front-end desaturase", which add double bond(s) to the carbon counting from the carboxyl end of the fatty acid molecule. Functional Δ5 and Δ6 desaturases can be found in organisms span through wide phylogenetic lineages such as fungi, protest, invertebrate and vertebrates (Alonso et al., 2003).

In human, the Δ5 and Δ6 desaturase are encoded by *Fads1* and *Fads2* respectively. These genes, together with *Fads3*, reside in the same chromosome (chromosome 11) and share a remarkably similar exon / intron organization (Marquardt et al., 2000). Function of mammalian Fads3 was not known at the time it

was isolated in 2010 (Pédrono et al., 2010). No Δ5, Δ6, Δ9 desaturation activity was reported for the Fads3 and therefore does not involve in PUFA biosynthesis.

From a functional point of view, the Δ5 desaturase genes should have evolved from a Δ6 ancestor as the substrate of Δ5 desaturase is produced by the Δ6 desaturase in the pathway (Alonso et al., 2003). Evidence showed that the occurrence of Δ5 desaturase was resulted from gene duplication from Δ6 desaturase and diverged independently (Michaelson et al., 1998) (Marquardt et al., 2000).

Like the mammalian desaturases, the nematode *Caenorhabditis elegans* Δ5 and Δ6 desaturase are encoded by distinct *Fads*-like genes that also reside in the same chromosome IV (Michaelson et al., 1998) (Napier et al., 1998). Nevertheless, the nematode *Fads* genes have higher sequence homology to each other than the mammalian *Fads* gene group. Phylogenetic analysis of *Fads*-like genes of various organisms demonstrated that the ancient Δ5 desaturase of unicellular slime mold, fungi, algae and mosses form a distinct branch (**Figure 2.6**) that does not include the nematode Δ5 desaturase, suggested that the nematode Δ5 desaturase evolved later from duplication of its Δ6 desaturase, similar to the context of human *Fads1* and *Fads2* (Sperling et al., 2003).

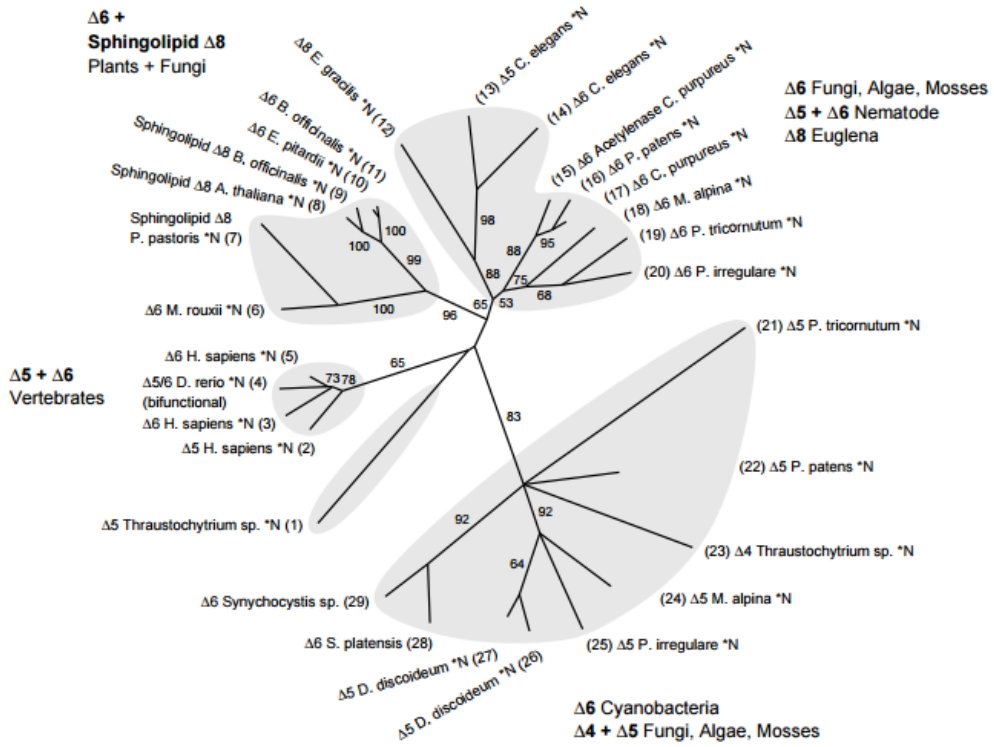


Figure 2.6 Unrooted phylogram for the grouping of front-end desaturases (taken from Sperling et al., 2003).

In teleosts, which has often been used as a model to study PUFA biosynthesis in vertebrate species, is showing a rather interesting evolutionary storyline. The scenario is different from the pattern found in mammals. Cloning and functional studies have revealed at least three distinct categories in fish. A single bifunctional Δ5 / Δ6 desaturase that shows sequence homology to human *Fads2* was first cloned from the zebrafish (*Danio rerio*) (Hastings et al., 2001). A much later study revealed a marine herbivore species, the rabbitfish (*Siganus canaliculatus*) possesses two bifunctional desaturase genes, with one having Δ5/Δ6 desaturation ability while the other has Δ5/Δ4 function. Both are *Fads2*-like (Li et al., 2010). Apart from the bifunctional desaturases, Atlantic salmon (*Salmon salar*) appear to have two separate *Fads2*-like Δ5 and *Fads2*-like Δ6 desaturase (Zheng et al., 2004) (Torcher et al., 2006) (Hastings et al., 2001). The third group comprises of most other teleost species, which only single *Fads2*-like Δ6 gene was isolated (Torcher, 2010) (Zheng et al., 2004)

It is interesting that all the fish desaturases characterized to date are *Fads2*-like regardless of Δ4, Δ5 or Δ6 functions. The *Fads1*-Δ5 appears to be mammalian specific. Comparative genomics study attempted to underpin the evolution of these genes found *Fads1*-Δ5 and *Fads2*-Δ6 in the ancient gnathostome (Castro et al., 2012). These ancient *Fads1* and *Fads2* subsequently went through a series of gene loss and expansion episodes that are mainly driven by habitat-specific LC-PUFAs abundance. The complete loss of *Fads1* in marine teleost was explained by the availability of preformed LC-PUFAs in their diet.

The appearance of sharks was dated back 400 Ma years ago before the thriving of diatoms and dinoflagellates. Thus both *Fads1* and *Fads2* genes may have been required to survive in this relatively LC-PUFAs poor environment. In contrast, the time of teleost emerged coincides with the rise of the diatoms and dinoflagellates, which explains the subsequent lost of *Fads1* Δ5 gene in teleost. (Castro et al., 2012)

High carnivores such as cats completely lost the ability to form DHA from ALA de novo (Rivers et al., 1975). Species inhabiting freshwater and terrestrial habitat that are fueled by green plants tend to have diversified desaturase genes to complement with the lack of LC-PUFAs in their diet (Leaver et al., 2008) (Sargent et al., 1989) (Sargent et al., 1995). However, despite being a marine species, the rabbitfish has a feeding preference towards some sea grasses, including eelgrass, which are naturally lack of LC-PUFAs (Gillan et al., 1984), has retained the genes to synthesize required LC-PUFAs endogenously.

Together with the findings of some fish desaturases are capable of Δ8 desaturation (Monroig et al., 2011b), the discovery of desaturase with Δ4 activity in rabbitfish (Li et al., 2010), Senegalese sole (Morais et al., 2015) and snakehead (Kuah et al., 2015) changed the overall perception of LC-PUFA biosynthesis

pathway. Δ4 desaturase enables a more direct route for DHA (22:6n-3) and DPA (22:5n-6) formation from their C_{20} n-3 and C_{20} n-6 precursors respectively. Instead of the single pathway as described in **Figure 2.5**, it has now been accepted that there are alternative pathways for C_{20} and C_{22} LC-PUFAs formation in verterbrates. (**Figure 2.7**)

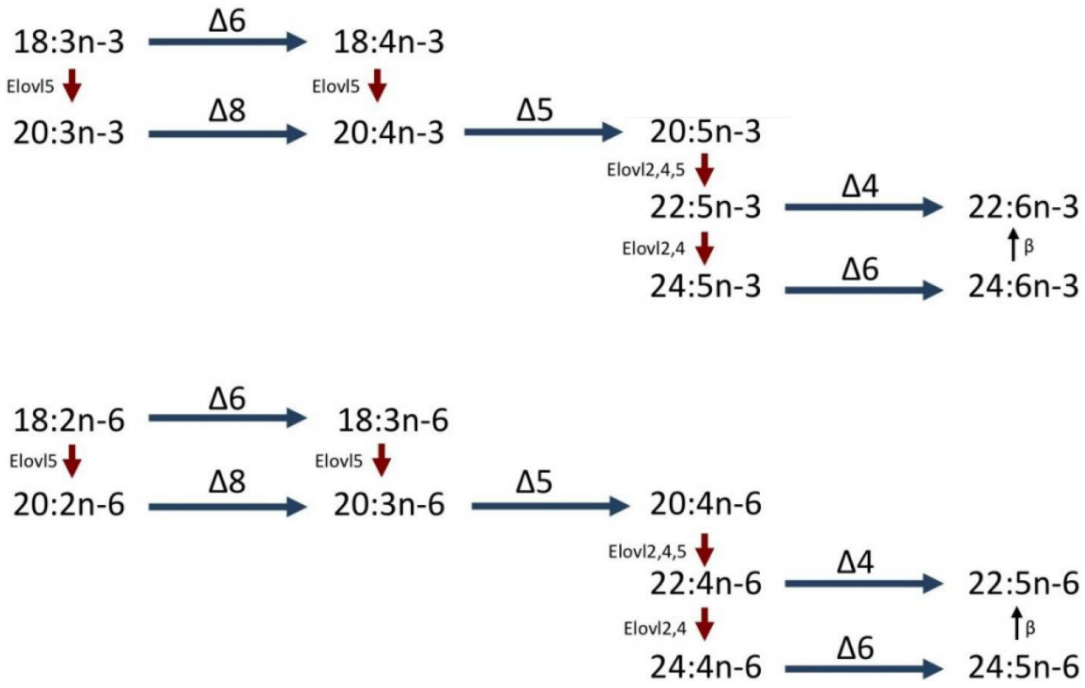


Figure 2.7 The synthesis of LC-PUFAs from LIN and ALA. Vertical downwards are catalysed by elongases (Elovl). Horizontal arrows represent desaturation at Δx position. Vertical upward arrow indicates β-oxidation shortening step (Sperling et al., 2003).