INFLUENCE OF DIETARY HUFA LEVELS ON EGG PRODUCTION, TISSUE FATTY ACID PROFILE AND DESATURASE AND ELONGASE mRNAs EXPRESSION IN FEMALE ZEBRAFISH (*Danio rerio*)

by

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I believe God provides an umbrella when it rains, and sends a rainbow after every storm. I'm blessed to have the opportunity to work with wonderful people who have been my umbrella and rainbow in times of adversity.

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~ Annette J.

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LIST OF ABBREVIATIONS

Acetyl CoA	acetyl Coenzyme A
ANOVA	analysis of variance
ARA	arachidonic acid
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
C_T	threshold cycle
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
dpf	day post fertilization
EFA	essential fatty acids
EPA	eicosapentaenoic acid
GLA	γ-linolenic acid
GSI	gonadosomatic index
FCR	feed conversion ratio
HUFA	highly unsaturated fatty acids
IPTG	isopropyl β-D-thiogalactopyranoside
LA	linoleic acid
LB	Luria-Bertani
LNA	α-linolenic acid
LO	100% linseed oil diet
LT	leukotrienes
LX	lipoxins
MMLV-RT	Moloney murine leukemia virus – reverse transcriptase
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger ribonucleic acid
PG	prostaglandin
PUFA	polyunsaturated fatty acid
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SEM	standard error of mean
SGR	specific growth rate
SO	100% squid oil diet

SLO	1:1 blend of squid oil:linseed oil diet
TX	thromboxanes
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

PENGARUH ARAS ASID LEMAK SANGAT TIDAK TEPU (HUFA) DALAM PEMAKANAN KE ATAS PENGHASILAN TELOR, PROFIL ASID LEMAK DALAM TISU DAN PENGEKSPRESAN mRNA GEN DESATURASE DAN ELONGASE DALAM IKAN ZEBRAFISH BETINA (*Danio rerio*)

ABSTRAK

Kajian ini telah dijalankan untuk meninjau kesan paras asid lemak sangat tidak tepu (HUFA) ke atas profil asid lemak dalam tisu dan prestasi pembiakan ikan zebrafish betina. Pengekspresan mRNA gen desaturase dan elongase di dalam tisu hati, otot dan ovari ikan zebrafish betina telah dianalisa. Tiga jenis makanan ikan yang mempunyai kandungan protein yang sama telah disediakan mengikut nisbah minyak sotong dan minyak linseed yang berbeza. Tiga makanan ikan tersebut mengandungi 100% minyak sotong (SO), campuran 1:1 minyak sotong dan minyak linseed (SLO) dan 100% minyak linseed (LO) sebagai sumber lipid. Eksperimen telah dijalankan selama 12 minggu dan ikan dibiakkan dua kali seminggu. Keputusan eksperimen menunjukkan bahawa profil asid lemak hati, otot, ovari dan telur ikan mencerminkan profil asid lemak makanan yang telah diberi. Secara amnya, peningkatan aras minyak linseed dalam makanan telah merendahkan pengendapan asid arakidonik (ARA, 20:4*n*-6), asid eikosapentanoik (EPA, 20:5*n*-3) dan asid dokosaheksanoik (DHA, 22:6*n*-3) dalam semua tisu yang dikaji. Bagi ikan yang telah diberi makanan yang mengandungi HUFA yang rendah, profil asid lemak hati menunjukkan aktiviti biosintesis yang meningkat dengan peningkatan dalam pengekspresan mRNA desaturase dan elongase hepatik. Walaubagaimanapun, peningkatan dalam aktiviti biosintesis HUFA tidak mampu mengimbangi paras ARA, EPA dan DHA yang rendah di dalam tisu hati ikan yang telah diberi makanan LO. Paras ARA dan EPA yang rendah juga di kesan dalam tisu otot dan ovari ikan yang diberi makanan LO. Tiada perbezaan yang signifikan telah dijumpai bagi

kandungan ARA dan EPA dalam telur ikan di antara ketiga-tiga jenis makanan, yang boleh menunjukkan akumulasi selektif kedua jenis HUFA ini di dalam telur. Peningkatan dalam pengekspresan gen desaturase dan elongase dikesan dalam ovari ikan yang telah diberi makanan HUFA yang rendah. Ikan yang diberi makanan SLO memberi jumlah telur yang paling tinggi. Ini menunjukkan kepentingan untuk memberi asid lemak tidak tepu *n*-3 dan *n*-6 dengan nisbah yang seimbang kepada ikan betina yang aktif membiak. Sebagai kesimpulan, kajian ini menunjukkan bahawa prestasi pembiakkan ikan zebrafish betina mendapat manfaat daripada penambahan HUFA dalam makanan, walaupun mempunyai kebolehan untuk meningkatkan transkripsi desaturase dan elongase dalam tisu semasa diberi makanan dengan paras HUFA yang rendah.

INFLUENCE OF DIETARY HUFA LEVELS ON EGG PRODUCTION, TISSUE FATTY ACID PROFILE AND DESATURASE AND ELONGASE mRNAs EXPRESSION IN FEMALE ZEBRAFISH (*Danio rerio*)

ABSTRACT

This study was conducted to investigate the effect of varying levels of dietary highly unsaturated fatty acids (HUFA) on tissue fatty acid profiles and reproductive performance in female zebrafish. Zebrafish were utilized as they are a good vertebrate model, easy to breed and care for, among other advantages. In addition, liver, muscle and ovarian tissues were analyzed to gauge the mRNA expression of desaturase and elongase genes. Three iso-nitrogenous experimental diets utilizing different ratios of squid oil and linseed oil were formulated and fed to female zebrafish. The diets contained 100% squid oil (SO), a 1:1 blend of squid and linseed oils (SLO) and 100% linseed oil (LO) as lipid sources. Breeding was carried out twice a week during the experimental feeding trial which lasted 12 weeks. Results revealed that fatty acid profiles of liver, muscle, ovary and egg reflected profiles of the corresponding dietary treatment. Basically, increasing levels of dietary linseed oil lowered deposition of arachidonic acid (ARA, 20:4*n*-6), eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22: 6*n*-3) in all tissues. For fish with low dietary HUFA intake, liver fatty acid profile implied increasing biosynthesis activities, which was supported by increased expression of hepatic desaturase and elongase mRNAs. However the increase in HUFA biosynthesis activities were unable to compensate for the inferior hepatic ARA, EPA and DHA levels in fish fed diet LO. Relatively lower concentrations of ARA and EPA were also obtained in muscle and ovary tissues of LO fed fish. Interestingly, no significant difference was found in ARA and EPA levels in eggs among all three dietary treatments, which imply selective accumulation of these HUFA in eggs. Desaturase and elongase genes expression showed an increasing trend in ovary during low dietary HUFA intake. Fish fed diet SLO had the highest egg production which indicates the need for the inclusion and a balanced ratio of dietary *n*-3 and *n*-6 unsaturated fatty acids in spawning females. In

conclusion, this study reveals that reproductive performance of female zebrafish benefits from the supply of dietary HUFA, despite possessing ability to increase transcription of desaturase and elongase in various tissues during low dietary HUFA intake.

CHAPTER 1

INTRODUCTION

1.1 Research background

The importance of fatty acids in aquaculture has gained much attention over the years. It is widely known that highly unsaturated fatty acids (HUFA) – arachidonic acid (ARA, 20: 4*n*-6), eicosapentaenoic acid (EPA, 20: 5*n*-3) and docosahexaenoic acid (DHA, 22: 6*n*-3) - play important roles in fish nutrition. In addition to being an energy source and components of cell membranes, HUFA have an influence on fish reproductive performance as well (Rennie *et al.*, 2005). Studies on broodstock nutrition of many species have revealed evidences that broodstock dietary fatty acids will eventually affect fecundity and egg and larvae fatty acid profiles. HUFA also act as precursors for eicosanoids, a physiologically dynamic group of molecules responsible for an array of cellular activities including gene regulation, signaling and maintenance of membrane integrity. Prostaglandins, the oxygenated metabolites of ARA and EPA have been shown to play essential roles in the development of vertebrates (Cha *et al.*, 2006).

Lipids in aquafeeds come from a variety of sources. The steady increase of aquaculture production over the last two decades has resulted in an increased utilization of fish meal and fish oil: the two dominant ingredients used in the production of aquaculture feeds (Tacon, 1996; Furuita *et al.*, 2007). As the saying goes, all good things must come to an end. The demand for fish oil is rapidly increasing along with the increase in farming activities, which compromises the future of aquaculture. The demand for fish oil by this industry will probably exceed available resources over the next decade (Mourente and Bell, 2006). These prospects have forced the industry to search for alternative lipid sources for use in fish feeds, with plant-based oils being the most interesting candidates. A large number of plant oils including linseed, palm, sunflower, borage, olive and soybean oil have been tried in experimental diets as possible alternatives to fish oil (Torstensen *et al.*, 2000; Tocher *et*

al., 2001, 2003; Rodríguez *et al.*, 2002, Montero *et al.*, 2005; Mourente *et al.*, 2005). Plant based oils pose a problem as they have different fatty acid composition compared to fish oil. Plant oils are rich in C18 polyunsaturated fatty acid (PUFA) but they lack *n*-3 HUFA which are abundant in fish oil. This indicates that the ability of the fish to compensate for oil composition differences is a very important factor in order to adapt to fish oil replacement. Accordingly, several studies involving freshwater and salmonid species have shown the possibility of high inclusion levels of plant oil without compromising growth and general physiological processes of fish (Bell *et al.*, 2002; Francis *et al.*, 2006; Manning *et al.*, 2006; Turchini *et al.*, 2007). At present, the benefits of dietary HUFA inclusion in freshwater broodstock reproduction remains to be confirmed as very little is known on the regulation of HUFA biosynthesis in these species, especially during the reproductive phase. Therefore, it is important to take into consideration the essential fatty acid (EFA) requirements of broodstock of the cultured species.

EFA requirements differ for marine and freshwater fish species. Although fish cannot synthesize C18 PUFA *de novo*, they can, to a certain extent, depending on species, convert PUFA from the diet into HUFA. Marine fish usually require EPA, DHA and ARA to be provided in their dietary regime. The natural diets of marine fish - crustacean and piscine preys - contain high levels of HUFA. These fish do not possess the ability to convert C18 PUFA, α-linolenic acid (LNA, 18: 3*n*-3) and linoleic acid (LA, 18: 2*n*-6) to their respective HUFA. It is speculated that the ability to desaturate and elongate PUFA have been lost in marine fish due to the readily available HUFA in their diets.

Freshwater fish on the other hand, have the ability to convert C18 PUFA into HUFA. They are able to convert LA into ARA and LNA into EPA and DHA (Bell *et al.*, 2003a). This attribute can be narrowed down to the possession of the desaturase and elongase genes which aid in the enzymatic HUFA synthesis pathways. The actual mechanisms and pathways of the regulation of teleost reproduction by dietary fatty acids involved are still largely unknown. There is considerable interest in seeking detailed understanding on the actual role of ARA, EPA and DHA in various aspects of reproduction,

such as oocyte maturation, ovulation, spawning, hatching success and larval quality (Sorbera *et al.*, 2001; Bell and Sargent, 2003; Patiño *et al.*, 2003). Elongase genes have been cloned from zebrafish, Atlantic salmon, Nile tilapia, African catfish and cod (Agaba *et al.*, 2004; Hastings *et al.*, 2004; Agaba *et al.*, 2005). As for Δ6 and Δ5 desaturase genes, extensive work has been done with fish such as carp, salmon, cod, tilapia, sea bream and rainbow trout (Seiliez *et al.*, 2001, 2003; Hastings *et al.*, 2004, Zheng *et al*., 2004a). All of these desaturases are primarily a single function enzyme, being either Δ6 or Δ5 desaturase. A bifunctional desaturase has been cloned in the zebrafish (Hastings *et al.*, 2001).

Zebrafish are gaining status as a useful vertebrate model system to study various cellular and molecular aspects of lipid metabolism in vertebrates. In addition to its status as a major model organism for developmental biology research, the versatility of zebrafish has also been extended to the field of reproductive biology due to its prolific spawning activities and ease of handling follicular cells under laboratory conditions (Patiño and Sullivan, 2002; Ge, 2005). Apart from this, advantages of working with zebrafish are aplenty. Small and easy to handle, zebrafish maintenance is considerably easy. Moreover, they readily spawn when given the suitable environment conditions. This particular factor makes zebrafish an excellent model to evaluate freshwater fish reproduction.

In this study, the effects of varying HUFA on tissue fatty acid deposition and reproductive performance of female zebrafish actively involved in spawning activities were evaluated. Dietary manipulations of fatty acid content were done with lipid sources from squid oil (source of HUFA) and linseed oil (source of PUFA). Furthermore, the influence of dietary HUFA levels on mRNA expression of desaturase and elongase in selected tissues were investigated to shed light on HUFA biosynthesis activities during reproduction.

1.2 Objectives

The aims of this study are

1. to evaluate effects of varying dietary HUFA levels on tissue fatty acid deposition and reproductive performance of actively spawning female zebrafish.
2. to determine the effects of varying dietary HUFA levels on transcriptional activities of zebrafish desaturase and elongase in different tissues.

CHAPTER 2

LITERATURE REVIEW

2.1 Lipid

Lipids are considered important nutrients in fish diets. They provide energy for growth, reproduction and migration, membrane structural components, essential fatty acids, precursors of eicosanoids required for regulatory processes and assist in the uptake of lipid soluble nutrients (McKenzie, 2001). Lipids are compounds soluble in organic solvents such as chloroform, hydrocarbons and alcohols. Fish lipids in particular, can be divided into two main groups – polar lipids composed mainly of phospholipids and neutral lipids composed principally of triglycerides (Tocher, 2003). There are five major classes of lipids: fatty acids, triacylglycerols, phospholipids, sphingolipids and sterols (De Silva, 1995).

Fatty acids form distinct series, and a shorthand system has been devised for the classification of these fatty acid series. Varieties in fatty acid molecules are contributed by the differences in the number of carbon atoms and in the number and positioning of the double bonds between the carbon atoms (Jobling, 1994). Further review of fatty acids can be found in Section 2.1.2.

Neutral lipids are formed by esterification of fatty acids with the alcohol, glycerol. Whilst mono- and diacylglycerols are found, triacylglycerols make up the great bulk of neutral lipids found in nature. Triacylglycerols consist of three fatty acid molecules esterified to three alcohol groups of glycerol, which can be made up of a single, two or three different fatty acids (Figure 2.1).

Phospholipids are esters of glycerol, whereby two of the alcohol groups are esterified with fatty acids and the third with phosphoric acid, which in turn is esterified by a nitrogenous base, the amino acid serine, the sugar alcohol inositol or by glycerol sulphate. The nature of the nitrogenous base provides the basis of the name of the specific families of phospholipids – phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and

phosphatidylinositol (Jobling, 1994). Phosphoglycerides are the most common of the phospholipids (Figure 2.3).

Sphingolipids are a group of complex polar lipids containing the long chain amino alcohol sphingosine or a related base as their backbone (Figure 2.4). The other group of lipid, sterols, is simple lipids in the form of tetracyclic hydrocarbon compounds. They can exist unesterified as an essential component of cell membranes or in a neutral lipid storage form esterified to a fatty acid. One example of sterols in fish is cholesterols (Figure 2.5) (Tocher, 2003).

2.1.1 Lipid metabolism

Metabolism includes catabolic and anabolic reactions which occur within an organism that results in nutrients being utilized for energy or growth. Dietary intake of fish contains the major nutrients – proteins, carbohydrates and lipids. Generally, this process involves digestion, absorption and transport of nutrients. Feed consumed by fish are digested in the gut, absorbed by the gut lining and appear in the bloodstream as their component molecules. Proteins are digested to release their component amino acids, which are subsequently used to synthesize new proteins or for energy. Similarly, carbohydrates will be broken down to simple sugars.

Lipid is broken down to fatty acids. Following absorption, fatty acids are then resynthesized into lipids which form droplets. These lipid droplets are circulated in the fish blood system. In order to be used, they must again be broken down to their constituent fatty acids. Fatty acids are then used for synthesis of membranes or further degraded for energy. Lipids contain more energy per unit weight than any other dietary component, and are used efficiently by fish as energy sources. Besides providing energy, they are source of hydrophobic components for the synthesis of macromolecules (Jump *et al.*, 1999).

The degradative pathways of amino acids, simple sugars and fatty acids will eventually reach a common intermediate compound – acetyl coenzyme A (acetyl CoA).

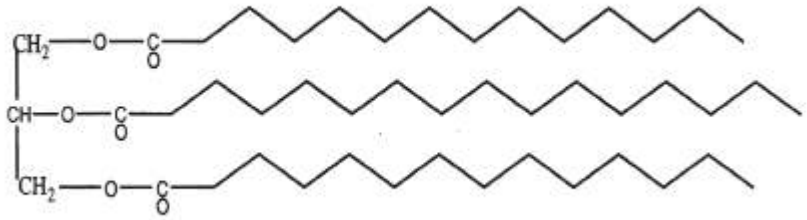


Figure 2.1: Tryacylglyceride: three fatty acids esterified to L-glycerol.
(Figure 2A, page 7. Tocher, 2003)

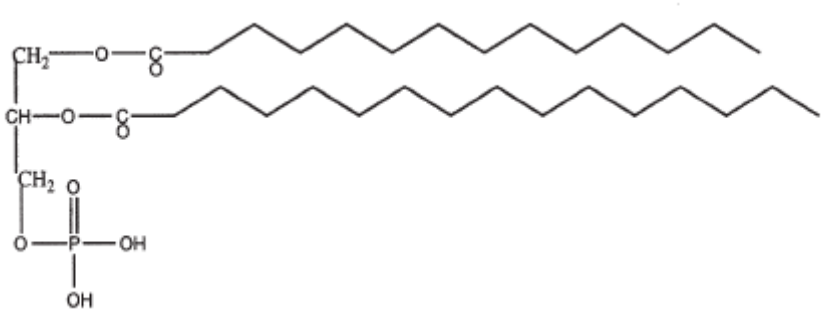


Figure 2.2: Phosphatidic acid, the backbone of the phosphoglycerides.
(Figure 2C, page 7. Tocher, 2003)

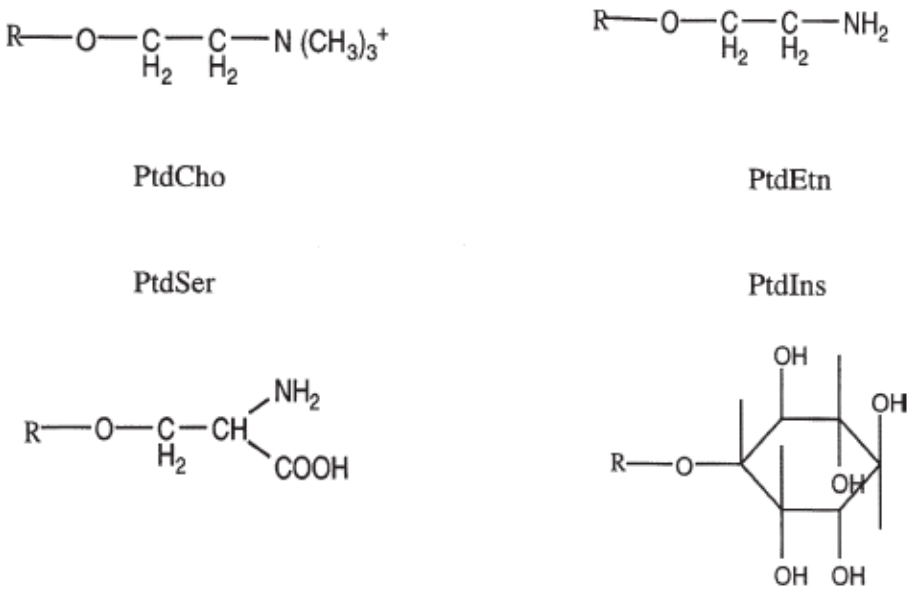


Figure 2.3: Structures of head groups in the major phosphoglycerides where R = the phosphatidyl group (see Figure 2.3). PtdCho, Phosphatidylcholine; PtdEtn, Phosphatidylethanolamine, PtdSer, Phosphatidylserine; PtdIns, phosphatidylinositol.
(Figure 3, page 9. Tocher, 2003)

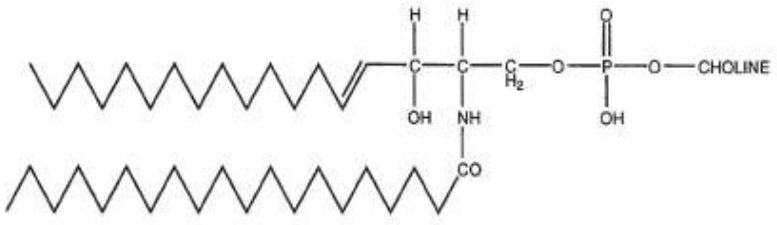


Figure 2.4: Sphingomyelin
(Figure 4A, page 10. Tocher, 2003)

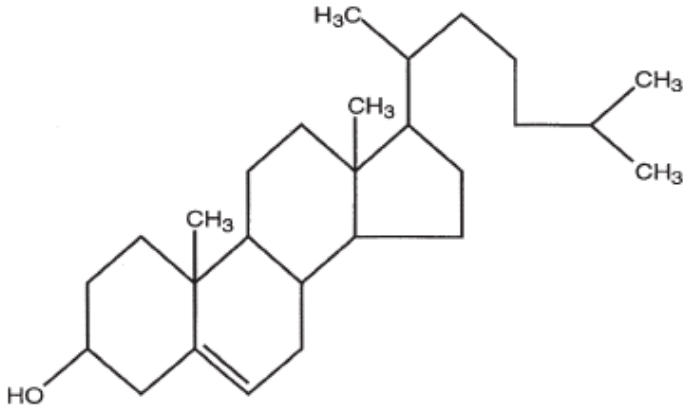


Figure 2.5: Cholesterol
(Figure 5, page 10. Tocher, 2003)

Acetyl CoA enters the citric acid cycle, which in turn is linked to the process of oxidative phosphorylation. The result is the production of CO_2, the consumption of O_2 and the liberation of energy, which is then stored as high-energy phosphate molecules, adenosine triphosphate (ATP) (De Silva, 1995).

2.1.2 Fatty acids

Fatty acids are carboxylic acids with long chain hydrocarbon side groups. They are designated on the basis of their chain lengths, degree of unsaturation (number of double bonds) and the position of the double bond. The nomenclature of fatty acids is as follows:

CX:Y*n*-Z

where, X shows the number of carbon atoms,

Y signifies the number or double bonds in the hydrocarbon chain

and Z denotes the carbon at which the first double bond appears, numbering from the methyl end (-CH3).

For example, 18:1*n*-7 and 18:1*n*-9 indicate that these fatty acids possess 18 carbon atoms and have one double bond (monounsaturated) at the position of carbon number 7 and 9 respectively, calculated from the methyl end of the molecule (Christie, 1973; Tocher, 2003).

An alternative way of naming the fatty acids:

CX:YΔK

where ΔK signifying the position of double bond from the carboxyl (-COOH) end of the molecule.

Using the same example as above, 18:1*n*-7 and 18:1*n*-9 would then be written as 18:1Δ11 and 18:1Δ9 (Figure 2.6B) respectively. Fatty acids without any double bonds are termed saturated fatty acids. For instance, they are represented as 16:0 and 18:0, which

denotes that these molecules have 16 and 18 carbon atoms (Figure 2.6A) (Christie, 1973; Tocher, 2003).

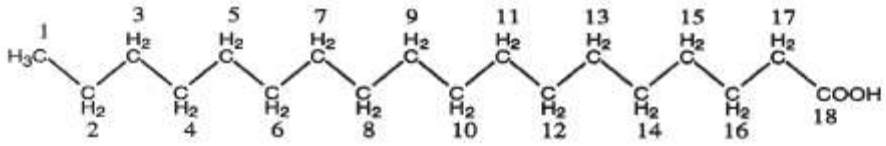
Polyunsaturated fatty acids (PUFA) may contain two or more double bonds. Consequently, the entire structure of a particular PUFA can be defined by specifying the position of the first double bond relative to the methyl end. Hence, in 18:3*n*-3 the first double bond is situated three carbon atoms from the methyl end of the molecule, which can also be written as 18:3Δ9,12,15 (Figure 2.6D). Another group also known as highly unsaturated fatty acids (HUFA), are fatty acids with carbon chain length more or equal to 20 carbon atoms and containing three or more double bonds. Important HUFA in fish are 20:4*n*-6 (arachidonic acid, ARA), 20:5*n*-3 (eicosapentaenoic acid, EPA) and 22:6*n*-3 (docosahexaenoic acid, DHA) (Christie, 1973; Tocher, 2003).

2.1.2.1 Unsaturated fatty acid biosynthesis pathway

PUFA cannot be synthesized *de novo* by any vertebrate species. Fish are no exception, as they too lack the Δ12 and Δ15 fatty acid desaturase enzymes required for the production of linoleic (LA, 18:2*n*-6) and α-linolenic (LNA, 18:3*n*-3) acids from oleic acid (18:1*n*-9) (Figure 2.7). However, it has been established that many vertebrates can convert dietary LA and LNA to long chain *n*-6 and *n*-3 highly unsaturated fatty acids (HUFA) respectively (Sprecher *et al.*, 1995; Zheng *et al.*, 2004a).

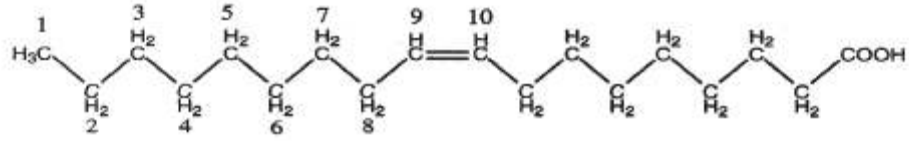
In marine fish, EPA and DHA are regarded as essential fatty acids (EFA) due to their inability to synthesize them. Marine fish have the reputation of being barely able to convert LNA to EPA and DHA, and LA to ARA. Conversely, freshwater fish differ from marine species in view of their dietary requirements; LA and LNA are regarded as their EFA (Bell and Sargent, 2003). The freshwater inhabitants have a capacity to desaturate and elongate the 18 carbon atom fatty acids into HUFA according to their needs (Seiliez *et al.*, 2003, Turchini *et al.*, 2006). Providing the correct EFA requirement to fish is crucial, as this will affect the growth and reproductive parameters of the cultured species.

A



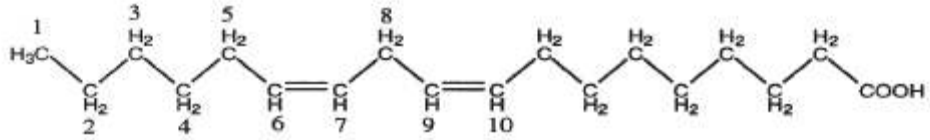
OCTADECANOIC ACID, STEARIC ACID, 18:0

B



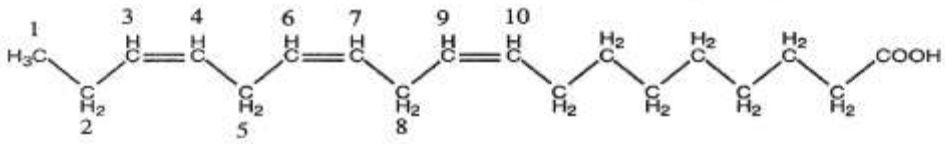
OCTADECENOIC ACD, OLEIC ACID, 18:1(n-9)

C



OCTADECADIENOIC ACID, LINOLEIC ACID, 18:2(n-6)

D



OCTADECATRIENOIC ACID, LINOLENIC ACID, 18:3(n-3)

Figure 2.6: Stuctures of the 18 carbon saturated and monounsaturated fatty acids, and representative polyunsaturated fatty acids of the *n*-6 and *n*-3 series. (Figure 1, page 4. Tocher, 2003)

LA and LNA can be converted to their respective HUFA *in vivo* by an alternating sequence of desaturation and elongation. The two pathways involved are depicted in Figure 2.8 (Agaba *et al.*, 2005). The pathways take place in the endoplasmic reticulum (Figure 2.9). Synthesis of ARA is achieved by Δ6 desaturation of LA to produce 18:3*n*-6 (γ-linolenic acid, GLA) which is then elongated to 20:3*n*-6 (dihomo-γ-linolenic acid, DGLA). This is finally desaturated at the Δ5 position to produce ARA. The pathway for EPA synthesis from LNA is essentially similar, but DHA synthesis requires two further elongation steps, a second Δ6 desaturation and a chain shortening step. Originally the insertion of the last, Δ4 double bond in 22:6*n*-3 was assumed to occur through direct Δ4 desaturation of its immediate precursor 22:5*n*-3 (Zheng *et al.*, 2004a; Agaba *et al.*, 2005). However, studies done on rat liver showed that the 22:5*n*-3 was further elongated to 24:5*n*-3 which is then converted by Δ6 desaturation to 24:6*n*-3 which goes through a chain shortening to form 22:6*n*-3 (Sprecher *et al.*, 1995). Various species have been studied to further elucidate these pathways, among some: rainbow trout, Atlantic salmon, tilapia and zebrafish (Buzzi *et al.*, 1997; Tocher *et al.*, 1997; Tocher *et al.*, 2002).

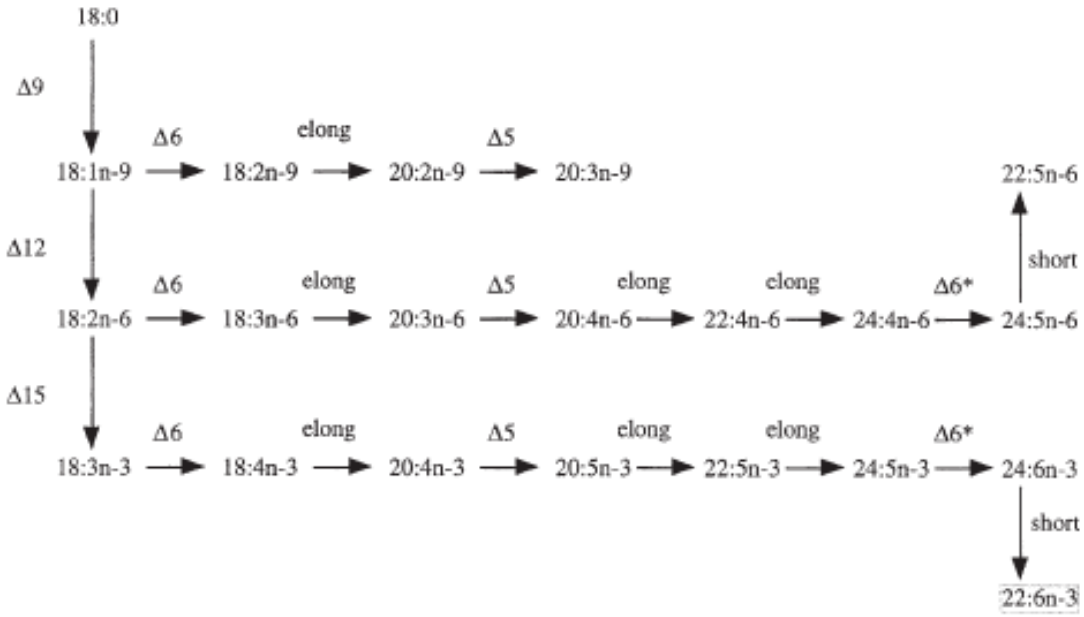
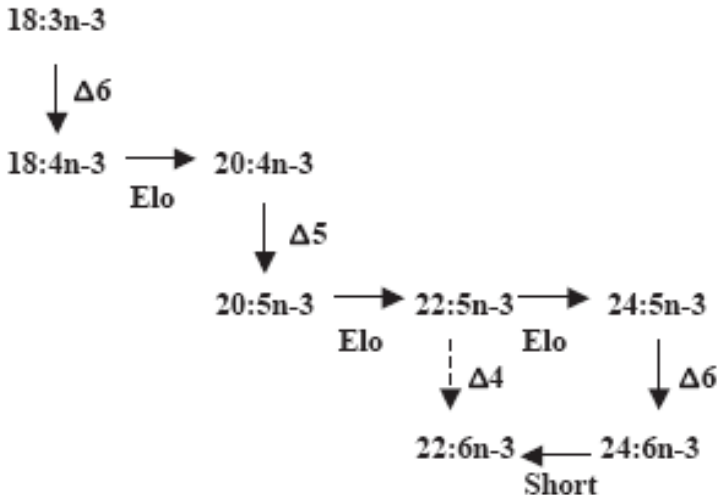


Figure 2.7: Pathways of biosynthesis of C20 and C22 HUFA from *n*-3, *n*-6 and *n*-9 C18 precursors. Δ5, Δ6, Δ9, Δ12, Fatty acid desaturases; Elong, Fatty acid elongases; Short, chain shortening. Δ9 desaturase is found in all animals and plants whereas Δ12 and Δ15 desaturases are generally only found in plants.
(Figure 6, page 26. Tocher, 2003)

A



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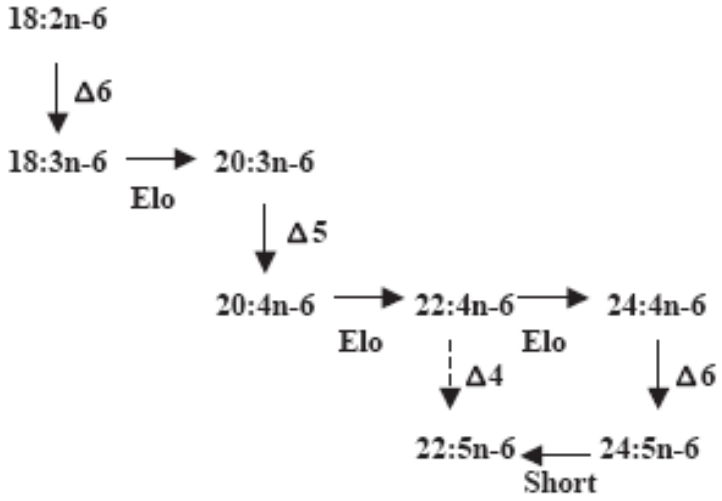

Figure 2.8: Pathways of highly unsaturated fatty acid (HUFA) biosynthesis from the C18 polyunsaturated fatty acids (PUFA), **A**: 18:3*n*-3 and **B**: 18:2*n*-6. Solid lines represent steps that have been shown to occur in fish. Dotted lines represent steps that have not been directly demonstrated in fish. Δ6, Δ5 and Δ4, fatty acid desaturases; Elo, fatty acid elongases; Short, peroxisomal chain shortening. (Figure 1, page 343. Agaba *et al.*, 2005)

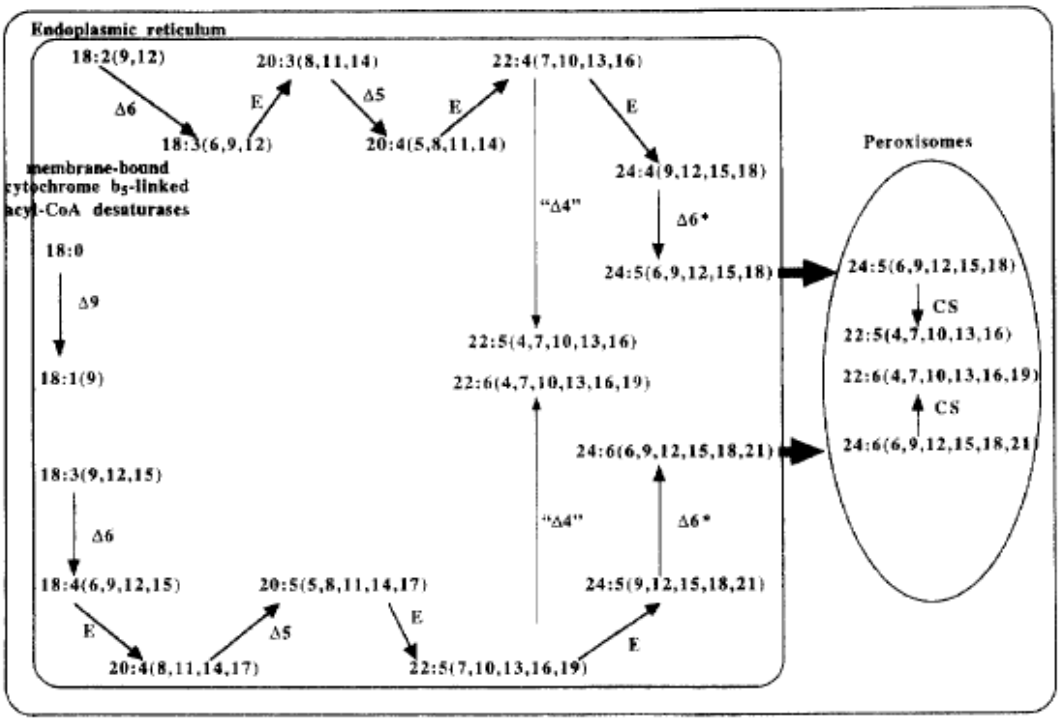

Figure 2.9: Summary of the main fatty acid desaturation reactions in animal cells. All fatty acid desaturation reactions in animals occur in the endoplasmic reticulum and are catalyzed by membrane-bound, cytochrome b5 linked desaturases that generally utilize coenzyme A linked substrates. Δ, Desaturase; E, Elongase. (Figure 3, page 76. Tocher *et al.*, 1998)

2.1.2.2 Desaturase and elongase genes in HUFA biosynthesis pathway

Over the last few years, major advances have been made in understanding the regulation and activities of fatty acid desaturase and elongase genes from an array of different organisms. Understanding the molecular basis of HUFA biosynthesis and its regulation in fish may enable the manipulation and optimization of the activity of the pathway to allow efficient and effective usage of plant-based oils as fish oil substitutes in aquaculture (Pereira *et al.*, 2003; Zheng *et al.*, 2004a; Agaba *et al.*, 2005).

Desaturase and elongase genes have been identified and cloned in a number of fish species. Elongase genes have been cloned from zebrafish, Atlantic salmon, Nile tilapia, African catfish and cod (Agaba *et al.*, 2004; Hastings *et al.*, 2004; Agaba *et al.*, 2005). As for Δ6 and Δ5 desaturase genes, extensive work has been done with fish such as carp, salmon, cod, tilapia, sea bream and rainbow trout (Seiliez *et al.*, 2001, 2003; Hastings *et al.*, 2004; Zheng et al, 2004a). All of these desaturases are primarily a single function enzyme. A bifunctional desaturase has been cloned in the zebrafish (Hastings *et al.*, 2001).

The zebrafish desaturase gene is unique because it encodes an enzyme having both Δ6 and Δ5 desaturase activities. It has been speculated that the bifunctional desaturase in zebrafish is a component of a prototypic vertebrate PUFA biosynthetic pathway that has persisted in a freshwater species. The deficit in the fatty acid desaturation pathway in marine fish could be attributed to their strictly piscivorous diet. As their diet is naturally rich in HUFA, they may have lost their ability to elongate and desaturate PUFA due to evolution (Hastings *et al.*, 2001). The cloning and characterization of fatty acid desaturase from carp, phylogenetically closely related to zebrafish, showed interesting results as it was basically unifunctional. Therefore, it is unclear whether the zebrafish desaturase represents an ancestral progenitor or a later evolutionary adaptation. This question may remain unsolved unless other bifunctional desaturases are identified (Zheng *et al.*, 2004a).

As the search for alternatives to fish oil in aquafeeds continues, studies done on desaturation and elongation of PUFA in fish fed various plant-based oils intensifies. Tissue fatty acids in fish fed vegetable oils are characterized by increased levels of C18 PUFA and

decreased levels of HUFA. Desaturase and elongase genes expression were affected by dietary treatments in fish. A recent study on salmon reported that expression of both Δ5 and Δ6 desaturase genes in liver were increased in fish fed the highest inclusion of vegetable oil though there was no dietary effect on the hepatic expression of elongase gene (Pratoomyot *et al.*, 2008). Generally, the increase in plant-based oils in diet had increased the expression of these genes in fish tissues (Tocher *et al.*, 1997; Tocher *et al.*, 2001; Zheng *et al.*, 2004a; Ling *et al.*, 2006). The use of cell culture systems and molecular gene expression study has elucidated some of the important pathway and biochemical reactions in HUFA biosynthesis.

2.2 Lipid and fatty acid requirements of fish

2.2.1 Dietary lipid requirement of fish

As lipids play an important role in fish nutrition, it has been studied widely in various aspects. Besides being energy source to fish, lipids also provide essential fatty acids (Sheik-Eldin *et al.*, 1996). Lipid requirements, more specifically EFA requirements, usually differ amongst fish species; even more so for fish from the marine environment and freshwater.

Most marine species usually require diets which contain HUFA [20:4*n*-6, 20:5*n*-3 and 22:6*n*-3] as they are unable to convert PUFA [18:3*n*-3 and 18:2*n*-6] via a series of desaturation and elongation (Tocher *et al.*, 2001). In contrast, most freshwater species are able to perform the PUFA conversion into HUFA, hence a diet rich in HUFA may not be necessary. However, there have also been speculations that marine fish may require such diet due to their adaptations to a carnivorous lifestyle. The hypothesis being that, consumption of a carnivorous diet, naturally rich in HUFA, results in an evolutionary down-regulation of the enzymes required for PUFA to HUFA conversion (Buzzi *et al.*, 1997; Tocher *et al.*, 1997, Seiliez *et al.*, 2003; Piedecausa *et al.*, 2007). Supporting this view, the pike (*Esox lucius*), a carnivorous freshwater species, displayed limited ability to convert PUFA to HUFA (Buzzi *et al.*, 1997).

In addition to being highly digestible and well metabolized by fish, lipids also increase diet palatability and stabilize feed pellet during manufacture, transportation and storage. Increasing dietary lipid may be and effective strategy to improve feed efficiency and reduce protein utilization as lipids is able to spare the use of protein as energy source.

Despite protein synthesis, some amino acids will be directed into energy liberating pathway to provide energy for basal metabolism and this will often limit fish growth. Lipid and carbohydrate will usually be included in the dietary regiment of fish to replace protein as the alternative energy source so that most of the amino acids will be utilized for protein synthesis. This is referred as the protein sparing effect. Sparing the usage of protein as energy source also reduces ammonia and nitrogen waste production from the metabolism of amino acids in the aquatic environment (Shyong *et al.*, 1998; Vergara *et al.*, 1999; Lopez *et al.*, 2006).

Usually, in aquaculture, fish meal is the main ingredient which is utilized to provide the protein source. The demand for fish oil and fish meal production as aquafeed ingredients is rapidly increasing, causing the depletion of world fish stocks. Besides finding alternatives to fish oil, substitutions to fish meal as energy providing protein should be used. Lipid is an effective non-protein energy source as it releases more energy per unit weight (De Silva and Anderson, 1995). It is easily digested and metabolized compared to carbohydrates, therefore serves as a better source of energy for protein sparing. Adequate levels of protein and lipid in diets should be carefully considered as an imbalance may have adverse effects of fish growth, nutrient utilization lipid deposition, increased production cost and deterioration in water quality (Vergara *et al.*, 1999; Tocher *et al.*, 2004).

Variations in protein sparing effect of lipid exist amongst fish species. In bagrid catfish (*Pseudobagrus fulvidraco*), increment of lipid sources with the constant amount of protein in diets improved protein utilization and growth (Lee and Sang, 2005). Increasing dietary lipid levels significantly improved hepatosomatic index, viscerosomatic index, protein efficiency ratio and protein retention in juvenile rockfish (*Sebastes schlegeli*), indicating protein sparing effect of lipid (Lee *et al.*, 2002). Conversely, increase in dietary

lipid did not induce protein sparing effect in studies conducted on white sea bream (*Diplodus sargus*), sharpsnout sea bream and dentex (Ozorio *et al.*, 2006; Hernandez *et al.*, 2001; Espinos *et al.*, 2003). High inclusion of carbohydrate may also inhibit absorption and reduced protein sparing effect by lipid (Ozorio *et al.*, 2006). Fish ingest little carbohydrate as part of their natural diet. Consequently they are incapable of metabolically utilizing high dietary levels of digestable carbohydrate. Thus lipids are the favoured form of non-protein metabolic energy (Huang *et al.*, 2007).

2.2.2 Dietary fatty acid requirement of fish

Fish require HUFA for their normal growth and development including reproduction. As mentioned previously, freshwater fish have the ability to convert C18 PUFA to HUFA, hence their EFA are LA and LNA. The EFA requirements of fish differ considerably from species to species. Inadequate provision of EFA may bring forth low growth rate, poor food conversion rate and affect reproductive performance of broodstock. They also act as precursors for a number of biologically active molecules like eicosanoids, pheromones, growth regulators and hormones (Pereira *et al.*, 2003). In fish, DHA and EPA are the major HUFA of the cell membranes while ARA is a minor component. Fish tissues have higher concentrations of DHA and EPA compared to ARA and fish have correspondingly higher dietary requirements for *n*-3 HUFA. However, ARA should not be disregarded as they play important roles in the formation of eicosanoids (Sargent *et al.*, 1999).

In general, HUFA play a role in maintaining the structural and functional integrity of cell membranes. Phosphoglycerides and their fatty acid compositions have a major and very well-established role in maintaining the structure and function of cellular biomembranes. There are few data that directly demonstrate a clearly defined role for specific fatty acids in membrane functions in fish. However, the importance of DHA in neural tissues of all vertebrates, including fish, has recently been the subject of considerable. Thus, in fish, dietary deficiency of DHA resulted in larval herring having an impaired ability to capture

prey at natural light intensities (Bell *et al.*, 1995) and impaired schooling behavior in yellowtail and Pacific threadfin (*Polydactylus sexfilis*) (Masuda *et al.*, 1998; Ishizaki *et al.*, 2001). These recent studies imply a critical role for DHA in the functioning of neural tissue (brain and eye) in fish and also demonstrate the importance of dietary DHA in marine fish (Tocher, 2003).

Eicosanoids are oxygenated derivatives of HUFA produced from membrane phospholipids by the action of phospholipases, cyclooxygenases and lipoxygenases (Ganga *et al.*, 2005). They are a range of highly active C20 compounds formed in small or trace amounts by virtually every tissue. In broad terms, eicosanoids are produced in response to stressful situations at a cellular and whole body level. ARA is the major precursor of eicosanoids in fish which are involved in a variety of physiological functions like cardiovascular functions, osmoregulation and functions of reproductive systems (Cejas *et al.*, 2004). EPA may also form eicosanoids but they are less biologically active than those formed from ARA. Moreover, EPA competitively inhibits the formation of eicosanoids from ARA (Figure 2.10). Likewise, eicosanoids by EPA also competitively interfere with the actions of eicosanoids formed from ARA (Sargent *et al.*, 1999).

The two main enzymes involved are cyclooxygenase that produces cyclic oxygenated derivatives or prostanoids, including prostaglandins (PG), prostacyclins (PG I) and thromboxanes (TX), and lipoxygenases that produce linear oxygenated derivatives, including hydroperoxy- and hydroxy fatty acids, leukotrienes (LT), and lipoxins (LX). Collectively, these fatty acid derivatives are termed eicosanoids, so named because they are derived primarily from the C20 PUFA DGLA, ARA and EPA. The eicosanoids are autocrines, that is, hormone–like compounds produced by cells to act in their immediate vicinity with a short half-life. Virtually every tissue produces eicosanoids, and they have a wide range of physiological actions, for example, in blood clotting, the immune response, the inflammatory response, cardiovascular tone, renal function, neural function and reproduction (Tocher, 2003). Influences of fatty acid in fish reproduction are further reviewed in Section 2.4.

Dietary fatty acids affect the composition and deposition of fatty acids in fish tissues (Dosanjh *et al.*, 1988; Torstensen *et al.*, 2000). Fish deposit significant quantities of fatty acids in their muscle. Fish like herring, sardines, salmon and trout provide a source of *n*-3 HUFA in human diets due to their deposition of their dietary fatty acids in muscle (Bell *et al.*, 2003b). As noted above, marine animals can contain very large levels of lipid in the form of oil. For example, many high-latitude zooplankton can routinely contain two-thirds or more of their dry body weight as oil, largely wax esters. Capelin (*Mallotus villosus*) can routinely contain 20% or more of their wet body weight as oil, largely triacylglycerols. Therefore, it is self evident, that fish consuming such oil-rich prey, for example, capelin consuming zooplankton, or cod or salmon consuming capelin, are capable of efficiently digesting and assimilating large quantities of lipid, and often of depositing large quantities of oil in their body tissues. All fish oils are highly polyunsaturated, characterized by high but variable levels of *n*-3 HUFA, predominantly EPA and DHA, with ARA as the major *n*-6 PUFA, and with 16:0 followed by 18:0 as the predominant saturated fatty acids, and all contain substantial amounts of the monoene 18:1*n*-9.

In freshwater fish, the LNA and LA ratios are major determinants of the final tissue ratios of EPA, DHA and ARA. Imbalanced levels and ratios of dietary EFA will cause competitive interactions between different series of fatty acids. A competitive reaction also exists between LA and LNA in the conversions to their end products of HUFA. Therefore the provision of an optimum ratio of *n*-3 and *n*-6 PUFA for fish are important, and sometimes may prove to be tricky as the requirements of different species vary (Mazorra *et al.*, 2003).

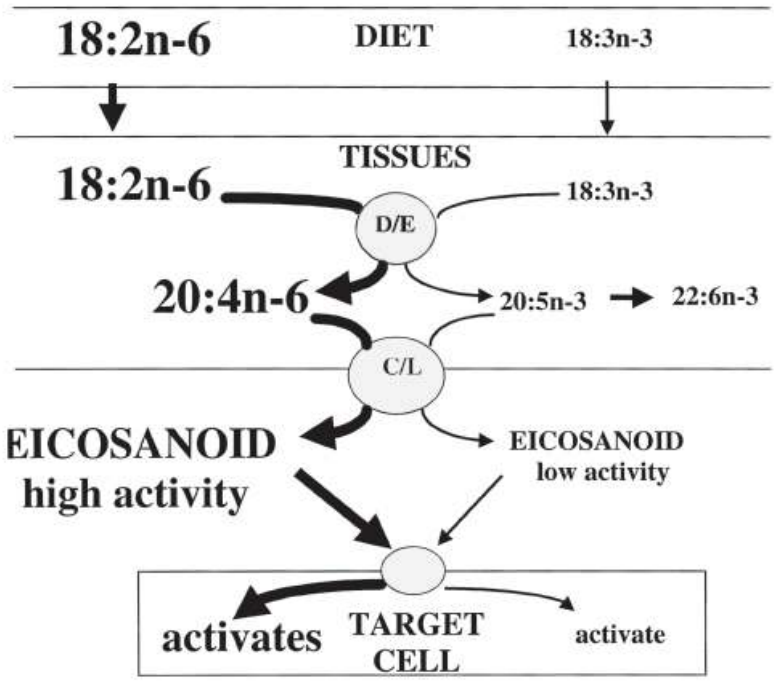

Figure 2.10: Links between dietary PUFA, tissue HUFA and eicosanoid production. Arachidonic acid, 20:4*n*-6 and eicosapentaenoic acid, 20:5*n*-3, produced by desaturation and elongation (D/E) of dietary 18:2*n*-6 and 18:3*n*-3 or obtained preformed in the diet, compete for the same cyclooxygenase and lipoxygenase enzymes (C/L) to produce, 2-series prostanoids and 4-series leukotrienes, and 3-series prostanoids and 5-series leukotrienes, respectively. Therefore the ratio of ARA:EPA determines the ratio of high activity : low activity eicosanoids. (Figure 7, page 50. Tocher, 2003)

2.3 Utilization of lipids in aquaculture

Fish farming has expanded and intensified rapidly, especially during the last two decades. In order to support the rise of this activity, particular attention has to be paid to the development of more suitable diets for each species (Bandarra *et al.*, 2006). Currently the dependence on fish meal and fish oil as major lipid sources is heavy. This dependency has raised concerns on the sustainability of the aquaculture sector. There is an urgent need to seek alternatives for utilization of marine-based oils, which are currently facing issues such as diminishing supplies and escalating cost (Ganga *et al.*, 2005; Ling *et al.*, 2006; Turchini *et al.*, 2006).

Plant-based oils have been suggested as alternate choices to fish oil as they are easily obtained and cheaper. The unfeasible criterion in plant oils is low or insufficient *n*-3 HUFA (Bell *et al.*, 2001, 2003a; Francis *et al.* 2006). Therefore it is crucial to possess the knowledge on the dietary fatty acid requirements of cultured species in order to successfully incorporate accurate levels of plant oils as the dietary lipid source.

Numerous studies on various marine and freshwater fish species have been conducted over the years in search for the suitable replacement or partial replacement of fish oil. Plant oils used as partial replacements for fish oil in marine fish diets have demonstrated promising results with respect to gilthead sea bream (Montero *et al.*, 2003; Izquierdo *et al.*, 2005), European sea bass (Izquierdo *et al.*, 2003; Mourente *et al.*, 2005) and turbot (Regost *et al.*, 2003).

Accordingly, several studies involving freshwater and salmonid species have demonstrated the possibility of high inclusion levels of plant oil without compromising growth rate of fish. Feeding Atlantic salmon (*Salmo salar*) with fish oil alone or blends with fish oil and sunflower oil, or linseed oil and sunflower oil did not show differences in growth (Menoyo *et al.*, 2007). Other studies utilizing soybean, linseed, rapeseed, palm and sunflower oils have been frequently evaluated and showed no reduction in growth or feed utilization, but affected tissue lipid composition, which reflected that of the dietary oils used (Bell *et al.*, 2003b; Bransden *et al.*, 2003; Francis *et al.*, 2006; Bahurmiz and Ng, 2007) .