MOLECULAR CLONING, FUNCTIONAL CHARACTERIZATION AND DEVELOPMENT EXPRESSION OF A NEW ELONGASE WITH POTENTIAL ROLE OF LONG-CHAIN POLYUNSATURATED FATTY ACID BIOSYNTHESIS IN A TELEOST (*Danio rerio*)

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by

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ALA	α-linolenic acid
ANOVA	analysis of variance
ARA	arachidonic acid
BF_3	boron trifluoride
BLAST	basic local alignment search tool
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
DGLA	dihomo-γ-linolenic acid
dH_2O	autoclaved sterile water
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonulease
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
DreElovl	*Danio rerio* (zebrafish) *Elovl*
EDTA	ethylenediaminetetraacetic acid
Ef1a	elongation factor 1-alpha
Elovl	elongation of very long chain fatty acids
EPA	eicosapentanoic acid
ER	endoplasmic reticulum
Fads	fatty acyl desaturase
FAME	fatty acid methyl ester

GC	gas chromatography
GLA	γ-linolenic acid
HUFA	highly unsaturated fatty acid
HYB^+	hybridization buffer with transfer ribonucleic acid
HYB^-	hybridization buffer without transfer
IPTG	isopropyl β-D-thiogalactopyranoside
ISH	*In situ* hybridization
JTT	Jones-Taylor-Thornton
KCl	potassium chloride
LA	linoleic acid
LB	Luria-Bertani
LCFA	long chain (saturated) fatty acid
LC-PUFA	long-chain polyunsatured fatty acid
MAB	Maleic acid buffer
MEGA	Molecular Evolutionary Genetic Analysis
$MgCl_2$	magnesium chloride
MUFAs	monounsaturated fatty acid
n or ω	omega
NCBI	National Center for Biotechnology Information
NaCl	sodium chloride
NaOH	sodium hydroxide
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction

PFA	paraformaldehyde
PTU	1-phenyl 2-thiourea
pH	potential of hydrogen (scale of acidity)
PUFA	polyunsaturated fatty acid
qPCR	quantitative real-time PC
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SCMM-U	*S.cerevisiae* minimal medium without uracil
SFAs	saturated fatty acid
SSC	saline sodium citrate
SPSS	Statiscal Package for the Social Science
TBE	Tri-borate-EDTA
Tris	Tris(hydroxylmethyl)aminomethane
UV	ultraviolet
VLC-PUFA	very long-chain PUFA
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPD	yeast extract peptone dextrose (medium for yeast growth)
Δ	position of the double bond from the carboxyl end of the molecule

PENGKLONAN MOLEKUL, PENCARIAN FUNGSI DAN EKSPRESI PERTUMBUHAN SUATU ELONGASE BAHARU DENGAN PERANAN POTENSI BIOSYNTHESIS ASID LEMAK POLITAKTEPU RANTAI PANJANG DALAM IKAN (*Danio rerio*)

ABSTRAK

Elongase rantaian panjang (ELOVL) amat penting untuk sintesis asid lemak politaktepu rantai panjang (LC-PUFA). Pada masa kini, tujuh ahli keluaga ELOVL (ELOVL1- ELOLV7) telah ditemui dalam mammalia. Kini, ahli yang baru dari ELOVL telah ditemui dalam ikan. Oleh itu, zebrafish (*Danio rerio*) telah dipilih sebagai subjek untuk penyelidikan ini. Zebrafish dipilih kerana ia merupakan model yang bagus untuk mengkaji metabolis lemak dan mempunyai laluan biosintesis LC-PUFA yang lengkap. Selain itu, zebrafish juga diiktiraf sebagai model yang baik untuk mengkaji pertumbuhan. Minat juga ditimbul untuk mengetahui kapasiti pemanjangan dan ekspresi elongase ini dalam pertumbuhan dan dalam tisu ikan dewasa. Gen lengkap untuk elongase A ini (NM_001076593.1) dan elongase B ini (BC095712.1) telah diklonkan dengan mengunakan rujukan yang boleh didapati dalam National Center for Biotechnology Information (NCBI). Pokok filogenetik telah dibina dan hasilnya menunjukan kedua-dua elongase ini merupakan ahli keluarga Elovl yang berbeza daripada ahli keluarga Elovl yang lain. Oleh itu, elongase A ini telah diberi nama Elovl8a dan elongase B ini telah diberi nama Elovl8b. Selain itu, pokok filogenetik juga mengumpulkan Elovl8 dengan gen Elovl4-like sebagai satu kumpulan. Pokok filogenetik telah menunjuk Elovl2, Elovl4, Elovl5 dan Elovl8 mempunyai moyang yang sama. Selain itu, Elovl2, Elovl4 dan Elovl5 mungkin merupakan hasil gen perkembangan daripada Elovl8. Pencarian fungsi menunjukan Elovl8b

mempunyai fungsi untuk memanjangkan substrak asid lemak monotaktepu (MUFA) dan juga C18 dan C20 substrak asid lemak politaktepu (PUFA) tetapi Elovl8a tiada fungsi yang demikian. Hasil analisis ekspresi gene dalam tisu dewasa ikan menujukan *elovl8a* mengekspres dengan tinggi dalam mata dan diikuti dengan otot, insang, otak dan usus. Sebagai perbandingan, *elovl8b* menunjukan ekspresi yang tinggi dalam usus dan diikuti dengan hati, otot, insang dan otak. Dalam pertumbuhan, *elovl8a*.tidak menunjukan perbezaan yang signifikan antara masa persenyawaan. Ekspresi *elovl8b* dalam pertumbuhan menunjuk corak perningkatan dan mencapai tahap pertinggian pada 120 jam selepas persenyawaan. Corak pengekspresen ruang-masa mencadangkan *elovl8a* mungkin terlibat dalam pertumbuhan neuromast dari 72 jam selepas persenyawaan sehingga 120 jam selepas persenyawaan. Selain itu, *elovl8b* mungkin terlibat dalam pertumbuhan hati dan usus. Hasil daripada kedua-dua penyelidikan menunjukan kepentingan *elovl8a* dan *elovl8b* semasa pertumbuhan. Kesimpulannya, hasil analisa dan penyelidikan menunjukan elovl8a merupakan kumpulan yang berbeza dengan ahli keluaga *elovl* yang lain. Oleh yang sedemikian, *elovl8* boleh diiktiraf sebagai ahli keluarga *elovl* yang baru.

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ABSTRACT

Elongase of very long-chained fatty acid (ELOVL) is very important in synthesising long-chained polyunsaturated fatty acid (LC-PUFA). At present, seven members of ELOVL family (ELOVL 1 – ELOVL 7) have been found in mammals. Recently, a putative new Elovl was found in teleost. In order to study and examine the function of this new Elovl, zebrafish (*Danio rerio*) was chosen. Zebrafish is a viable lipid metabolism model with a complete LC-PUFA biosynthesis pathway and also a very good biological development model. An interest has arisen to determine the elongation capacity and also expression of this elongase of interest during development and in adult tissues. The full length of elongase of interest A (NM_001076593.1) and elongase of interest B (BC095712.1) genes were successfully cloned from the sequence available in National Center for Biotechnology Information (NCBI). A phylogenetic tree was constructed and revealed elongases of interest were grouped as a distinct group from the others elongases (Elovl1-7). Therefore they were named as *elovl8a* and *elovl8b* based on the result obtained from the phylogenetic tree. Other than that, Elovl2, Elovl4, Elovl5 and Elovl8 were found originated from the same ancestor and Elovl2, Elovl4 and Elov5 could be the result of gene expansion from Elovl8. Functional characterization of both elongases showed zebrafish Elovl8b converted monounsaturated fatty acid (MUFA), together with C18 and C20 polyunsaturated fatty acid (PUFA) substrates while Elovl8a lacked in these activities. Result from Weblogo analysis showed the elongase motif region of Elovl8 is rigid and

consistent. Expression of both elovl in adult tissues were determined by using quantitative PCR (qPCR), where *elovl8a* showed high expression in eyes, followed by muscle, gills, brain and intestine. In comparison, *elovl8b* showed high expression in intestine followed by liver, muscle, gills and brain. In development, *elovl8a* showed no significant different between stages while expression level of *elovl8b* showed gradually increase and reached the highest in 120hpf. The result from spatio-temporal expression pattern suggested that *elovl8a* gene involved in development of neuromast starting from 72hpf to 120hpf while *elovl8b* gene involved in development of intestine and liver from 72hpf to 120hpf. Both results from gene expression and spatio-temporal expression were similar which showed the possibility of expression and importance of *elovl8a* and *elovl8b* during development. Overall, the results from present study strongly suggested that zebrafish *elovl8* is distinct from current seven *elovl* family member. Besides that, this research also reduced the possibility of *elovl8* to be group as paralog gene of *elovl4* as suggested by other researcher. Therefore, this group of *elovl8* are prepared to be classified as new *elovl* member.

CHAPTER ONE: INTRODUCTION

1.1 Research background

Fatty acids are carbon chained with methyl group and carboxyl group at two different ends (Rustan & Drevon, 2001). Fatty acids are classified into saturated fatty acids, monounsaturated fatty acid, and polyunsaturated fatty acid. Categorise under polyunsaturated fatty acid, long chained polyunsaturated fatty acids (LC-PUFAs) are one of the essential element for normal growth and great function of the central nervous system (Hamosh & Salem, 1998; Rustan & Drevon, 2005; Schuchardtl *et al*., 2010). LC-PUFAs can be further categorised into two families: n-3 (omega 3) and n-6 (omega 6) with n-3 derives from α-linolenic (ALA) acid while n-6 derives from linoleic acid (LA) (Hamosh & Salem Jr, 1998). Examples of LC-PUFAs include arachidonic acid (ARA: 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (Ferraz *et al*., 2019). Lack of LC-PUFAs might affect lipid profile, cause blood coagulation and inflammatory disorders (Schuchardt *et al.*, 2010). Other than that, DHAs are vital for the proper development of retina and brain (Abedi & Sahari, 2014).

In vertebrate, LC-PUFAs are gained through diet or synthesise endogenously from C18 polyunsaturated fatty acid (PUFA). Unfortunately, PUFA can only be obtained through daily food consumption as vertebrate cannot biosynthesis such PUFA *de novo* in the body (Ferraz *et al*., 2019). In mammals, PUFA cannot synthesise directly from precursor oleic acid and the efficiency rate of conversion into their respective LC-PUFA are low (Abedi & Sahari, 2014). Therefore, direct uptake of LC-PUFA through the daily meal is more effective (Abedi & Sahari, 2014). LA is mostly found in vegetable oil products while ALA is mostly found in green plants, botanical oils, and nuts (Chilton *et al.*, 2017). According to Ferraz *et al*. (2019), biosynthesis of

LC-PUFAs from the dietary essential C18 precursors need a synchronized action of the membrane-bound enzymes located in the endoplasmic reticulum. These enzymes employed are fatty acid desaturases (Fads) and elongation of very long chain fatty acid (Elovl) protein.

The Elovl enzyme elongates the fatty acid chain by adding two carbons into the carboxyl end and this process consists of four sequential reactions which are condensation, reduction, dehydration and reduction (Sassa & Kihara, 2014). To date, there are seven isozymes (ELOVL1 to ELOVL7) in Elovl family (Sassa & Kihara, 2014). Xue *et al.* (2014) mentioned that ELOVL1, ELOVL3, ELOVL6, and ELOVL7 prefer substrate like saturated and monounsaturated fatty acid (MUFA) while ELOVL2, ELOVL4 and ELOVL5 prefer PUFA as substrate. Other than that, *elovl* genes are also expressed differently across different tissues at the transcript level (Xue *et al*., 2014).

A research carried out by Xue and colleague in 2014 successfully cloned two *elovl* genes from Atlantic cod (*Gadus morhua*). Based on their phylogenetic tree result, they believed that both genes were two paralogous genes for *elovl4* gene and named them as *elovl4c1* and *elovl4c2*. Around the same time, our lab started to clone a corresponding homologous of this gene from striped snakehead (*Channa striata*). However, the result from the constructed phylogenetic tree was contradicted with work from Xue *et al.* (2014). This is because the putative labelled *elovl4c* was grouped as a distinct clade from *elovl4* (Kuah, 2015). This putative labelled *elovl4c* was opposed to be the paralogous gene for *elovl4*. Further research was carried out to examine the functional characterization of labelled putative *elovl4c* in striped snakehead. For further understanding, the homologous gene of this labelled putative *elovl4c* was cloned and functional characterization study was examined from siakap (*Lates*

calcarifer) and Nile tilapia (*Oreochromis niloticus*) also (Kuah, 2015; Han, 2017). All these cloned homologous genes showed similarity with less than 50% identical to any member of the ELOVL family. Functionally, the elongation activities of this putative *elovl4c* was different from other ELOVL family member.

In order to have a better understanding in this labelled putative *elovl4c* gene, a significant and representative biological model should be used. Zebrafish (*Danio rerio*) is a good model to study development, lipid metabolism and embryogenesis (Bhandari *et al.*, 2016). Molecular cloning, functional characterization and expression profile of several *elovl* genes during embryogenesis have been reported in zebrafish (Tay *et al.*, 2018). Other than that, zebrafish tissues also contain higher concentrations of PUFA than other mammalian tissues (Tay *et al.*, 2018). Last but not least, the biosynthesis pathway of LC-PUFA in zebrafish was accomplished (Tan *et al.*, 2010). These reasons make zebrafish a viable model for further understanding of the identity and also the function of the putative *elovl4c*.

1.2 Objectives

The aims of this study are:

- To determine the phylogenetic status of the putative *elovl4c* gene from zebrafish.
- To study the functional characterization of the putative Elovl4c protein elongase from zebrafish through *in-vitro* assay in yeast.
- To identify the expression distribution of putative *elovl4c* gene in different tissues of zebrafish.
- To determine the spatio- temporal expression pattern of putative *elovl4c* gene throughout the zebrafish embryogenesis.

CHAPTER TWO: LITERATURE REVIEW

2.1 Fatty acid

Naturally occurring fatty acids are originated from either triglycerides or phospholipids. Fatty acids are a group of aliphatic monocarboxylic acids (R-COOH) that make up part of a lipid molecule (Kuah, 2015). Most of the fatty acids have straight hydrocarbon chain, have even number of carbon atoms and rarely have branched. Fatty acids vary with 2 to 36 carbons and contain one or more double bonds in their hydrocarbon chain (Kuah, 2015). According to Rustan and Drevon (2005), fatty acids are carbon that chained with a methyl group and a carboxyl group at two different ends. The naming of fatty acids is determined by the position of the double bond that nearest to the methyl end and also the location of double bonds that counted from the carboxyl group (Figure 2.1) (Rustan & Drevon, 2005).

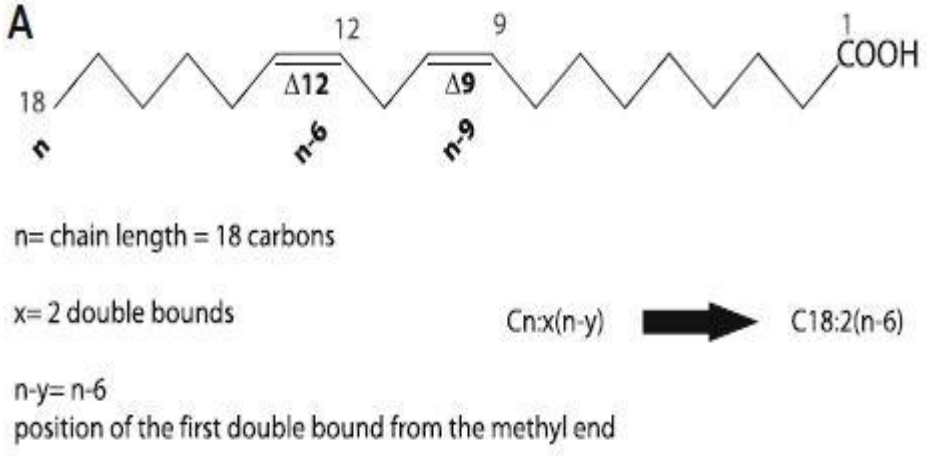

Figure 2.1: Example of fatty acid nomenclature (Retrieved from Guillou *et al*., 2010).

The number of double bonds and the length of the carbon chain are the ways to classify fatty acids (Sassa & Kihara, 2014). From Sassa and Kihara (2014) point of view, fatty acids with carbon chain length from 11 to 20 should classify as long-chain fatty acids (LCFAs) while fatty acids that have more than 20 carbon chain-length can

classify as very long fatty acids (VLFCs). There are also fatty acids that have more than 26 carbons chain-length which classify as ultra-long-chain fatty acids (ULCFAs). Other than that, fatty acids can further be classified as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) which based on the number of double bonds (Guillou *et al.*, 2010).

Table 2.1: Example of fatty acids classification that based on the number of carbon bond (Retrieved from Guillou *et al*., 2010)

	Symbol	**Common name**
Saturated fatty acids	C16:0	Palmitic acid
	C18:0	Stearic acid
	C20:0	Arachidic acid
	C22:0	Behenic acid
	C24:0	Lignoceric acid
Non essential unsaturated fatty acids	C16:1(n-7)	Palmitoleic acid
	C16:1(n-10)	Sapienic acid
	C18:1(n-9)	Oleic acid
	C20:1(n-9)	Gadaleic acid
	C22:1(n-9)	Erucic acid
	C20:3(n-9)	Mead acid
n-6 and n-3 polyunsaturated fatty acids	C18:2(n-6)	Linoleic acid
	C20:4(n-6)	Arachidonic acid
	C22:5(n-6)	Docosapentaenoic acid
	C18:3(n-3)	α – Linolenic acid
	C20:5(n-3)	Eicosapentaenoic acid
	C22:6(n-3)	Docosahexaenoic acid

2.2 Long-chain polyunsaturated fatty acid (LC-PUFAs)

Fatty acids with carbon chain-length between 18 to 20 or more are categorized as long-chain polyunsaturated fatty acids (LC-PUFAs). Fatty acids that fall under this category can be further categorized into two sub-family: ω3 (n-3) and ω6 (n-6), depends on the location of the first double bond count from the methyl end group (Abedi & Sahari, 2014). There are a few major and important examples of n-3 LC-PUFA such as α-linolenic acid (ALA), docosahexaenoic acid (DHA),

eicosapentaenoic acid (EPA) while the example of n-6 LC-PUFA include linoleic acid (LA) and arachidonic acid (ARA) (Abedi & Sahari, 2014).

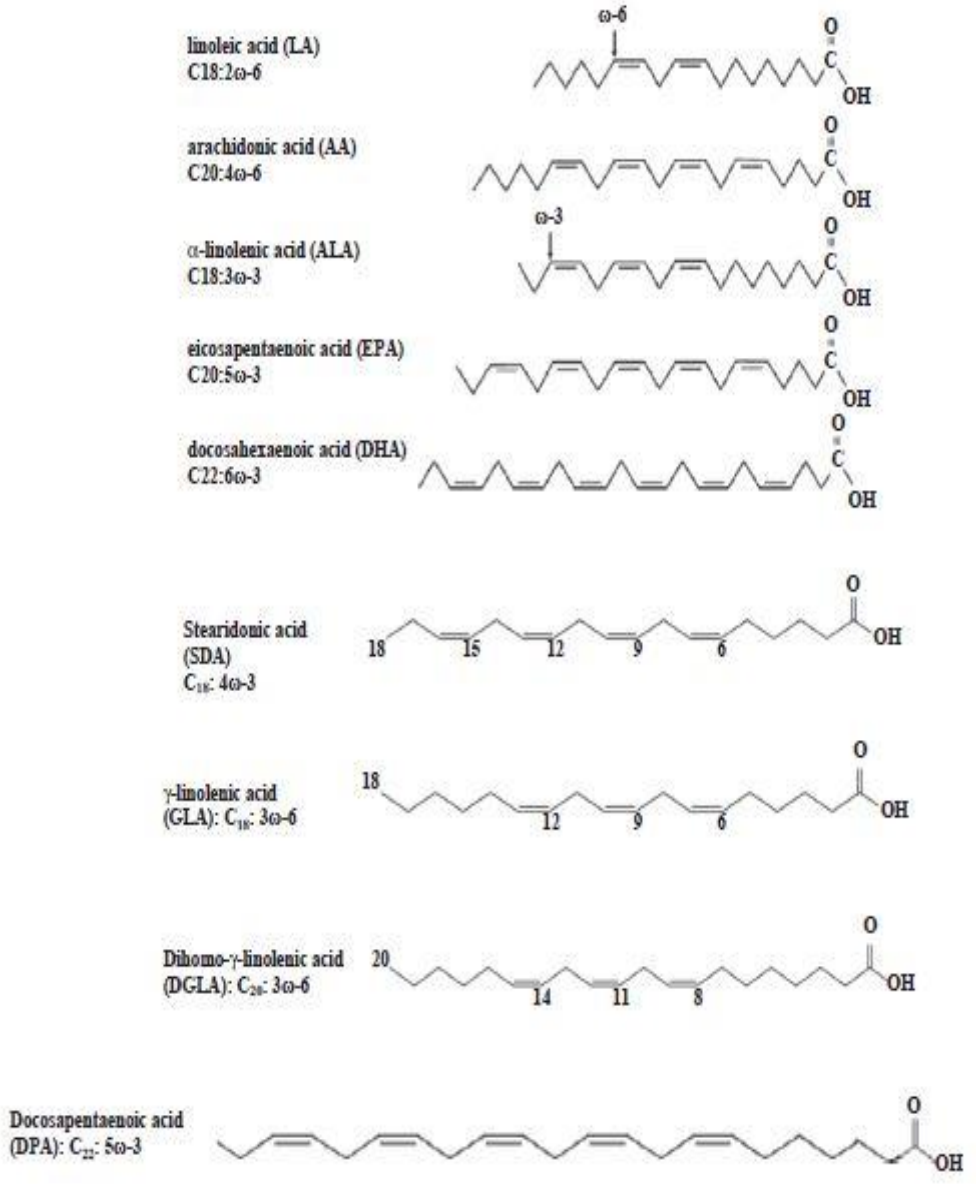

Figure 2.2: Structure of long-chain polyunsaturated fatty acid (Retrieved from Abedi & Sahari, 2014)

2.3 Importance and role of LC-PUFAs

Fatty acids are vital and essential for life as major metabolic energy sources, significant structural components for the membrane and as gene regulators (Guillou *et al*., 2010). Other than that, fatty acids also contribute to molecule signalling and metabolic regulation (Rangan & Smith, 2002). LC-PUFAs play significant roles in

ensuring normal cellular function (Monroig *et al*., 2009). Besides that, n-3 LC-PUFAs not only can promote health for cardiovascular and immune function but also can protect human against neurological and inflammatory condition (Monroig *et al*., 2011). Arachidonic acids (ARA) and eicosapentaenoic acids (EPA) act as precursors for biologically active compounds that initiate physiological processes such as inflammation, haemostasis, and reproduction (Monroig *et al*., 2009). Other than that, LC-PUFAs can reduce blood pressure, inhibit the synthesis of vaso aggressive low-density lipoprotein (LDL), and have beneficial effects on skin disease (Abedi & Sahari, 2014).

2.3.1 Importance of LC-PUFAs in aquaculture

Fishes are nutritionally high-quality food as fishes are a major source of omega-3 long chain polyunsaturated fatty acid (LC-PUFA) for human diet (Strobel *et al.*, 2012). Fish has been referred as one of the excellent and irreplaceable sources of LC-PUFA for man (de Souza, 2010). This lead to a global raised demand on fish consumptions. However, highly demanding of fish cause stagnant in worldwide marine fisheries. So, the human has to depend on farmed fish. Breeders not only try to increase production of fish but also try to ensure a better quality of fish for consumers. Traditionally, fish meal and fish oil are important ingredients for fish feed. However, feed manufacturers never stop to seek for other sources like poultry, soybeans and corns for replacement as the production cost is expensive (Nowosad & Kucharczyk, 2017).

According to Patil *et al*., (2005) PUFAs are essential fatty acids for the development of fish and fish oils are still the least expensive natural source of PUFA for a human. In aquaculture, PUFAs are essential to increase the probability of

spawning success (Ishak *et al.*, 2008). Besides that, having adequate and balance dietary ratio of ARA, EPA, and DHA are important for fish in order to have higher fertility and better reproductive performance (Ishak *et al*., 2008). Broodstock that feeds with sufficient LC-PUFAs concentration can improve egg morphology and viability (Watters *et al*., 2012). At the larval stage, LC-PUFAs are needed for neurological development, higher survival rate and better growth (Watters *et al*., 2012). Other than that, the high content of LC-PUFA in fish can aid in disease resistance by boosting and helping in immune system. However, the fish farmer tries to maintain the high nutritional quality of LC-PUFA in fish flesh just for human consumption.

2.3.2 Importance of LC-PUFA toward aquatic life

In a marine environment, primary production of PUFA occurs in photosynthetic microalgae, bacteria and heterotrophic protists (Monroig *et al*., 2013). Unlike the freshwater environment, the marine environment is rich with n-3 LC-PUFA as they are occupied with single-cell microorganism (Monroig & Kabeya, 2018). This is because microalgae have the ability to introduce first unsaturation into saturated fatty acid to produce monounsaturated fatty acid (Monroig & Kabeya, 2018). Besides that, the higher trophic level organism in marine environment act as 'trophic upgrader' where PUFAs are modified when transfer along the food chain (Monroig & Kabeya, 2018).

PUFA are essentially important toward marine bivalve, crustaceans, echinoderms and vertebrate. They are required for growth and for metabolites. Other than that, PUFA is important for reproduction, immunity, ion balance regulation and also for buoyancy control. LC-PUFA such as ARA is required to improve growth in scallop species for membrane fluidity (Parrish, 2013). Besides that, some LC-PUFA

is thought to work synergistically with well-known toxins such as domoic acid, diarrheic shellfish poison or neurotoxin brevetoxin (Parrish, 2013). These toxins are important as one of the protection feature or special characteristic for certain fish and dinoflagellates.

2.4 Biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFAs)

Biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFAs) is responsible by two main enzymes: elongases of very long fatty acid (Elovl) and fatty acyl desaturase (Fad) (Monroig *et al*., 2009).

According to Agaba *et al.* (2005), in vertebrate, biosynthesis of LC-PUFAs is started by sequential elongation and desaturation of linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3). The arachidonic acid (ARA, 20:4n-6) is synthesised firstly from LA to γ-linolenic acid (GLA, 18:3n-6) by $\Delta 6$ desaturation (Agaba *et al.*, 2005). Then elongates to dihomo-γ-linolenic acid (DGLA, 20:3n-6) and finally, ARA is synthesised from DGLA by desaturates at the $\Delta 5$ position (Agaba *et al*., 2005). The synthesis pathway for eicosapentaenoic acid (EPA, 20:5n-3) is quite similar to the synthesis of ARA but the synthesis of docosahexaenoic acid (DHA, 22:6n-3) requires further two steps of elongation (Agaba *et al*., 2005).

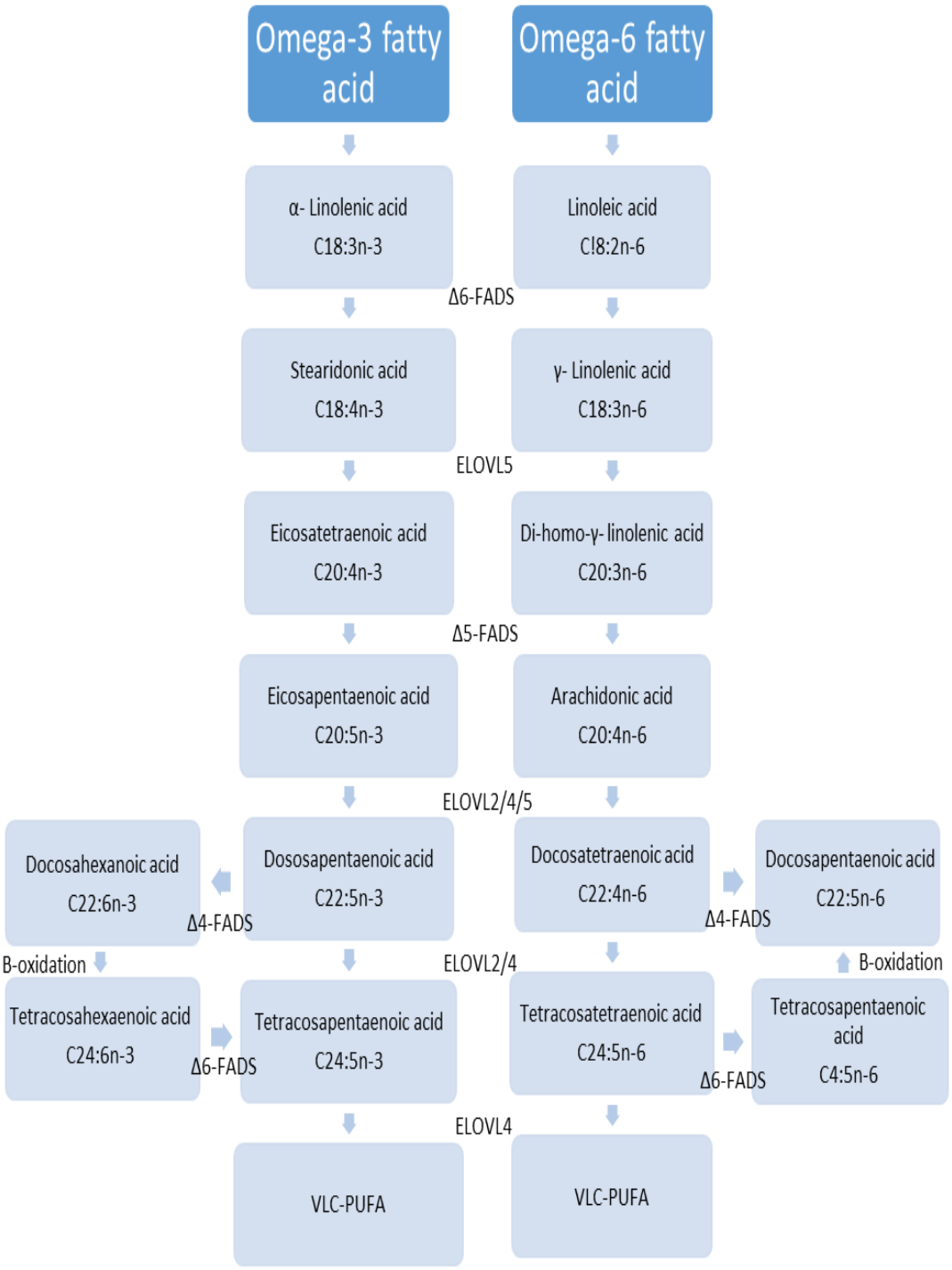

Figure 2.3: Biosynthesis pathways of LC-PUFA for a teleost from their respective precursors. (Adapted from Castro *et al.*, 2012).

2.6 Elongation of fatty acid

Elongation of fatty acids with more than 16 carbons chain occurs in endoplasmic reticulum which absorbs from food or synthesises *de novo* in the cytoplasm (Sassa & Kihara, 2014). According to Zhang *et al*. (2016), elongation of fatty acids occurs also in mitochondria and predominant microsomes. In simple word, elongation of fatty acids is a process where two carbons are added to the carboxyl end in each cycle (Sassa & Kihara, 2014). Fatty acid elongation is a serial process that consists of four main steps which include condensation, reduction, dehydration and reduction (Sassa & Kihara, 2014). Elongation of fatty acid is initiated by elongation of primer such as acetyl or propionyl with 2 carbon units that donated from malonyl-CoA and use nicotinamide adenine dinucleotide phosphate (NADPH) as reductant (Jakobsson, *et al*., 2006).

The first step of fatty acid elongation involves the condensation of an acyl-CoA molecule with malonyl-CoA and resulting in a β-ketoacyl-CoA molecule (Jakobsson *et al*., 2006). This involves a two-step reaction: the carboxylation of biotinlyl moiety then followed by transfer of carboxyl to acetyl-CoA acceptor (Rangan & Smith, 2002). This carboxylation process is a rate-limiting step (Brolinson, 2009). This process is catalysed by fatty acid elongase. This protein belongs to the ELOVL family with seven isozymes (ELOVL1-7) in mammals (Sassa & Kihara, 2014).

In the reduction step, the β-ketoacyl-CoA transforms to β-hydroxyacyl-CoA by 3-ketoacyl-CoA and uses NADPH as a cofactor (Brolinson, 2009). 3-ketoacyl-CoA reductase is the enzyme responsible for this reaction (Sassa & Kihara, 2014). Reduction from NADPH to β-keto reductase seems to involve cytochrome *b5* and cytochrome *P-450* reductase (Cook & McMaster, 2002).

In the third step or the dehydration step, the β-hydroxyacyl-CoA is dehydrated and become trans-2-enoyl-CoA (Jakobsson *et al.*, 2006). This step is catalysed by 3-hydroxyacyl-CoA dehydratase (HACD) with the presences of NADPH (Brolinson, 2009). This step appears to be another rate-limiting step as 3-hydroxyacyl-CoAs is significant but low in level (Sassa & Kihara, 2014). In the very last step which is another reduction step, trans-2-enoyl-CoA is converted to acyl-CoA (Sassa & Kihara, 2014). In this step, NADPH is required as a cofactor (Sassa & Kihara, 2014). The elongation cycle is now completed with extended acyl-chain.

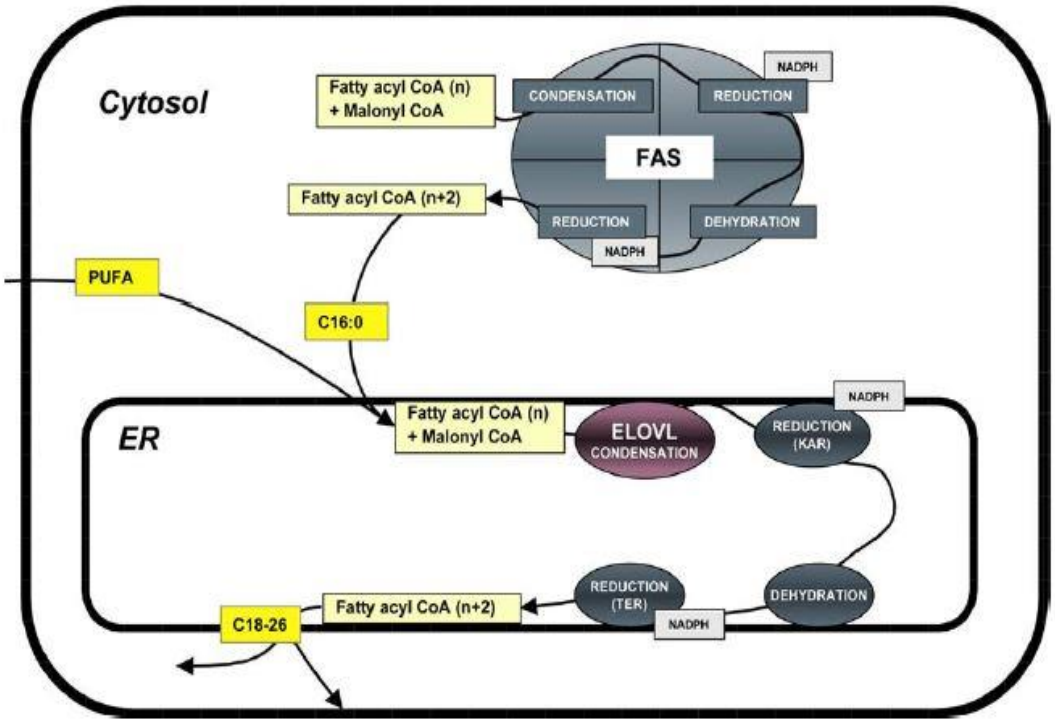

Figure 2.4: Elongation of fatty acid in mammals (Retrieved from Jakobsson *et al.*, 2006).

2.6 Elongation of very long chain fatty acid (ELOVL)

Elongation of very long fatty acids, ELONGASEs or ELOVLs are enzymes needed to perform condensation reaction in the first step of the elongation cycle (Jakobsson *et al.*, 2006; Jump, 2009). To date, there are seven family members in

ELOVL family naming from ELOVL1 to ELOVL7 (Jump, 2009). These enzymes are group together based on their similar motifs in gene or protein such as KxxExxDT, histidine box (HXXHH), QxxFLHxYHH, NxxxHxxNYxYY and TxxQxxQ (Jakobsson *et al*., 2006). Other than that, ELOVL proteins also have seven transmembrane regions, lysine or arginine deposits at the carboxyl terminus which act as ER retrieval signals (Jakobsson *et al*., 2006). At present, Elovl1, Elovl3, Elovl6, and Elovl7 have not been characterized in fish but Elovl2, Elovl4, and Elovl5 have been widely studied in a range of fish (Han, 2017).

2.6.1 Functional analysis of ELOVL enzymes

In fatty acid elongation, ELOVL1, ELOVL3, ELOVL6 and ELOVL7 prefer monounsaturated fatty acid (MUFA) and saturated fatty acid (SFA) as substrate. ELOVL2 and ELOVL5 prefer polyunsaturated fatty acid (PUFA) while ELOVL4 prefer SFA and very long chain PUFA especially those with carbons chain-length more than 28 (Zhang *et al*., 2016). This statement is supported by Ferraz *et al*., (2019) who mentioned that ELOVL2, ELOVL4 and ELOVL5 can elongate PUFA substrate, therefore, play important roles in the synthesis of LC-PUFA. Other than that, Kihara (2012) stated that ELOVL3 and ELOVL7 elongate both saturated and unsaturated C16 to C22 fatty acid but the highest toward C18 fatty acid. Besides that, ELVOL1 elongates saturated C18, monounsaturated C20 and C22 (Kihara, 2012).

Discovery of ELOVL1 is related to ELOVL3 due to its sequence homology to ELOVL3 (Jakobsson *et al*., 2006). According to Brolinson (2009), some researcher would treat ELOVL1 as a “housekeeping elongase” due to its similar gene expression pattern that can be found in all murine tissues tested. Functional studies of ELOVL1 shows it has preferences toward saturated fatty acids up to 26 carbons in length and

play a significant role in forming membrane-related functions especially sphingolipids (Jakobsson *et al*., 2006). In teleost, ELOVL1 expresses in swim bladder and kidney during early development through *in-situ* hybridization of zebrafish (Bhandari *et al*., 2016).

ELOVL2 is discovered by its similar sequence with ELOVL 3 (Brolinson, 2009). Unlikely, ELOVL2 shows more restricts gene expression pattern then ELOVL3. ELOVL2 is found abundance in testis and liver while lesser in brain, kidney and white adipose tissue (Brolinson, 2009). In both human and mouse, ELOVL2 has the ability to elongate arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), docosatetraenoic acid (AdA; 22:4n-6) and docosapentaenoic acid (DPA; 22:5n-3) in transfected yeast. However, their function is absent in elongate ultra-long-chain fatty acid (ULC-PUFA) (Ferraz *et al*., 2019). In mouse, ELOVL2 shows the ability to elongate γ-linolenic acid (GLA; 18:3n-6) which is an overlapping function of ELOVL5 (Jakobsson *et al*., 2006). In early embryogenesis, ELOVL2 is expressed in intestine and brain of zebrafish (Monroig *et al*., 2009).

ELOVL3 is the first introduced member in ELOVL family. This is because of its high expression in brown adipose tissue (BAT) of a cold-exposed mouse (Brolinson, 2009). Other than that, during perinatal development, ELOVL3 is expressed highly with maximum level immediately after birth. This indicates the expression of ELOVL3 is highly related to the recruitment of brown fat (Jakobsson *et al*., 2006). Studies showed that ELOVL3 is preferred in elongating saturated and monounsaturated fatty acid that up to 24 carbons in length (Brolinson, 2009). Studies from Jakobsson *et al*. (2006) suggested the ELOVL3 has function in synthesizing saturated and/or monounsaturated LC-PUFA in order to generate lipid droplets. Besides that, studies

through *in-situ* hybridization revealed that ELOVL3 is expressed in sebocytes of sebaceous glands and also in the hair follicles (Jakobsson *et al*., 2006).

ELOVL4 is one of the most studies members in elongase family because of its association with Stargardt-like macular dystrophy, an eye disorder which leads to loss of vision (Brolinson, 2009). ELOVL4 is required to generate saturated very long chain fatty acid (VLC-FA) and also contribute in the synthesis of very long-chain PUFAs (VLC-PUFAs) (Monroig *et al*., 2010). In marine fish, Elovl4 has functional similarities to Elovl2, therefore, Elovl4 might replace the apparent absence of Elovl2 (Monroig *et al*., 2012). According to Jakobsson *et al*. (2006), ELOVL4 is highly expressed in the human retina than in brain and testis. This indicated that ELOVL4 is involved in the synthesis of DHA. *In-situ* hybridization of zebrafish that carried out by Monroig *et al*. (2010) showed that Elovl4 is expressed in liver, eyes, ovary, and testis.

As one of the most studied elongase member, ELOVL5 also receives lots of attention for its high expression in testis, adrenal glands, and liver where these tissues contained high levels of docosapentaenoic acid (DPA; 22:5n-6) (Brolinson, 2009). According to Tan *et al.,* (2010) Elovl5 is widely studied in mouse, rat, and human is because of its significant roles in the biosynthesis of EPA and DHA. While DHA, EPA, and ARA are widely known to play important roles in vertebrate reproduction and development (Jaya-Ram *et al.,* 2008). ELOVL5 involves in elongation of various LC-PUFA with carbon chain length from 18 to 20. However, it does not has the ability to elongate LC-PUFA beyond 22 carbon chain-length as it is a strictly PUFA-specific elongase (Kihara, 2012). In marine fish, ELOVL5 is detected in liver, brain, intestine, eyes, and spleen (Monroig *et al*., 2012). *In situ* hybridization reveals that ELOVL5 is expressed in the brain region for zebrafish embryogenesis from 24hpf to 72hpf (Tan *et al*., 2010).

ELOVL6 elongates shorter saturated fatty acid if compared with other ELOVL where it prefers to elongate fatty acid with 12 to 16 carbons (Sassa & Kihara, 2014). ELOVL6 often expresses highly in mouse liver and adipose tissue (Brolinson, 2009). According to Jakobsson *et al*. (2006), ELOVL6 is expressed in tissues with high lipid content such as BAT, white adipose tissue (WAT) and brain. ELOVL7 often has a preference in elongating fatty acid with carbon chain-length 16 to 20 with the highest activity toward carbon chain-length of 18 (Naganuma *et al*., 2011). ELOVL7 is reported involved in prostate cancer growth (Naganuma *et al*, 2011).

Table 2.2: Fatty acid substrate preferences by each identified ELOVL. (Retrieved from Han, 2017).

ELOVL	FA substrate preference
1	C20-C22 SFA and MUFA
2	C20-C22 PUFA
3	≤ C24 SFA and MUFA
4	> C24 SFA and C22 PUFA
5	C18-C22 PUFA
6	C12-C16 SFA
7	C18-C22 SFA

In the year 2014, Xue and colleagues cloned four *elovl4* paralogous gene, named *elovl4a*, *elovl4b*, *elovl4c1* and *elovl4c2* from Atlantic cod (*Gadus morhua*). A phylogenetic tree was constructed using Elovl1 to Elovl7 protein sequences from pufferfish (*Takifugu rubripes*), Atlantic cod, zebrafish (*Danio rerio*), and salmon (*Salmo salar*). The predicted sequence of Elovl4c from zebrafish and pufferfish are also included in this phylogenetic tree. However, protein sequences of Elovl4c for cod, zebrafish, and pufferfish are grouped as another clade away from the protein sequence of Elovl4a and Elovl4b in the phylogenetic tree. Elovl4c1 and Elovl4c2 are suspected to be the results of gene duplication in the cod lineage (Xue *et al*., 2014). The exact

function and expression pattern of these paralogues Elovl4c1 and Elovl4c2 genes remain unknown as Xue and colleagues (2014) did not further analyse them.

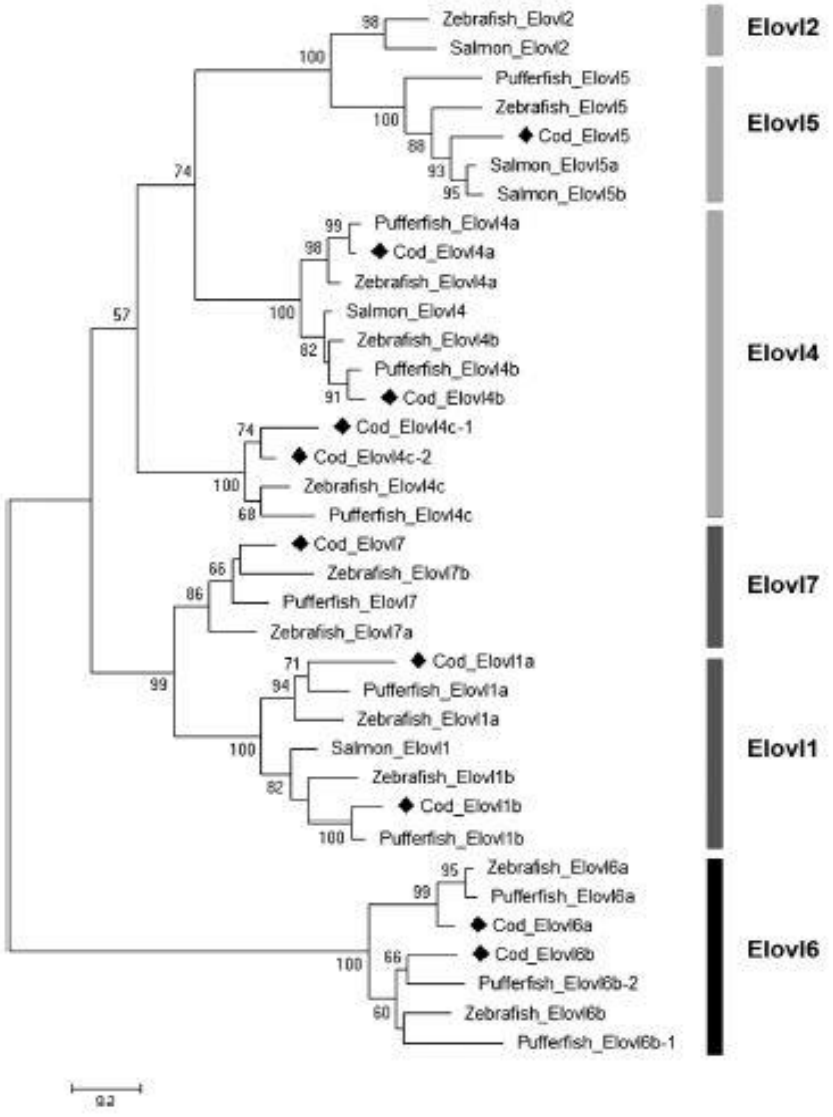

Figure 2.5: Phylogenetic tree constructed by Xue and colleague. They considered Elovl4c1 and Elovl4c2 were paralogous gene for Elovl4 although the Elovl4c group was clearly separated from Elovl4 family. (Retrieved from Xue *et al.*, 2014).

During the same year, the homologous gene of labelled putative *Elovl4c* from snakehead (*Channa striate*), siakap (*Lates calcarifer*) and Nile tilapia (*Oreochromis niloticus*) are cloned by a group of researchers (Kuah, 2015; Han, 2017). The cloned homologous genes had shown similarity with less than 50% identical to any member of the ELOVL family. The gene expression result and the functional characterization

result in synthesizing LC-PUFA of the homologous gene also show less similarity with other ELOVL family member. These results increase the possibility of the homologous gene to be a new family member of ELOVL family (Kuah, 2015; Han, 2017).

2.7 Zebrafish (*Danio rerio*)

Zebrafish (*Danio rerio*), a small tropical freshwater fish that belongs to Cyprinidae family. Zebrafish are grouped under bony fishes, Teleostei (Carpio & Estrada, 2006). They are distributed throughout South and Southeast Asia with highest species diversity in north-eastern India, Myanmar, Bangladesh, Nepal, and Bhutan. (Spence *et al.*, 2008). The name *Danio* is derived from the Bengali name "Dhani" which mean "the rice field" (Spence *et al*., 2008). Zebrafish stay in the slow-moving stream, ditches and shallow ponds that connected to paddy field (Spence *et al*., 2008).

Zebrafish was introduced by Francis Hamilton, a British East India company's surgeon (Spence *et al*., 2008). Adult zebrafish is approximate 2 to 3 cm in length and has distinguishing colour pattern with alternating dark and light horizontal stripes like zebra (Spence *et al*., 2008). The sex of the zebrafish is easy to differentiate. Male zebrafish is slender and torpedo-shaped, with black longitudinal striped, and usually has gold colouration on the belly and fins (Wixon, 2009). While female zebrafish is fat when laden with eggs and has very little gold on their undersides (Wixon, 2000). Zebrafish is described as fish with an oblique mouth, has an incomplete lateral line extending to the pelvic fin-based, has two pairs of barbels and has five to seven dark blue striped extending from behind of the operculum to the caudal fin (Spence *et al*., 2008).

Figure 2.6: Illustrated image of zebrafish (*Danio rerio*) from (Markowski, 2019) Animal Diversity Wed (Retrieved from: https://animaldiversity.org/accounts/Danio_rerio/)

2.7.1 Zebrafish as a model organism

George Streisinger, the first person who uses zebrafish as model organisms in his experiment and later receives a lot of love from other researchers as zebrafish is a convenient and cost-effective model. (Gerhard, 2003). Apart from that, the versatility of zebrafish had also extended to reproductive biology due to its ease handling embryo under laboratory condition (Jaya-Ram *et al*., 2008). A single zebrafish female has high fecundity which can lay more than 200 eggs per week under laboratory condition throughout the year which really suitable for large-scale genetic approaches (Carpio & Estrada, 2006). Furthermore, the embryos of zebrafish are also strong enough to cope with experimental manipulation like microinjection (Carpio & Estrada, 2006). Besides, the embryo of zebrafish is small in size which can be incubated in a microplate and observed under the microscope.

Apart from that, the embryos develop very fast as they take only 24 hours for embryogenesis and 5 days for organogenesis which allow fast completion for the experiment that relates to development (Gerhard, 2003). Transparent embryo allows easy visualization of the formation and function of an internal organ (Carpio & Estrada,

2006). Short generation time (3 to 4 months to reach adulthood) of zebrafish ease the studies of chronic and genetic (Segner, 2009). According to Ishak *et al.* (2008) zebrafish also a useful model in the understanding of oogenesis and folliculogenesis as the oocytes of female zebrafish can be easily differentiated to 4-5 distinct follicle stages. Another important characteristic of zebrafish is the availability of mutant zebrafish embryos with deformity of morphology or organ dysfunction allow studies for human disease (Bopp *et al*., 2006).

2.7.2 Zebrafish as a genetic model organism

Zebrafish and human are vertebrate therefore they do share a close structural and physiological relationship. The linkage between zebrafish and human diverged approximate 450 million years ago, therefore, their genome is similar in size (Lardelli, 2008). Zebrafish has 30 000 to 80 000 genes which have more than 80% similarity with mouse and also human (Ho *et al*., 2004). Other than that, zebrafish has reported having multiple homologous genomes with other vertebrates (Tay *et al.,* 2018). Therefore, orthologues of most human genes can be found in zebrafish where they commonly show similar expression pattern (Lardelli, 2008). This advantage makes zebrafish a powerful genetic system to study the physiology of vertebrate (Ho *et al*., 2004). The ability of zebrafish to accomplish forward genetic studies by mutagenizing the entire genome and screening for certain phenotypes also made zebrafish a popular model (Ho *et al.,* 2004).

2.7.3 Zebrafish as a lipid metabolism model

Zebrafish has gained a reputation as a competent model to examine the role of lipids and fatty acids during development (Tay *et al.*, 2018). Zebrafish also contributes great effort in studies of obesity, dietary lipid absorption, metabolic disease, diabetes,

fatty liver disease, atherosclerosis, cardiovascular disease, and lipoprotein biology (Miyares *et al*., 2014; Tay *et al*., 2018).

Furthermore, Ho *et al.* (2004) mentioned that endogenous lipid transport and lipolysis in fish is similar to mammals but with slight differences in absorption and deposition process. Zebrafish is a popular lipid metabolism model because it has the same gastrointestinal organs like a human where zebrafish also has liver, intestine, exocrine and endocrine pancreas, and gallbladder (Carten & Farber, 2009). Apart from that, zebrafish is a great model for LC-PUFAs studies. This is because the biosynthesis pathway of LC-PUFAs was accomplished with the identification of key enzymes (Tan *et al.*, 2010). This is very important as the lipid metabolism system in zebrafish and in human are similar and they do share the same biochemical level (Carten & Farber, 2009). This advantage allows the researcher to relate the *de novo* biosynthesis pathway of LC-PUFAs in zebrafish with human due to the importance of LC-PUFAs in various physiological events.

Other than that, zebrafish relies on its yolk contents for nutrition throughout the development until they reach an early larval stage which is around 5 days post fertilization (dhp) (Fraher *et al*., 2016). This makes them a very good model for the study of lipid metabolism as they can be considered a 'closed system'. This is because their nutrient intake is not yet influenced by outer factors, therefore, the potential experiment variables due to feeding can be excluded during embryogenesis (Fraher *et al*., 2016).

There are a few great finding are achieved by using zebrafish as the model of study. Research conducted by Jaya-Ram et al. (2008) stated that the female zebrafish that fed with 1:1 squid oil: linseed oil (SLO) and 100% linseed oil (LO) produced an

expressive higher amount of eggs. This proved that HUFA is required in spawning females. This finding also suggested that zebrafish is a great model for investigating the effect of maternal HUFA toward embryo development. Research of Ishak *et al.* (2008) demonstrated different expression pattern of desaturase and elongase genes in different zebrafish follicular stage. Their finding suggested that HUFA biosynthesis in zebrafish ovary is much localized. This showed that zebrafish is a useful model to understand oogenesis and folliculogenesis. Other than that, research of Enyu and Shu-Chien (2011) highlighted several mitochondria-related responses in zebrafish liver after 15 days of starvation and followed by normal feeding activities. They found out that during starvation and after refed, both carbohydrate and noncarbohydrate-based metabolic pathways are different. This demonstrated that zebrafish is suitable to detect the effect of fasting and caloric restriction. Besides that, research from Tay *et al.* (2018) successfully identified several response elements that are significant to initiate activation of transcription for a *fad2* promoter of zebrafish. This not only showed that zebrafish is a useful model to understand the role of lipid and fatty acid but also allowed expression tracking in developing zebrafish embryos.

2.8 Whole-mount *in situ* hybridization (ISH)

According to Jensen (2014), *in situ* hybridization is a scientific technique that detects and identified the location of specific DNA or RNA sequences in cells, preserved tissue sections or entire sample (whole mount *in situ* hybridization) by hybridizing the anti-sense RNA probe to a particular sequence. From Nakamura (1990) point of view, *in situ* hybridization can be used for: (1) detect DNA target; (2) amplify RNA genes that inside a cell nuclei; (3) identify location of specific gene within chromosome; (4) detect location of mRNA within the cells; (5) locate RNA or DNA of viral nucleic acid. *In situ* hybridization is developed in the 1980s by using radio-

active labelled DNA probes to detect the location of a gene expressed on histological sections (Thisse & Thisse, 2008). In the 1990s, RNA probes with chemically labelled allow quicker and easier analysis of gene expression on whole-mount tissues or embryos (Thisse & Thisse, 2008).

This application can be used for two complementary strands of DNA, for RNA-to-DNA and for RNA-to-RNA (Jensen, 2014). The main advantage of using whole-mount ISH is because this technique is a quick and efficient method to identify spatial and temporal gene expression pattern in early larvae and/or embryos (Thisse & Thisse, 2008). Other advantages of ISH are allowed maximum use of tissue that difficult to get and different hybridizations can be achieved on the same tissue (Jensen, 2014).

CHAPTER THREE: MATERIALS AND METHOD

3.1 Cloning and sequencing of ORF sequence of *Danio rerio* elongase

3.1.1 Sequence search

First fragment of elongases of interest were obtained from Dr Kuah Meng Kiat's previous work for zebrafish. This elongases of interest were BLAST through GenBank and full length of elongase of interest was found deposited in GenBank. The first fragment of elongase of interest A was found 99% identical with *Danio rerio* ELOVL fatty acid elongase 8a (elovl8a), mRNA (NM_001076593.1) (Appendix A) while first fragment of elongase of interest B was found 100% identical with *Danio rerio* zgc:112263, mRNA (cDNA clone MGC:112263 IMAGE:7242429), complete cds (BC095712.1) (Appendix B).

3.1.2 Primer design

Specific primers were designed in pair for both elongase of interest A (NM_001076593.1) and elongase of interest B (BC_095712.1) according to the nucleotide sequence obtained from GenBank. A restriction enzyme site of *Bam*HI was added at the forward primer while *Xho*I was added at the reverse primer in order to amplify the open reading frame (ORF) of elongase cDNA from zebrafish's liver and intestine tissue. The sequences of the primer designed were shown in Table 3.1

Table 3.1: Primers sequence used to amplify the ORF of elongase of interest A and elongase of interest B from zebrafish with their respective restriction site shown in underline.

	Primer sequence
Dre8aCDS F1	5'- CCCGGATCCAAAATGGTGTCTGTATCACCG-3'
Dre8aCDS R1	5'- CGGCTCGAGTTAGGCTAGCTTGGTCTTCTT-3'
Dre8bCDS F1	5'- CAAGGATCCAAAATGGAATCCGCATGG-3'
Dre8bCDS R1	5'- CGGCTCGAGTTAAGACTTCTTGTTCT-3'