EFFECTS OF DIETARY ALPHA LINOLENIC ACID ON BIOSYNTHESIS OF N-3 LONG CHAIN POLYUNSATURATED FATTY ACIDS IN ANIMALS

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Abstract

Omega-3 (n-3) long chain polyunsaturated fatty acids (LCPUFA), particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are important for normal health as well as growth and brain development in humans. These fatty acids can be consumed in the diet directly, or synthesised from short chain PUFA consumed in the diet. Fish, particularly in species with a high fat content like salmon, are a major source of these beneficial fatty acids in the human diet.

Fish production from aquaculture continues to expand due to a growing human population and demand for fish. Currently there is a reliance on fish oil and fish meal derived exclusively from wild fish as the primary lipid and protein source in fish feeds. Depleted wild fish stocks have made this source of n-3 LCPUFA unsustainable and alternative sources of n-3 LCPUFA are required to fill the void.

Most animal species can convert the plant derived 18 carbon (C18) n-6 linoleic acid (LA, 18:2 n-6) and n-3 α-linolenic acid (ALA, 18:3 n-3) to 20 and 22 carbon (C20-C22) LCPUFA by using a series of enzymes to extend and alter the saturation level. There are two types of enzymes responsible for desaturating and elongating fatty acids are desaturases and elongases. The genes associated with these processes appear to be regulated by the extremes of dietary PUFA intake but the extent is currently unclear.

This thesis is aimed to examine the effect of dietary PUFA on tissue n-3 LCPUFA levels in animals (rat and fish) after the consumption of diets with increasing levels of ALA, and to investigate whether the expression of desaturases and elongases is involved in the regulation of lipid metabolism and therefore LCPUFA biosynthesis. Furthermore, this thesis also investigated the potential enzyme functions of barramundi Δ6 desaturase and elongase using a yeast heterologous system.

Experiments showed that while high ALA diets consistently produced higher levels of n-3 LCPUFA in rat tissues than low ALA diets, mRNA abundance of the Δ6 desaturase (FADS2) and elongase 2 (ELOVL2) genes were increased only in animals fed the low PUFA reference diet compared to those fed diets with adequate to high PUFA levels. There was no correlation between the gene expression of desaturases, elongases or transcription factors and the levels

of EPA, docosapentaenoic acid (DPA, 22:5 n-3) or DHA in rat blood, liver and other tissues as a result of feeding increasing levels of ALA.

In barramundi however, while vegetable oils induced significant increases in mRNA abundance of FADS2 and ELOVL genes compared with those fed the fish oil-based commercial diet, the tissue EPA, DPA and DHA levels were not increased. It is therefore hypothesised that the enzyme activity of barramundi Δ6 desaturase was low and therefore limited the effectiveness of the enzymes in the LCPUFA pathway to produce EPA and DHA. Furthermore, a large amount of variation between individual fish in DHA levels among those fed the vegetable oil-based diets was found, and this may provide a possibility for a future breeding program of barramundi for better DHA production.

Barramundi FADS2 and ELOVL genes were also cloned into yeast cells and performed functional expression of the two enzymes. Results revealed that the barramundi Δ6 desaturase also showed Δ8 desaturase activity and the elongase showed a broad range of fatty acid specificity with the greatest activity with EPA. In addition, a significant amount of the desaturation and elongation fatty acid products could be detected in the culture medium at various time points after the addition of fatty acid substrates, and that it was important to take the levels of fatty acids in the medium into account when it came to calculating enzyme activity.

Declaration

This is to certify that the data contained in this thesis is my own work and the thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Wei-Chun Tu and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Publications:

Omega-3 long chain fatty acid synthesis is regulated more by substrate levels than gene expression. 2010. Prostaglandins Leukot Essent Fatty Acids 83: 61-68.
***Wei-Chun Tu**, Rebecca Cook-Johnson, Michael James, Beverly Mühlhäusler and Robert Gibson*

Abstracts:

Study of barramundi (*Lates calcarifer*) Δ6 desaturase and elongase functions and activities using a yeast heterologous expression system. Experimental Biology, Washington D.C. USA. April 2011.
***Wei-Chun Tu**, Michael James, Rebecca Cook-Johnson, Beverly Mühlhäusler, David Stone and Robert Gibson*

Barramundi desaturase and elongase prefer omega-3 fatty acids as substrates and the Δ6 desaturase has Δ8 Activity. New Investigators Award, The 9th Conference of the International Society for the Study of Fatty Acids and Lipids, Maastricht, Netherlands, May 2010.
***Wei-Chun Tu**, Rebecca Cook-Johnson, Michael James, David Stone and Robert Gibson*

The effect of dietary α-linolenic Acid levels on regulation of omega-3 lipid synthesis in rat. World Congress on Oils and Fats & 28th ISF Congress, Sydney, Australia, September 2009.
***Wei-Chun Tu**, Rebecca Cook-Johnson, Michael James, Beverly Mühlhäusler and Robert Gibson*

Omega-3 LCPUFA levels in rat tissue after consumption of omega-3 rich vegetable oils. The Max Tate Prize for the best presentation in Plant and Food Science, 1st Annual Postgraduate Student Symposium, School of Agriculture, Food and Wine, University of Adelaide, Australia, September 2008.
***Wei-Chun Tu**, Rebecca Cook-Johnson, Michael James and Robert Gibson*

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Chapter 1
Introduction and Literature Review

Dietary lipids play an important role in the health and well being of humans and animals alike. Long chain n-3 PUFA in particular have been found to be beneficial for a range of chronic diseases including heart disease, arthritis and diabetes. While one method of increasing the n-3 LCPUFA levels in our tissues is to consume two fatty fish meals per week, most Australians actually eat less than one meal of low fat fish per fortnight, and as a result fail to achieve an adequate intake of the n-3 LCPUFA. EPA and DHA are the two most important n-3 LCPUFA. Although these LCPUFA can be obtained most readily from fish, these marine-derived fatty acids can also be derived via conversion of the short-chain precursor n-3 PUFA, ALA, in animals including humans, though there is variation between animals in their ability to make this conversion (1). Therefore, the two possible strategies for improving the n-3 status of the population are to consume diets enriched with fish or fish oil containing n-3 LCPUFA or plant derived n-3 PUFA such as ALA and stearidonic acid (18:4 n-3).

The conversion of ALA to EPA and DHA occurs through the LCPUFA synthetic pathway (Figure 1.1) and involves a series of desaturation and elongation steps. The presence of this metabolically active fatty acid conversion pathwayhas raised the question of whether the endogenous conversion of short chain n-3 precursors (derived from plants) in humans and most other animals could enable them to obtain their n-3 LCPUFA requirements from plant sources. Specifically, whether the essentiality of ALA in the diets of humans and other animals directly reflects the biological actions of ALA itself or of the LCPUFA that are derived from ALA remains contentious (2, 3). Due to the fact that the rate-limiting enzyme in the pathway, the Δ6 desaturase, is shared by both n-3 and n-6 C18 PUFA precursors and also 24 carbon (C24) n-3 LCPUFA in the process of DHA biosynthesis, the challenge is to determine the appropriate balance of dietary fats which can minimise competitive inhibition, and thereby maximise the efficiency of conversion of ALA and 18:4 n-3 to n-3 LCPUFA in humans and other animals.

Studies in this thesis were designed to demonstrate the effects of n-3 rich ALA vegetable oils on n-3 LCPUFA accumulation in the tissues of rat (as a model animal for mammals) and a popular fish (barramundi) eaten by humans and to determine the factors that regulate n-3

LCPUFA biosynthesis. Cloning and heterologous expression of barramundi desaturase and elongase genes, the two key enzymes in LCPUFA biosynthesis, are also included in this thesis. The overall aim of this project was to develop a sustainable oil blend that could be directly incorporated into feed of meat animals, such as farmed fish, to increase the n-3 LCPUFA levels in their tissues, and to demonstrate the possibility of making farmed fish a major source of n-3 LCPUFA without the need to include fish oil and fish meal in aquaculture feeds.

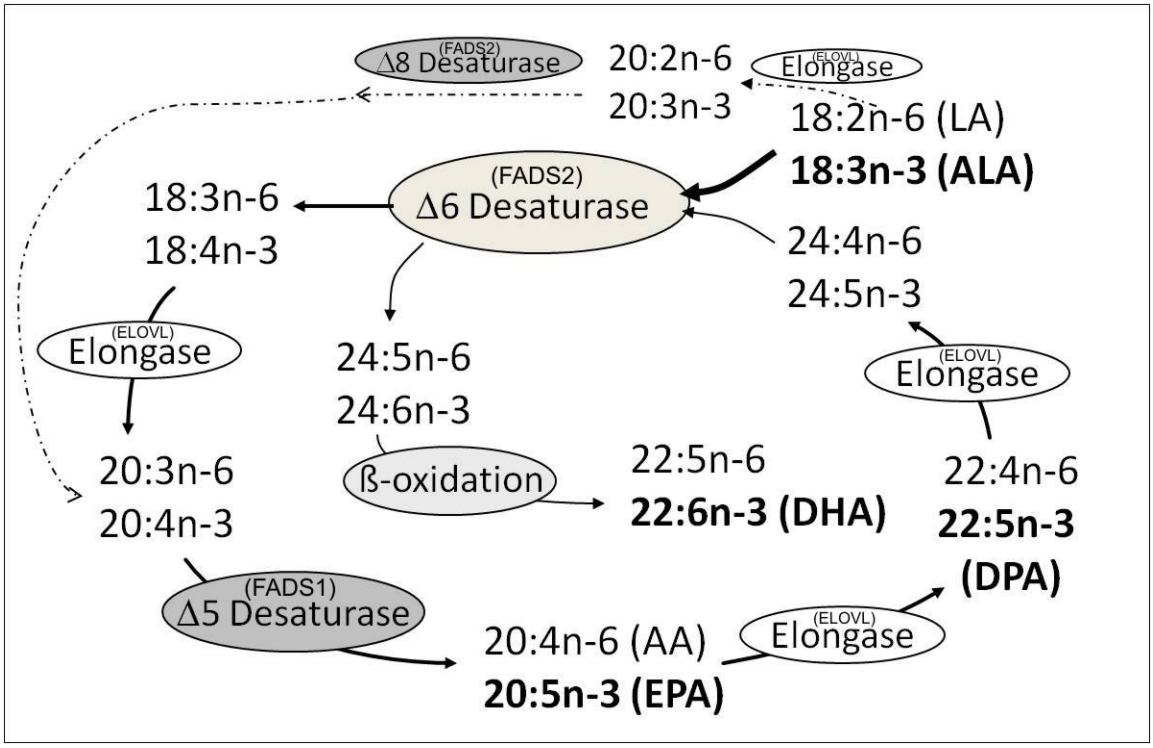


Figure 1. 1 Schematic overview of conversion of dietary essential fatty acids (EFA) (ALA and linoleic acid (LA, 18:2 n-6)) to LCPUFA (4, 5).

1.1 Dietary lipids and n-3 (LC) PUFA

Lipids are important constituents of diets and essential structural components required by the body for membrane integrity and cell function, and lipids stored in the adipose tissue are a major energy source for meeting metabolic requirements. Lipids are also a source of precursor compounds for the synthesis of eicosanoids and docosanoids, which are signalling molecules made by oxygenation of either C20 fatty acids for eicosanoids, such as prostaglandins, prostacyclins, thromboxanes and leukotrienes and for C22 fatty acids for docosanoids which include some resolvins and the docosatrienes (6). Cholesterol, glycosphingolipids and phospholipids are three types of constitutive lipids involved in regulating membrane related

functions (7). Many cellular functions and expression of membrane-bound molecules including enzymes, receptors and carrier-mediated transporters are influenced by alterations in the composition of membrane lipids (8).

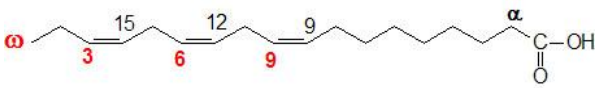
Most lipids contain at least one fatty acid of varying chain lengths, degrees of saturation and different configurations. Fatty acids of microbial, plant and animal origin commonly contain even numbers of carbon atoms in a carbon chain with a functional carboxyl group (–COOH) at one end. The fatty acids in nature can be classified into three subtypes, saturates, monounsaturates (monoenes) and polyunsaturates. Saturates have no double bonds between the individual carbon atoms of the fatty acid chain. Myristic acid (14:0), palmitic acid (16:0) and stearic acid (18:0) are the most common dietary saturates, and are found in animal and dairy fats (9). Monoenes, such as palmitoleic acid (16:1 n-7) and oleic acid (18:1 n-9) contain a single double bond between two methylene interrupted carbon atoms. Oleic acid is the most common monoene, and accounts for ~90% of dietary monounsaturated fatty acids. Monoenes are important components of membrane structural lipids, particularly nervous tissue myelin. Unlike the essential fatty acids, the body has the capacity to synthesise all the monoenes it requires in a process catalysed by the enzyme fatty acid synthase. As a result, they are not considered to be essential components in the diet. Monoenes are present in many animal- and plant-derived foods and contribute to roughly 15% of the fatty acids in the diet (10-12).

PUFA are fatty acids that contain more than two double bonds along their long carbon chain backbones. Two biologically important PUFA subgroups, n-3 and n-6 PUFA, are defined according to position of the first methylene-interrupted double bond from the methyl (omega) end of the fatty acid. Thus, n-3 fatty acids such as ALA are denoted by the nomenclature 18:3 n-3, indicating that the molecule contains three carbon-carbon double bonds with a carbon chain length of C18, and the position of first unsaturated bond is at the third carbon from the omega terminal end (Figure 1. 2 A). The n-6 fatty acids, such as LA, 18:2 n-6, also have at least two double bonds but in this class of fatty acids the first unsaturated bond is inserted at the sixth carbon from the omega terminal end (Figure 1. 2 B). The two n-3 and n-6 C18 PUFA are considered essential because vertebrates cannot synthesize these lipids *de novo* and rely on the diet as their sole source. Longer chain n-3 PUFA such as EPA and DHA are synthesized in animal and human tissues via multiple steps of desaturation and elongation of ALA. Another physiologically important n-6 LCPUFA, arachidonic acid (AA, 20:4 n-6), is converted from LA by the same set of enzymes. Both ALA and LA are primarily found in

green leaves and vegetable oils used in cooking but the main dietary source for n-3 LCPUFA is fish and other marine animals. This is because the n-3 LCPUFA are synthesized by phytoplankton at the base of the oceanic food chain which are consumed by fish and other marine organisms and accumulate in their tissues (13). AA is found in most animal products, including beef, pork, chicken, egg and dairy products (14). Some fish, particularly those found in warmer tropical waters, may also contain high amounts of AA (15).

The biosynthesis of LCPUFA, particularly the n-3 family, has attracted much attention because of their importance in human nutrition. Interest in the n-3 fatty acids originally stemmed from the results of an epidemiological survey of the prevalence of chronic diseases in traditional Eskimos living in Greenland relative to Western-European populations (16). The Eskimos were found to have a higher frequency of apoplexy and epilepsy but a much lower frequency, and virtual absence, of acute myocardial infarction, diabetes mellitus, thyrotoxicosis (or hyperthyroidism), bronchial asthma, multiple sclerosis and psoriasis (20). The high consumption of fish, and consequent increase in the dietary content of the n-3 LCPUFA, EPA and DHA, in the traditional Eskimo diets was considered to be the source of the reduced incidence of these diseases (17). Multiple interrelated mechanisms could explain the effects of EPA and DHA on disease risk, including 1) a shift from the AA-derived eicosanoid pathway to the parallel (less inflammatory) EPA-derived pathway (18); 2) transcriptional regulation of cytokine production (18); 3) direct effects on the expression of genes involved in the immune response, inflammation, lipid metabolism, and energy utilization (19, 20). As a consequence of these effects, diets rich in EPA and DHA are associated with a reduced blood concentration of a number of biomarkers of chronic disease risk (17). DHA is the major n-3 LCPUFA in the brain and retina, and it is believed that DHA is used continuously for the biogenesis and maintenance of neuronal and photoreceptor membranes (21-24). However, in the typical Western diets, more than 20 times more n-6 lipids are consumed than n-3 lipids (25). The predominance of n-6 fatty acids in diets has resulted in an imbalance in the levels of n-3 and n-6 in the diet, and an increase in the ratio of n-6 to n-3 fatty acids. As a result, ALA and LA compete for desaturation and chain elongation for synthesizing EPA and AA as well as other fatty acid metabolites. The relative dietary amounts of n-3 and n-6 fatty acids are important determinants of the relative cellular amounts of ALA and LA, and an appropriate balance of these fats in the diet is essential to optimize AA, EPA and DHA content in the membranes (4, 26).

A



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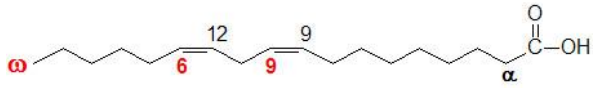


Figure 1. 2 Chemical structures of ALA (A) and LA (B).

1.2 Metabolism of dietary n-3 PUFA

After consumption of ALA in the diet, it is absorbed by the gastrointestinal tract and then secreted into the bloodstream for delivery to the liver (2). The availability of dietary ALA for conversion to n-3 LCPUFA is determined by the efficiency of uptake and the degree of partitioning towards degradation (β-oxidation) as well as incorporation into structural organs and storage reservoirs (2). The n-3 PUFA and LCPUFA which are incorporated into adipose tissue (particularly white adipose tissue) represent a reserve lipid pool which is available for mobilization during periods of increased demand such as fasting (27-29). Apart from the assimilation of ALA into adipose tissue, there are two other basic metabolic fates for dietary ALA in humans and other animals. The first is the use of ALA for energy production through β-oxidation, which can result in extensive carbon recycling (30, 31), and the second is as a substrate for the synthesis of EPA and DHA via desaturation, elongation and peroxisomal β-oxidation steps (32). This conversion occurs mainly in the endoplasmic reticulum of the liver with the final β-oxidation step, in which tetracosahexaenoic acid (24:6 n-3) is oxidised to the C22 product, DHA, occurring in the peroxisomes.

1.2.1 Degradation of dietary n-3 PUFA: β-oxidation cycle

A high proportion of PUFA consumed in the diet is immediately directed toward oxidation after consumption, resulting in low concentrations of PUFA in the blood and limited availability of these fatty acids for conversion to LCPUFA. Fatty acids can be degraded via different mechanisms, including α-, β- and ω-oxidation. In humans α-oxidation, which involves the chain-shortening of fatty acids by one carbon atom, takes place in peroxisomes only, whereas β-oxidation occurs in both peroxisomes and mitochondria (33). Fatty acid ω-oxidation consists of three sequential steps in which the terminal methyl group is converted

into a carboxyl group, and this is regarded as a minor pathway which occurs in the smooth endoplasmic reticulum, accounting for only 5-10% of total fatty acid oxidation in the rat liver under normal physiological conditions (34). The dicarboxylic acids that are produced via the ω-oxidation pathway can either be excreted into the urine or undergo further β-oxidation in mitochondria or peroxisomes (35, 36). The preferred pathway for oxidizing fatty acids is via β-oxidation in mitochondria. Mitochondrial fatty acid β-oxidation represents a physiological response to tissue energy depletion such as fasting and increased muscular activity, and this form of oxidation is believed to provide the major source of energy (as much as 80%) for the heart and skeletal muscle (37). The peroxisomes are the sole site of β-oxidation in fungi (yeast) and plants, following a process similar to that in mitochondria, but the individual reactions of the peroxisome β-oxidation pathway are catalyzed by different enzymes compared with the mitochondrial β-oxidation system (38). Importantly, in animals, peroxisomes oxidize a unique set of fatty acids including C22 to 26 carbon (C26) long chain fatty acids which cannot undergo β-oxidation in mitochondria. However, peroxisomes are unable to fully β-oxidize fatty acids; the fatty acids are initially oxidized to short chain fatty acids in peroxisomes and then transferred to mitochondria in the form of acyl-CoA esters for the completion of β-oxidation (39).

ALA is a precursor for n-3 LCPUFA synthesis and also a preferred substrate for β-oxidation in humans and other animals such as rat and fish, and ALA is reported to be rapidly β-oxidized within liver mitochondria (40, 41). The proportion of consumed ^{13}C-ALA used in energy production can be estimated from the appearance of liberated $^{13}CO_2$ in breath (42). In humans, the C18 fatty acids (ALA and LA) are the two most highly oxidized fatty acids (43). In tracer studies with labelled ^{13}C-ALA, diets containing high amounts of ALA (7% of energy, %en) resulted in more recovered tracer in breath 9 hr after tracer ingestion than in low ALA (0.4%en) diets (26). The complete oxidation of dietary ALA to CO_2 accounts for 25% of dietary ALA within 24 hr and 60% after 7 days in humans (44). The cumulative recovery of $^{13}CO_2$ from ^{13}C-ALA in the breath of humans during the first 12 hr after tracer ingestion has been shown to be negatively correlated with the maximal amounts of plasma ^{13}C-EPA and ^{13}C-DPA (45). Moreover, the proportion of ingested ALA that undergoes β-oxidation is also different between males and females, accounting for 22% of the consumed dose in females compared to 30% in male (46, 47). This may reflect the lower muscle mass for energy consumption or greater amounts of adipose tissue in females, and the lower oxidation of ALA in females contributes to the increased synthesis of LCPUFA from ALA in females compared

to males (2). Therefore, β-oxidation of ALA in humans ranges from 16 to 33% of the ingested tracer dosage, and this is dependent on the time since ingestion and gender (48). One of the reasons that ALA conversion is relatively poor is due to high proportion of ALA in diets immediately directed toward β-oxidation after consumption. The same phenomenon has been reported in rodents; in rats fed a diet containing about 2%en LA and 2%en ALA for 26 days, only 3% of LA and 1.4% of ALA were converted to LCPUFA, and the remaining ALA and LA (~80%) could not be accounted for, suggesting that it was either converted to energy by β-oxidation or stored in visceral fat (49). Lin and Salem (50) conducted a study in male rats using defined diets containing 15% of total fatty acids as LA and 3% as ALA and then fed single oral dose of both $^{2}H_5$-ALA and $^{2}H_5$-LA to the animals. This study showed that, on average, around 16-18% of the deuterium-labeled $^{2}H_5$-ALA and $^{2}H_5$-LA was accumulated in a number of tissues and approximately twice as much of the $^{2}H_5$-ALA (6%) was converted to its longer chain metabolites than was the $^{2}H_5$-LA (2.6%); whereas the remaining 78% of both C18 precursors was apparently catabolized or excreted. These studies indicate that significant amount of both ALA and LA from the diets are utilised for energy metabolism, leaving limited amounts of ALA available for conversion to n-3 LCPUFA.

1.2.2 Conversion of ALA

1.2.2.1 Conversion of ALA in cell cultures

Microsomes isolated from liver contain all the enzymes which are required for conversion of ALA to n-3 LCPUFA (51, 52). When HepG2 cells, the human liver hepatocellular carcinoma cell line, are incubated with ALA, the accumulation of ALA, EPA and DPA in the cell phospholipid fraction increases in a dose-dependent manner, whilst DHA accumulation is more limited; an accumulation of the intermediate Δ6 desaturase substrate, 24:5 n-3, is also linear in response to increasing levels of ALA, suggesting that the elongases involved in the conversion of EPA to DPA and of DPA to 24:5 n-3 were not saturated. In contrast, the accumulation of 24:6 n-3 and DHA with increasing dietary ALA does not follow a linear pattern, suggesting that the competition between 24:5 n-3 and ALA for Δ6 desaturase may contribute to the limited accumulation of DHA in cell membranes (53). Similar results to those in HepG2 cells have been obtained in brain cells. Incorporation of DHA into SH-SY5Y neuroblastoma cells under conditions that mimic n-3 physiological influx was analyzed after supplementation with ALA, EPA, DPA and preformed DHA. The results show that the incorporation of EPA, DPA, and preformed DHA followed a dose-response curve until the tissue reached saturation, whereas that of DHA converted either from ALA, EPA, or DPA

peaked at low concentrations of precursors and decreased at higher doses, in spite of the ability of the cells to desaturate fatty acids and synthesize DHA from each of the upstream precursors (54). The limited incorporation of DHA into phospholipids suggests that DHA competes with EPA and DPA for phospholipid acylation, and that there may be an impairment of activities of the enzymes that are involved in the final peroxisomal β-oxidation step of DHA production. All of these result in fairly inefficient incorporation of DHA derived from ALA into phospholipids compared with the incorporation of DHA that is obtained preformed from the diet (54). ALA conversion using human cell cultures indicate that cells have the capacity to biosynthesize and accumulate n-3 LCPUFA from their precursors, ALA or longer chain fatty acids. However, the conversion from ALA through to DHA is generally limited.

1.2.2.2 Conversion of ALA in mammals other than humans

In baboons, ^{13}C-tracer studies have shown that fetuses have the capacity to convert ALA to DHA *in vivo* in liver; and that the liver preferentially secretes long chain metabolites for delivery to other organs such as the brain and retina (55). The synthesis of n-3 LCPUFA from the short chain precursors depends on the concentration of the n-3 short-chain precursor, ALA, in the diet. In primates which are fed ALA deficient diets, tissue DHA levels were depleted and this was accompanied by a significant compensatory increase in longer chain n-6 fatty acids (56). Similarly, in rats fed ALA deficient diets (ALA 0.2% of total fatty acid) for 15 weeks, the levels of liver ALA, EPA, DPA, DHA and overall n-3 PUFA in total lipid were decreased by ~94% for each of the n-3 fatty acids, when compared with a group of rats fed an ALA sufficient diets (ALA 4.6% of total fatty acid) for the same period (57). The n-3 PUFA deficient diets decreased rat brain DHA levels by 37% and increased brain AA and n-6 DPA (22:5 n-6) concentrations (58). Thus, ALA conversion to n-3 LCPUFA can be modified by either increasing dietary ALA intake or decreasing LA intake. Excessive amounts of n-6 PUFA together with a high LA:ALA ratio, that is found in current Western diets, could therefore have negative effects on the n-3 LCPUFA content of tissues and thus the associated health benefits (59-62).

Is the n-6 to n-3 ratio or the absolute levels of ALA in the diet the main determinant of ALA conversion to n-3 LCPUFA in mammals other than humans?

The efficiency of LCPUFA synthesis diminishes with the progression of ALA down the fatty acid pathway, and thus DHA conversion from ALA is more restricted than that of EPA (3, 44,

63). A pioneering study by Mohrhauer and Holman reported that the level of AA in tissues decreased with increasing amounts of dietary ALA (64). This finding led to further studies of the interrelationship between LA and ALA, in particular the competition between these substrates for conversion to longer chain fatty acids when both are present in the diet. When rats were fed a fat-free diet supplemented with PUFA containing different combinations of ALA (0.05 to 1.8%en for 6 levels) and LA (0.08%en, 0.3%en and 0.6%en), ie, 18 dietary treatments with a range of various LA:ALA ratios (12:1 to 0.04:1), the percentage of AA in liver lipids of rats fed high levels of ALA (1.8%en) was less than 50% of the levels observed in livers of rats fed the lowest amounts of ALA (0.05%en). This effect was seen irrespective of the LA content of the diet, suggesting that the conversion of LA to its n-6 LCPUFA is suppressed by increasing dietary ALA (65). In the same study, the amount of the n-6 fatty acids, LA, AA and n-6 DPA in rat liver were increased significantly with increasing dietary LA levels, but there were no accompanying changes in the concentration of the n-3 family, ALA, EPA, DPA and DHA. These results suggested, therefore, that the ratio of LA to ALA in the diet has less of an impact on n-3 LCPUFA synthesis compared with n-6 PUFA synthesis and conversion, and that the synthesis of n-3 LCPUFA is regulated to a greater extent by dietary ALA content (65).

The effect of dietary ALA levels on n-3 LCPUFA production is similar in other animal species. In a piglet study in which the level of dietary LA was kept constant at 13% of total fatty acids whilst ALA levels were increased from 1% up to 26% of total fatty acids, i.e. total PUFA content varied from ~15 to 40% of the total fatty acids, tissue levels of ALA, EPA and DPA were all elevated with increasing dietary ALA content and decreasing LA:ALA ratio. However, the incorporation of DHA into piglet tissues followed a curvilinear pattern; the maximum incorporation of DHA occurred at LA:ALA ratios of 4:1 and 2:1, or total PUFA of 16% and 19% of total fatty acids, respectively (4). A similar study also suggested that DHA accretion requirements in the piglet brain and retina could be met with a supply of dietary ALA at 1.7%en (equal to 3.9% of total fatty acids) and an LA:ALA ratio of 4:1 (total PUFA levels of about 20% of total fatty acids) (66). Woods *et. al.* (67) examined a broader range of dietary PUFA levels in rat pups, by lowering the LA:ALA ratio from 10:1 to 1:1 and further down to 1:12 using ALA and LA ethyl esters and fixing the total PUFA levels at 30% of total fatty acids. They found significant increases in the accumulation of ALA, EPA and DPA in the brain and retina with increasing proportion of dietary ALA, whereas there was only a small effect of increasing dietary ALA on brain DHA accumulation and no effect on the

proportion of retinal DHA. Similarly, another study showed that varying the LA:ALA ratio from 22:1 to 1:1, and total PUFA levels from 19 to 36% of total fatty acids, resulted in a curvilinear relationship between dietary ALA and the proportion of neuronal cell phosphatidyl-serine and phosphatidyl-inositol DHA, with the highest DHA incorporation in both lipid fractions occurring at an LA:ALA ratio of between 7.8:1 and 4.4:1, and a total dietary PUFA content of 20% (68).

Based on the results of the studies described above in rats and piglets, it has become clear that the dietary LA:ALA ratio is not the only determinant of n-3 LCPUFA conversion, but that both the ALA and total PUFA content of the diet also have a role in determining the overall effect of a diet on n-3 LCPUFA synthesis, particularly the synthesis of DHA.

1.2.2.3 Conversion of ALA in humans

As in other mammals, the liver is the major site of lipid metabolism in humans. Hepatic AA, EPA and DHA levels in patients with non-alcoholic fatty liver disease (steatosis and steatohepatitis) were significantly decreased by 59-86% compared with that in normal liver, and this appeared to be a result of impaired activity of the enzymes required for desaturation and elongation of the dietary LA and ALA (69). When humans consume n-3 lipid rich diets, the ingested or synthesized n-3 LCPUFA partially replace the n-6 fatty acids, particularly AA, in cell membranes. However, the modern Western diet typically contains high levels of n-6, particularly LA and AA, and low amounts of n-3 fatty acids (70). As a result, n-6 fatty acids are usually present at much higher levels than n-3, and this is reflected in the composition of blood and tissue phospholipids.

It is generally agreed that the synthesis of DHA in humans is relatively inefficient, and a number of studies have demonstrated that the effects of providing pre-formed EPA or DHA in the diet cannot be reproduced by providing the equivalent amount of ALA (46, 47, 71). A double-blind, randomized human trial reported that the dietary ALA-rich flaxseed oil had no effect on plasma DHA levels, but increased circulating plasma EPA and DPA levels by 60% and 25%, respectively, after 12 weeks (72). Moreover, a similar study in lactating women showed that the consumption of 20 g of flaxseed oil, providing the equivalent of ~11 g of ALA per day, was able to elevate the levels of ALA, EPA and DPA in breast-milk, plasma and erythrocytes but had no effect on DHA concentrations (73). Another similar human study showed that diets containing flaxseed oil resulted in a significant increase in EPA in the

plasma lipid fractions and neutrophil phospholipids; but no significant increase in DHA content (74). One of the reasons for this relatively poor conversion efficiency of ALA to DHA is thought to be due to competitive inhibition between substrates for the enzymes in the fatty acid pathway which converts ALA and LA to longer chain fatty acids (13). An excess of dietary LA in human diets increases competition between LA and ALA for the same desaturases and elongases activities, and limits n-3 LCPUFA synthesis (53, 75, 76). As a result, there have been many attempts to increase the levels of EPA and DHA in humans by altering the ratio of dietary LA:ALA (77, 78).

Is the n-6 to n-3 ratio or the absolute levels of ALA in the diet the main determinant of ALA conversion to n-3 LCPUFA in humans?

There is evidence that the balance of n-6 and n-3 fatty acids in the diet plays an important role in ALA conversion in humans as it does in other species. In a study in human infants, those infants who received an infant formula which contained 3.2% of total fatty acid as ALA and an LA:ALA ratio of 4.8:1, had higher DHA levels in plasma phospholipids but lower levels of AA compared to those that received formula containing 0.4% of total fatty acids as ALA with LA:ALA ratio of 44:1, when the LA content was kept constant (79). Thus, the highest ALA intake, or the lowest LA:ALA ratio, resulted in higher plasma phospholipid levels of DHA, and the lowest ALA intake, or the highest LA:ALA ratio, resulted in higher levels of AA. A more recent trial indicated that infants fed formula with a ratio of LA:ALA of 5:1 had greater DHA levels in plasma and erythrocyte phospholipids than infants receiving a formula with an LA:ALA ratio of 10:1, again, the LA level was kept constant (80). Both of these studies suggested that higher intakes of ALA, and the accompanying decrease in the LA:ALA ratio, results in enhanced synthesis of n-3 LCPUFA.

The first tracer study in humans to quantify the effects of changes in dietary LA intake on the conversion of ALA to its long chain metabolites showed that the average conversion of ALA to n-3 LCPUFA was reduced by 40% when LA intake increased from 4.7 to 9.3%en (81). However, the simultaneous change in both ALA intake and the LA:ALA ratio could have affected n-3 fatty acid biosynthesis because as the LA level of the diet was changing, ALA intake was decreasing from 0.6 to 0.3%en and the LA:ALA ratio increasing from 8:1 to 31:1 (81). Another human study, compared two dietary groups with the same LA:ALA ratio (7:1) but different in amounts of LA%en and ALA%en with a reference group in which the diet had a higher LA:ALA ratio (LA 7%en, ALA 0.4%en with LA:ALA of 19:1). Their results showed

that the incorporation of dietary ALA into phospholipids increased by 3.6% in the low LA group (LA 3%en, ALA 0.4%en with LA:ALA of 7:1) and decreased by 8% in the high ALA group (LA 7%en, ALA 1.1%en with LA:ALA of 7:1) compared to the reference diet. In absolute amounts, ALA content increased by 34 mg in the low-LA group but there were no obvious changes in the high-ALA group, in which virtually all of the ALA in the plasma phospholipids was converted to EPA and DPA. Whilst the DHA content of phospholipids as a % of total fatty acids was not significantly different between the 3 diets, if DHA content was converted to absolute amounts, it increased slightly, from 0.7 mg in the low ALA group to 1.9 mg in the high ALA dietary group (26). Hence, these results suggest in order to achieve the maximum n-3 LCPUFA conversion in humans, one cannot focus only on altering the ratio of LA:ALA, but also need to consider the individual amounts of the dietary PUFA, ALA and LA, present in the diet.

In summary, the background diet influences the conversion of n-3 and n-6 fatty acids because the enzymatic catalysis system involved in the n-3 series is also used for n-6 fatty acid conversion. Simply changing the LA:ALA ratio in the absence of any change in dietary fat content, can have some effect on DHA concentrations, but these will be minor, since it will not reduce the total amount of LA and ALA which competes with their C24 long chain products, and limits their conversion to DHA and its counterpart (22:5 n-6). An *in vivo* human study suggested that dietary LA competes with ALA for conversion to their respective downstream n-6 and n-3 LCPUFA and their incorporation into or partitioning between membrane and plasma lipid stores (82). Increases in EPA synthesis can be obtained by lowering the intake of LA whereas increases in DHA synthesis can only be achieved by increasing ALA intake. Thus, reducing LA in diets together with an increase in ALA consumption is likely to be the optimal strategy for promoting EPA and DHA synthesis (26).

1.3 Desaturations and elongations in the n-3 fatty acid synthetic pathway

Biosynthesis of the n-3 and n-6 LCPUFA series occurs via the same consecutive desaturation, elongation and partial degradation steps (76, 83). Vertebrates are capable of synthesizing double bonds at Δ4, Δ5, Δ6, Δ8 and Δ9 positions[1] of fatty acid molecules, however they lack the Δ12 and Δ15 desaturase enzymes which are present in insects and most higher plants. As

[1]Δ for indicating the number and position of the double bonds; eg, Δ9 indicates a double bond between carbons 9 and 10 of the fatty acid; the Δ system which is the reverse of the ω- nomenclature. ω-9 indicates a double bond on the ninth carbon counting from the ω-carbon.

a result, fatty acids that contain unsaturated carbons beyond the Δ9 position, such as ALA and LA, must be obtained from the diet (84). Howton *et. al.* (85) fed 1-^{14}C-LA to rats and first showed that this fatty acid was transformed into AA through desaturation and elongation. The same group further demonstrated that 1-^{14}C-18:3 n-6 was transformed into AA, suggesting a route from LA to 20:4 n-6 via 18:3 n-6. Furthermore, methyl 2,3-$^{14}C_2$-20:3 n-6 administered to rats was dehydrogenated to 2,3-$^{14}C_2$-20:4 n-6, thus proving the existence of the pathway from LA to AA via synthesis of 18:3 n-6 and 20:3 n-6 (86, 87). The same route is also utilised for the metabolism of ALA to EPA (88). The pathway from ALA to EPA and further to DHA has been extensively studied and it is generally accepted that C22 fatty acids, DPA and 22:4 n-6, are first converted to C24 LCPUFA in the endoplasmic reticulum, and then undergo a second desaturation by Δ6 desaturase to form 24:6 n-3 and 24:5 n-6, before finally moving to peroxisomes for partial β-oxidative chain-shortening to produce DHA (32, 83, 89, 90).

1.3.1 Desaturases

The enzymes involved in LCPUFA biosynthesis, including the Δ4, Δ5, Δ6 and Δ8 desaturases, are named 'front-end' desaturases. The 'front-end' desaturases introduce a double bond between a pre-existing olefinic bond and the carboxyl (front)-terminal end of the fatty acid (91). Cloning and sequence analysis of deduced amino acid sequences has shown that animal desaturases share several structural features, including a N-terminal cytochrome *b*5 domain carrying heme-binding motifs, membrane-spanning domains and three histidine-rich regions that are characteristic of membrane-bound desaturases (5, 92-99). The expression of Δ9 desaturase activity is also present in the majority of mammalian cells, however the Δ9 are categorized in the stearyl-CoA (SCDs) subgroup of desaturases, which introduce the first *cis*-double bond at the C9 and C10 position from the carboxyl terminal end of fatty acids and synthesize monounsaturated fatty acids from saturated fatty acids that are either synthesized *de novo* or derived from the diet (100, 101). The Δ9 desaturase has a conserved motif consisting of 8 histidine rich regions.

1.3.1.1 Δ4 and Δ8 desaturases

The existence of Δ4 fatty acid desaturation in the biosynthesis of DHA has been reported in protozoan trypanosomes (102), the photosynthetic freshwater protist *Euglena gracilis* (103) and marine microalgae *Pavlova lutheria* (104) and *Thraustochytrium* sp (105). When expressed in a yeast system, Δ4 desaturase introduces a double bond at position 4 of DPA and 22:4 n-6 resulting in the production of DHA and n-6 DPA (105). The FADS2 gene encodes

the Δ6 desaturase. A putative FADS2 gene isolated from a marine fish species, *Siganus canaliculatus* (white-spotted spinefoot or rabbitfish) was able to confer yeast cells the ability to convert DPA to DHA, indicating that this gene encoded an enzyme with Δ4 desaturase activity (99). The presence of Δ4 desaturase activity in fish indicates that they have a simpler route for the conversion of EPA to DHA than that in the classic LCPUFA pathway, however, not all fish have Δ4 desaturase activity for the LCPUFA synthesis and the Δ4 desaturase activity has yet to be found in any mammalian species. However, a study in which mammalian glioma cells were shown to be able to convert exogneously added deuterated fatty acid substrates 20:3 n-3 to 20:4 n-3 raises the possibility of the existence of Δ8 desaturase activity[2] in mammals (106). This is supported by an *in vivo* study in mouse liver, which suggested that a small percentage of the products identified were formed by Δ8 desaturation (107). A recent report has also demonstrated that the primate Δ6 desaturase also has Δ8 activity, since it can desaturate 20:3 n-3 and 20:2 n-6 to 20:4 n-3 and 20:3 n-6, respectively, when expressed in yeast cells (5). Together, these findings indicate the existence of an alternative pathway for conversion of ALA and LA in which they are first converted to C20 intermediates and then further destaturated by Δ8 desaturase into 20:4 n-3 or 20:3 n-6, respectively. However, whether these alternate routes of PUFA metabolism have physiological significance in humans and other animals remains unclear.

1.3.1.2 Δ5 and Δ6 desaturases

Of all the desaturases involved in the LCPUFA pathway, the Δ5 and Δ6 desaturases (encoded by the FADS1 and FADS2 genes respectively) are the most well recognized as playing key roles in LCPUFA synthesis. Although the existence of FADS genes has long been known (108, 109), the Δ5 and Δ6 desaturases, unlike the Δ9 desaturase (110), have never been isolated, purified and assayed in a reproducible fashion. The gene coding for Δ6 desaturase from the cyanobacterium *Synechocystis* (111), *Borage officinalis* (112) and *Caenorabditis elegans* (113) and Δ5 desaturases from *C. elegans* (114) and the fungus *Mortierella alpina* (115, 116) were cloned and identified using a gain-of-function approach to identify the desaturase gene that coded for an enzyme involved in fatty acid metabolism. Taking advantage of the sequence information for the desaturases in eukaryotes, cDNA banks then allowed mouse, human and rat Δ5 and Δ6 desaturases to be successfully cloned (92-96, 98, 117). In addition to mammals, the Δ6 desaturases have also been isolated and functionally

[2]The Δ8 desaturase introduces a double bond at the position 8 in C20 fatty acids (20:3 n-3 and 20:2 n-6) that have an existing Δ11 unsaturation.

characterized in various fish species, including Atlantic salmon (*Salmo salar*) (118), zebrafish (*Danio rerio*) (119), carp (*Cyrinus carpio*), turbot (*Psetta maxima*) (97), rainbow trout (*Oncorhynchus mykiss*) (97, 120), gilthead sea bream (*Sparus aurata*) (97, 121), cobia (*Rachycentron canadum*) (122), Atlantic cod (*Gadus morhua*) (123) and barramundi (*Lates calcarifer*) (124). Among these fish species, the only desaturase that has been functionally identified as having predominately Δ5 activity is from Atlantic salmon (125) and zebrafish possesses a single protein with both Δ6 and Δ5 desaturase activities (119).

The availability of unsaturated fatty acids in cells depends on the activity of enzymes involved in fatty acid synthesis and metabolism. The Δ6 desaturase converts ALA into 18:4 n-3 and LA into 18:3 n-6. The two desaturation products are then further converted into EPA and AA, respectively, through the actions of the Δ5 desaturase, followed by elongation steps which form part of the principal LCPUFA synthetic pathway (83) (Figure 1.1). The two desaturase enzymes can be found in a variety of animal tissues, but are present at highest levels in the liver. Expression of Δ5 desaturase is highest in the human liver and it is also expressed at modest levels in heart and brain and lower, but still detectable, levels in lung, kidney, placenta, pancreas and skeletal muscle (93). The expression of Δ6 desaturase mRNA shows the same distribution pattern as Δ5 desaturase but with relatively higher levels in liver compared with those of Δ5 desaturase mRNA.

The Δ6 desaturase catalyzes the initial and rate-limiting desaturation of the two C18 EFA for the conversion to other LCPUFA (92, 94). Studies on the two strains of FADS2 (Δ6 desaturase) knockout mice have provided convincing evidence that no other desaturase can catalyse the initial step in the conversion of ALA and LA, since deletion of FADS2 gene expression abolishes the initial step in the enzymatic cascade of LCPUFA synthesis in all tissues. FADS2 knockout does not impair the normal viability and lifespan of the animals. The knockout mice do, however, exhibit altered membrane integrity and fluidity, and not able to reproduce (21, 126). Interestingly, residual LCPUFA were detected in all tissues examined in FADS2 knockout animals, suggesting that FADS2 deficiency does not result in complete depletion of LCPUFA (126). Like FADS2, FADS1 (Δ5 desaturase) is also involved in the LCPUFA pathway, and converts both n-3 and n-6 LCPUFA (20:4 n-3 and 20:3 n-6) into EPA and AA, respectively (93, 94). An unusual Δ5 desaturation activity has been indentified in FADS2 knockout mice which resulted in synthesis of 20:3 n-6 (Δ7,11,14) or 20:4 n-3 (Δ7,11,14,17) two fatty acids metabolites produced from LA and ALA, respectively. This

process was identified in mice after oral administration with radio-labeled ALA or LA and occurred through desaturation of ALA and LA by Δ5 desaturase followed by elongation, and suggests that the Δ5 desaturation of LA and ALA can occur when expression of Δ6 desaturase is suppressed (126).

1.3.1.3 Competition effect

An early study indicated that the Δ6 desaturase has a higher affinity for ALA compared to LA, implying that ALA would be desaturated in preference to LA if both were present at equal concentrations, and that these fatty acids might not be metabolized at the same rate. In the same study, incubation of labeled LA with increasing amounts of ALA resulted in a inverse relationship between the conversion of LA to 18:3 n-6 and the amount of ALA added, implying that ALA and LA compete for the same desaturation enzyme (108). Thus, the study above suggested that the competitive phenomena observed between n-3 and n-6 PUFA is likely to occur principally at the initial desaturation step. The conversion of 20:3 n-6 from LA through the Δ5 desaturase has been revealed in both FADS2 knockout and also in wild-type mice, although wild-type mice did not accumulate 20:3 n-6 (Δ7,11,14) in liver phospholipids as Δ6 desaturase-null mice do. Furthermore, no significant changes in Δ5 desaturase mRNA expression was observed in the livers of Δ6 desaturase-null mice, indicating that the accumulation of 20:4 n-3 or 20:3 n-6 in FADS2 knockout animals was not dependent on upregulation of Δ5 desaturase and that is likely due to loss of competition from Δ6 desaturase at the step of ALA or LA desaturation, as well as loss of competition at the step of incorporation of fatty acids into phospholipids (126).

A functional Δ4 desaturase has recently been cloned from a marine species, white-spotted spinefoot, showing that the FADS2 gene can display bifunctional Δ4/Δ5 activities in fish (99). However, the presence of the microsomal Δ4 desaturase in other animals has not yet been established and therefore the ability to desaturate 24:5 n-3 is important because this fatty acid is an intermediate in DHA synthesis, at least in rat and human tissues (90). The same Δ6 desaturase is able to catalyse both the desaturation of ALA to 18:4 n-3 and of 24:5 n-3 to 24:6 n-3 (127, 128), suggesting that the C24 fatty acids must undergo desaturation by Δ6 desaturase to form 24:6 n-3, before they can move to peroxisomes to undergo chain-shortening and produce DHA. Furthermore, studies of relative Δ6 desaturase activities in response to different substrates showed that the enzyme has a higher affinity for C18 than C24 fatty acids (127), suggesting that there is likely to be substrate competition for Δ6

desaturase when both C18 and C24 substrates are present, and that high levels of C18 substrates in the diet inhibit the further desaturation of C24 substrates, and thus DHA production.

In the LCPUFA synthetic pathway, an elongation step adds two carbons to synthesize 20:4 n-3 and 20:3 n-6 from 18:4 n-3 and 18:3 n-6, respectively. A second elongation step then converts EPA to DPA and AA to 22:4 n-6. The combination of desaturation and elongation steps in the fatty acid pathway makes possible the formation of LCPUFA with different characteristics and functions.

1.3.2 Elongases

Fatty acid elongation occurs in the cytosol, mitochondria and endoplasmic reticulum (microsomes), with the microsome considered the predominant site for the elongation of fatty acids with 12 carbons (C12) and longer chain lengths in eukaryotic cells. This elongation involves the addition of two carbon units to a fatty acyl-CoA, employing malonyl-CoA as the donor and NADPH as the reducing agent (129-132). Seven fatty acid elongases have been identified in the mouse (96, 133-135), rat (136), and human genomes (117, 137, 138) and are termed very long chain fatty acid gene (ELOVL) subtypes 1 to 7. Among the mammalian elongase family, ELOVL2, ELOVL4 and ELOVL5 are capable of elongating C18 PUFA to C22 LCPUFA. ELOVL5 is also able to elongate a more broad range of substrates including 16 carbon (C16) monoenes (133, 136) and ELOVL2 preferentially elongates C22 LCPUFA such as DPA to longer chain derivatives (130, 131). Together, ELOVL5 and ELOVL2 are the key elongases involved in the conversion of the n-3 and n-6 C18 PUFA to C20-C24 LCPUFA and, ultimately, the generation of the end product, DHA (32, 89). All ELOVL proteins contains a histidine-rich domain (HxxHH) and several transmembrane regions in mice (133, 135), rats (139, 140), humans (117, 138) and fish such as zebrafish (141), Atlantic salmon (125, 142), rainbow trout (143), cobia (122) and barramundi (124). All of the functionally characterised fish elongases were considered to be ELOVL5-like until recently when a putative ELOVL2 gene was isolated from Atlantic salmon (142) and zebrafish (144) and when characterised in yeast this enzymes was found to have a high specificity for C20 and C22 LCPUFA substrates.

Analysis of human ELOVL5 expression in 22 human tissues revealed that the highest levels of the ELOVL5 mRNA were found in the testis and adrenal gland, with significant amounts

of the transcript also detected in liver, brain, lung and prostate tissue at approximately equivalent levels (117). Studies using liver microsomal protein extracts from wild-type and ELOVL5 knockout mice demonstrated that the elongation of 18:4 n-3 to 20:4 n-3 and 18:3 n-6 to 20:3 n-6 is ELOVL5 dependent. In this same study, ELOVL5 knockout mice exhibited decreased cellular AA and DHA levels in liver, and consequently, an increase in the expression of sterol regulatory element-binding protein-1c (SREBP-1c) and its target genes involved in fatty acid and triglyceride synthesis, which culminated in the development of hepatic steatosis (145). The liver of ELOVL5 knockout mice also exhibited significant increases in the activity of ELOVL2 on C20 substrates EPA and AA compared to wild-type mice, suggesting the presence of a compensatory mechanism whereby ELOVL2 can increase its capacity for C20 elongation in order to counteract the decrease in DHA in the ELOVL5 knockout animals (145). The expression of the ELOVL2 gene is highest in the rat liver but it is also detected in the kidney, lung and brain tissues, at significant levels (146). ELOVL5 and ELOVL2 expression is regulated differently by nutritional status. ELOVL5 expression is altered in response to fasting and refeeding or by high levels of fish oil or olive oil in the diet, whilst expression of ELOVL2 in the rat liver is not affected even by extreme changes in nutritional status (146).

Although it is now clear that Δ5, Δ6 desaturases, elongase 2 and 5 are major enzymes involved in the biosynthesis of n-3 and n-6 LCPUFA, it remains unclear to what extent alterations in dietary PUFA level result in altered gene expression of these enzymes and, ultimately, increases or decreases in the rate of the LCPUFA synthesis.

1.4 Dietary regulation of desaturase and elongase genes

Cloning of animal Δ5 and Δ6 desaturase genes has demonstrated that both n-3 and n-6 fatty acids utilize the same enzyme system to synthesize their long chain metabolites. It has been suggested that the expression of the Δ5 and Δ6 desaturase genes, and hence the rate of LCPUFA synthesis, is regulated by prevailing substrate concentration. Both *in vivo* and *in vitro* studies have reported that Δ5 and Δ6 desaturase mRNA expression and enzyme activity are suppressed in the presence of adequate n-3 and n-6 PUFA in the diet relative to the level of expression when dietary n-3 and n-6 PUFA intakes is inadequate (93, 94). One study by Xiang *et. al.* (152) reported that dietary intake of higher levels of saturated and monounsaturated fatty acids in healthy humans was associated with increased expression of the Δ5 and Δ6 desaturase gene expression in peripheral blood mononuclear cells (PBMC),

whilst these was a negative correlation between dietary LA and ALA content and the Δ5 and Δ6 desaturase gene expression in these same cells (147). Furthermore, studies in the marine gilthead sea bream larvae, demonstrated that feeding rapeseed (monoenes 7.9%, LA 3.4%, ALA 1.0% of total fatty acids in diet) and soybean (monoenes 3.7%, LA 5.7%, ALA 0.6% of total fatty acids in diet) oil diets was associated with higher expression of the Δ6 desaturase gene compared to feeding flaxseed oil (monoenes 2.6%, LA 2.5%, ALA 5.3% of total fatty acids in diet) and fish oil. Furthermore, doubling the amount of flaxseed oil (monoenes 3.6%, LA 3.3%, ALA 9.1% of total fatty acids in diet) in the diets led to further inhibition of desaturase gene expression in these fish (148). Conversely, feeding freshwater fish, such as zebra fish and tilapia with vegetable oils containing a 1:1 ratio of LA (9.1% of total fatty acids) and ALA (8.7% of total fatty acids) increased Δ6 desaturase enzyme activity, resulting in increased levels of 18:4 n-3, EPA and DHA, although the desaturation of ALA was insufficient to maintain tissue proportions of EPA and DHA in fish fed the vegetable oil diet at the same level as in fish fed the fish oil diet (149). Thus, dietary ALA and LA are the main dietary components that regulate Δ6 desaturase activities, however, differences in the genes themselves or in the regulation of their gene/enzyme expression may relate to the different responses of desaturase to variation in dietary PUFA levels.

In addition to the effect of substrate concentrations on desaturase gene expression, there are also sex-differences in the baseline level of expression. In a study of rats which were born with a low-DHA status, both the phospholipid DHA levels and hepatic expression of Δ5 and Δ6 desaturase genes were higher in females compared to males (150). Female rats had a higher expression of liver desaturases to replete their DHA status more readily than males (150). In humans, tracer studies have shown that men (46) and women (47) have a different capacities to convert ALA to DHA. These studies showed that there was minimal conversion of ALA to DHA in men but a significantly higher level of radio-labeled DHA was observed in women (2). Higher DHA synthesis in women could be explained by gender-related differences in sexual hormone status and, therefore, differential hormonal regulation of PUFA metabolism. This hypothesis is supported by clinical trials, which have shown that higher concentrations of estrogen are associated with higher levels of AA and DHA in the blood lipids (151, 152).

Adult rat liver expresses four ELOVL subtypes (ELOVL1, 2, 5 and 6) with ELOVL5 being the most abundant (146). Fish oil or fish oil-enriched diets feeding suppresses lipogenic gene

expression (153), hepatic elongase activity as well as decreasing Δ5, Δ6 desaturases and ELOVL5/6 mRNA levels (146). Many of the effects of fish oil on hepatic gene expression can be attributed to the suppression of SREBP-1c or the activation of peroxisome proliferator-activated receptor-α (PPARα). Wang *et. al.* (146) suggested that PPARα agonists could induce LCPUFA synthesis because WY14,643, a PPARα agonist, increased liver ELOVL5, FADS1 and FADS2 gene expression. However, in rats fed an n-3 PUFA deficient (ALA 0.2% of total fatty acids, no DHA) compared with the adequate (ALA 4.6% of total fatty acid, no DHA) diet, mRNA and activity levels of Δ5, Δ6 desaturases and elongases 2/5 were upregulated in liver, whilst liver PPARα and SREBP-1 mRNA levels were unaffected, so the relationship between the expression of these different groups of enzymes is still unclear (57).

In fish, feeding ALA and 18:4 n-3 containing vegetable oil diets to saltwater Atlantic salmon increased hepatic gene expression of Δ5, Δ6 desaturases and elongases compared to fish fed with fish oil-based diets. This increase in desaturase and elongase gene expression was reflected in an increase in the concentration of tissue LCPUFA, in particular in 20:4 n-3 and 20:3 n-6 compared to those of in fish fed fish oil-based diets, but was not able to achieve concentrations of EPA and DHA equivalent to those observed in fish who were fed preformed EPA and DHA (154).

Therefore, dietary n-3 and n-6 fatty acid status appears to be the main factor that affects the expression of genes involved in the LCPUFA pathway and the concentration of products, EPA and DHA. Notably, according to previous studies, the supply of excessive precursors and metabolites may actually cause a decrease in the rate of Δ5 and Δ6 desaturation by suppressing the gene expression of these enzymes and therefore limit the rate of PUFA conversion to LCPUFA. Taken together, this leads to the hypothesis that an optimized level of precursors (LA and ALA) and low levels of long chain products (EPA and DHA) in the diet may have the capacity to induce increased mRNA expression of the Δ5 and Δ6 desaturases, increase the conversion of ALA and ultimately elevate EPA and DHA status in animals.

Lipid is a critical component in animals and diets not only because it provides EFA and fat soluble vitamins, but also because of its major contribution to energy supply. Lipids provide approximately twice the energy of carbohydrates and proteins in the typical human diet, and lipids can be added to animal feeds via supplementation with pure oils, oil seeds or oil-rich cereals. The choice of lipid and the form in which it is included in the farmed animal feed is

decided by a number of factors, which include 1) the availability and cost of the raw material, both locally and on the world market, 2) the impact of lipid form (oilseed or extracted oil) and its fatty acid composition on feed digestibility and 3) the influence of consumers and retailers regarding the introduction of genetically modified material (such as genetically modified soybean) into the food chain (155). Other factors, including sensory properties of the meat, food intake and feed conversion efficiency also need to be considered.

1.5 Dietary approaches for increasing n-3 status in humans

In order to increase LCPUFA content of human diets, we must first identify which foods are naturally rich in n-3 lipids, and secondly, determine whether modulating the lipid composition of the feeds of food animals can increase the n-3 LCPUFA levels in their tissues, and hence the meat consumed by humans (156). Fish, especially oily fish, is the richest source of EPA and DHA in human diets. Algae and seaweed are the primary producers of DHA and EPA in the ecosystem, and the high n-3 LCPUFA content of many fish varieties is ultimately due to the high intake of algae by fish at the bottom of the marine food chain, which are subsequently eaten by larger fish such that the n-3 LCPUFA accumulate in these fish and are found at highest levels in fish at the top of the food chain (13). In Australia, fish consumption remains relatively low and the majority of EPA and DHA are derived from non-fish sources, in particular meat and eggs. Eggs provide a reasonable amount of DHA (<50 mg/egg) but little EPA; for eggs from chickens fed flaxseed generally provide 60-100 mg DHA/egg (157). Beef contains a higher amount of saturated fatty acids than other meats because more than 90% of the dietary unsaturated fatty acids consumed are hydrogenated to saturated fatty acid in the rumen during the digestion process (158). Beef is also the major contributor to dietary n-3 LCPUFA intake for Australians, contributing 22.3% of n-3 LCPUFA intake for adults compared with lamb (5.9%), pork (3.9%), and poultry (10%). Importantly, most of the n-3 LCPUFA in the diet are obtained from fresh cuts of meat, with limited amounts obtained from processed food (159). In addition to meat and eggs, an increasing number of n-3 enriched products, to which n-3 PUFA or LCPUFA are added during the manufacturing process can also be purchased from major Australian supermarkets. The number of the n-3 PUFA enriched foods on the market has increased considerably in recent years, and now includes products ranging from juice, breakfast cereals and bread to butter, milk, dairy products and eggs.

The heightened interest in n-3 LCPUFA status of human populations has been accompanied by an increased interest in increasing n-3 fatty acid content of animal products that are consumed as part of the typical Australian diet such as eggs and popular meat animals. The major supply of n-3 LCPUFA in animal feeds is limited to fish and marine seaweed oil. However, low yields and high costs of oil extractions of algae limit the usefulness of algae as a major n-3 lipid source (160, 161). Ocean fish are the last wild animals that are still hunted by humans on a large scale. It was once thought that the bounty from the ocean was endless, however, it is becoming clear that the ocean's resources are rapidly being depleted and that there is an urgent need to identify ways to take pressure off global marine resources. Depletion of global marine fisheries has also been a force driving an expansion of seafood production through aquaculture. However, aquaculture currently still relies heavily on wild fish for the production of feed for farmed fish and therefore does not relieve the pressure on global fish stocks and is not a sustainable practice (162). Consequently, the increasing price of fish and fish oil due to growing human populations, decreasing marine fisheries and increasing consumer demand are forcing the aquaculture industry, as well as poultry and pig industries which incorporate fish oil and fish meal into their feeds, to source less costly oils and protein meals for manufacturing animal diets (163). The development of alternative and sustainable crops or plant sources with high levels of ALA or other n-3 PUFA to be used as precursors for synthesis of LCPUFA in farmed animals has thus become an area of intense research interest (164).

ALA, the C18 precursor of n-3 LCPUFA, is derived from plant cell membranes and widely distributed in plant sources, and are commonly consumed in the form of vegetable oils (165). Analysis of the fatty acid composition of major commercial vegetable oils, including soybean, corn, canola, rapeseed, olive and coconut oils, showed that soybean, canola and rapeseed oils contain significant amounts of ALA with 7-9%, 7.6-10% and 10-12% of total fatty acids, respectively (166, 167) and also LA (20-54% of total fatty acids), and have a LA to ALA ratio in a range of 3-7:1. However, to further increase or optimise ALA content in the dietary formulations, sourcing oils containing both high levels of ALA and low levels of LA is essential. Flaxseed oil has long been known as the major and most sustainable source of ALA (168, 169), whilst other ALA-rich plant oils include camelina (*Camelina sativa*) (167), perilla (*Perilla frutescens*) (166, 168), and chia (*Salvia hispanica*) (170). Of these, flaxseed, perilla and chia oils have the highest levels of n-3 precursors, with values reaching 55-61% (with LA:ALA ratio of 0.3:1) versus 38% (LA:ALA ratio of 0.4) of total fatty acids for camelina,

respectively (171). An alternate approach for increasing n-3 fatty acid levels through the diet is to achieve an optimal ratio and energy level of dietary LA and ALA, by decreasing the dietary intake of n-6 PUFA-rich oils (corn, sunflower, safflower, cottonseed, and soybean oil) and simultaneously increasing oils rich in n-3 PUFA (flaxseed, perilla, and chia seed) (172, 173).

As mentioned previously, most land animals and some freshwater fish are able to produce LCPUFA from C18 parental fatty acids in diets, albeit inefficiently (174, 175). Studies have indicated that the n-3 status of animal meats consumed by humans can be increased by increasing the level of n-3 fatty acids in animal feeds (4, 176); however, this is complicated by the need to consider the impact of digestion on the transfer of fatty acids from the diets into tissues. The major site for dietary lipid digestion is the small intestine for ruminant and non-ruminant animals; however, the absorption of fatty acids from the diet and, ultimately, there incorporation into tissue phospholipids differs significantly between ruminant and non-ruminant species. In non-ruminant species, dietary lipids are absorbed unchanged before incorporation into the tissue lipids, whilst in ruminants, rumen microorganisms isomerise and hydrolyse the dietary lipids before absorption in the small intestine (155). Thus, providing additional n-3 lipids in ruminant diets does not necessarily elevate tissue n-3 LCPUFA. Feeding lambs (post-weaning, at 90 days of age) with ALA-rich flaxseed oil diets, for example, had no significant effect on n-3 LCPUFA level when compared with lambs fed a standard (low ALA) diet but did elevate the n-3 to n-6 fatty acid ratio (177). In contrast, increasing dietary ALA content in non-ruminant species, such as chickens (178) and pigs (179), results in enrichment of n-3 LCPUFA in their meats. By way of example, supplementing chicken diets with ALA rich vegetable oils resulted in an increase in ALA levels in thigh meat from 1.6 to 13.9% of total fatty acids, whereas the contents of n-3 LCPUFA increased only modestly from 0.2 to 0.39% for EPA, 0.12 to 0.24% for DPA and 0.1 to 0.25% for DHA (180). Another study indicated that using a genetically engineered 18:4 n-3-rich soybean oil in chicken feeds resulted in increased levels of 18:4 n-3, EPA and DPA in the breast and leg meat of the chickens, without affecting the sensory properties of the freshly cooked meat (181). Pigs fed with diets containing either 0%, 5%, 10% or 15% flaxseed oil had dose-dependent increases in ALA and EPA content of the back fat, kidney fat, liver and heart tissues with increasing dietary flaxseed oil but no accompanying increase in DHA levels. Furthermore, there was no significant difference between the 10% and 15% flaxseed oil on the levels of ALA and EPA contents in most tissues (185). Similarly, pigs fed

a diet supplemented with 0.5% (g/kg diet) flaxseed oil in combination with 1.5% sunflower or olive oil, exhibited higher proportions of ALA (~3 fold increase) and EPA (~2 fold increase) in the dorsi muscle compared to pigs fed diets with low n-3 PUFA (179). Other pig studies have shown that, in general, ALA rich diets (up to 5% flaxseed oil) modestly increase EPA and DPA, but at the same time increase ALA up to 10 fold in adipose tissue and 3 fold or more in muscle, but have no effects on DHA concentrations in either tissue (182-184). In summary, increasing n-3 status in animal meats can be achieved by including ALA rich oil in the diets to increase ALA, EPA and DPA levels in the meats, but in most cases ALA rich diets have no or limited effect on muscle DHA levels. Therefore, increasing DHA levels in meats at present still relies on the inclusion of fish oil or fishmeal in the animal diets (185).

A number of fish studies have shown that both fishmeal and fish oil can be partially/completely replaced by other protein and oil sources without compromising growth or feed conversion efficiency (186-188). However, similar to studies on dietary ALA supplementation of land animals, most fish studies have shown that the fatty acid profiles of fish reflect dietary lipid composition, and that farmed fish fed fish oil or fishmeal replacements generally have low n-3 LCPUFA levels in their flesh and other tissues (154, 189, 190). Importantly, many marine animals, including fish and shell fish, have a limited ability to synthesize LCPUFA from ALA (123, 191). Feeding sea bream and sea bass four different diets including soybean, rapeseed and flaxseed oil or a mixture of the three vegetable oils with a proportion of 1:3:6 to substitute 60% of the fish oil in their diets had no negative effects on fish growth and flesh quality, but was associated with significant increases in the liver and muscle content of C18 PUFA and significant reductions in the n-3 LCPUFA content in both of these tissues (192). In a further study in Atlantic salmon, substitution of 33% fish oil with vegetable oils resulted in decreases in the EPA and DHA content in the flesh of 30% and 25%, respectively, compared to the those in fish fed 100% fish oil (193). Interestingly, switching fish previously fed 100% vegetable oil back to a 100% fish oil diet for 5 months resulted in the restoration of EPA and DHA to approximately 80% of the value in fish fed 100% fish oil for 1.5 years, suggesting that the decrease in n-3 LCPUFA produced by feeding fish on vegetable oils could be partially restored by feeding a diet containing fish oil for a period before harvest (193). In summary, as the reliance on aquaculture for sustainable fish production continues to grow to meet consumer's demand for fish products, there will need to be an increased use of non-marine lipid and protein source to reduce pressure on global fish stocks. However, the use of alternative materials brings new challenges, such as negative

effects on flesh quality, reductions in n-3 LCPUFA status and reduced growth rates. This dilemma requires extensive research on the capacity of different marine species and different types of fish, particularly those that are most popular with consumers, to synthesise their own n-3 LCPUFA from C18 precursors in response to different dietary compositions in order to develop diets which optimise/enhance this process.

1.6 Recommendations for EFA and n-3 LCPUFA

Significant progress has been made recently in relation to setting recommendations for levels of EFA and n-3 LCPUFA intake for the general population and for the level of intake required for the prevention or treatment of chronic diseases, in particular cardiovascular disease. The recommendations from various health authorities are summarised in Table 1.1. However, the actual levels of intake required to achieve physiological effects may need to be adjusted for specific individuals due to the large inter-individual variability in n-3 PUFA metabolism based on genetics, gender and age (ie, the concept of personalised nutrition) (173, 194). Furthermore, it is important to consider the fat composition of the background diet, since high intakes of saturates and/or n-6 PUFA are likely to interfere with the biological effects of n-3 PUFA (195).

The n-6 fatty acids have potential physiological functions (196, 197), however, n-6 PUFA compete with n-3 PUFA for conversion and for incorporation into tissues, such that excessive intake of n-6 PUFA has the potential to limit the production of anti-inflammatory n-3 PUFA derived mediators and n-3 LCPUFA. Therefore, diets must contain an appropriate balance of n-3 and n-6 EFA to achieve optimal biological effects (173). It has been suggested that increased intake of n-3 PUFA need to be accompanied by reductions in the intake of saturates and n-6 PUFA, in order to achieve optimal effects in reducing the risk of chronic disease while still providing adequate intakes of essential nutrients (197).

To avoid deficiency, ALA and LA should be present at 0.4%en and 2.4%en in the diet (200 mg and 1200 mg/100 g diet), respectively, according to the results of studies in humans and other animals (198). There is still a lack of robust dose-response data relating EPA and DHA intake to health benefits and protection against chronic disease, and the relative efficacy of EPA, DPA and DHA in improving symptoms in different disease states remains to be defined (199). However, it is becoming common to relate the outcomes of epidemiological studies to estimates of n-3 LCPUFA intakes (particularly combined intake of EPA and DHA) or to

blood n-3 LCPUFA levels in different age groups within human populations (200). For example, in a meta-analysis, a 37% reduction in the relative risk of coronary heart disease (CHD) in the general population was observed with a daily consumption of n-3 LCPUFA (EPA+DHA) of 566 mg (201). Many health authorities and organizations now recommend daily dietary intakes for total n-3 PUFA of 0.8 to 3 g/day (0.4 to 1%en), with specific recommendations for the intake of EPA and DHA ranging from 90 to 2000 mg/day depending on the authority issuing guidelines. The Australian National Health and Medical Research Council (NHMRC) recommends an intake of n-3 LCPUFA of 90-160 mg/day for the general adult population, with intakes of ~500 mg/day required for obtaining beneficial effects on reducing CHD risk (Table 1.1). Consumption of 1 fatty fish meal/day (or alternatively, a fish oil supplement) typically results in an intake of around 900 mg/day of EPA and DHA, therefore, consumption of at least 2 fish servings per week is now recommended by American Heart Association (AHA) (202).

Table 1. 1 Most recent recommended dietary intakes for fatty acid intake for adults by different organizations

References	Subjects	Adequate daily intake			Potential effects
		LA	ALA	n-3 LCPUFA	
FNB: IOM[1] (2002) (203)	Adults (19-50)	(5-6%en)			
	Men	17g/day	1.6g/day	-	General health
	Women	12g/day	1.1g/day	-	
AHA[2] (2003) (204)	Adults		1.5-3g/day (randomized clinical trials are needed)	Fatty fish at least 2 times a week	General health
	Patients with documented CHD			1000mg/day (EPA+DHA)	Maintaining cardiovascular health
	Patients with hypertriglycerid -emia			2000-4000mg/day (EPA+DHA) (under a physician's care)	Lower triglyceride 20-40%
ISSFAL[3] (2004) (205)	Adults	2%en	0.7%en	-	For general health
	Adults	-	-	500mg/day (EPA+DHA)	Maintaining cardiovascular health
NHMRC[4] and MoH[5] (2006) (206)	Men (19+)	13g/day	1.3g/day	160mg/day (EPA+DPA+DHA)	For general health
	Women (19+)	8g/day	0.8g/day	90mg/day (EPA+DPA+DHA)	
	Pregnancy (19-50)	10g/day	1.0g/day	115mg/day (EPA+DPA+DHA)	
	Lactation (19-50)	12g/day	1.2g/day	145mg/day (EPA+DPA+DHA)	
NHMRC and MoH (2006) (206)	Adults	4-10%en	0.4-1%en		Optimising chronic disease risk, notably CHD
	Men			610mg/day	Reducing chronic disease risk
	Women			430mg/day	
FAO[6]/WHO[7] (2008) (207)	Adults	2.5-9%en (AMDR[8])	≥ 0.5%en (L-AMDR[9])	0.25-2g/day (AMDR, EPA+DHA)	General health, probably preventing CHD and some cancers and degerative diseases of aging
AHA (2009) (197)	Adults	5-10%en			Reduces the risk of CHD

[1]FNB: Food and Nutrition Board, Institute of Medicine.
[2]AHA: American Heart Association.
[3]ISSFAL: International Society for the Study of Fatty Acids and Lipids.
[4]NHMRC: National Health and Medical Research Council.
[5]MOH: Ministry of Health.
[6]FAO: Food and Agricultural Organization of the United Nations.
[7]WHO: World Health Organization of the United Nations.
[8]AMDR: Acceptable macronutrient distribution range.
[9]L-AMDR: Lower level of acceptable macronutrient distribution range.

1.7 Rationale and significance of this thesis

Attempts to increase the level of the n-3 LCPUFA in human and animal tissues by altering the ratio of LA to ALA in the diet have met with limited success. Results in a previous piglet study done by our group (4) with modest to high levels of PUFA showed that while the level of EPA increased in a linear manner as the dietary LA:ALA ratio was lowered, the level of DHA was not significantly increased. Based on data pointing to existence of the competition between n-3 and n-6 PUFA for enzymes in the fatty acid pathway, particularly Δ6 desaturase, it has been hypothesized that simply changing the ratios of LA:ALA is likely to have only a minor positive effect on the levels of C22 LCPUFA. Thus, in order to increase the production of DHA from ALA, both the total amount of PUFA in the diet and the LA:ALA ratio need to be reduced. To achieve the goal of optimising health outcomes for humans by dietary modifications, the experiments presented in this thesis are divided into three separate components: 1) utilizing a rat model to demonstrate the effects of a narrow range of dietary treatments on EPA and DHA accumulation in mammals, 2) using a popular farmed fish (barramundi) to examine the efficacy of dietary oil blends in feeds on EPA and DHA accumulation in a fish commonly consumed by humans and 3) characterising barramundi Δ6 desaturase and elongase 5 genes by a yeast heterologous expression system in order to clarify enzyme activities and their functions on LCPUFA production.

The Hooded-Wistar weanling rat is a vertebrate species of experimental animal commonly used to predict the response of humans to dietary treatments. They are a popular mammalian model for biosynthetic and nutritional experiments (208). For fish, n-3 LCPUFA, particularly EPA and DHA in fish diets have been shown to be required to retain EPA and DHA levels in various marine fish species as well as for normal growth and development (209-212). Vegetable oils have been reported to significantly decrease EPA and DHA contents in salmon fish tissues compared to those fed 100% fish oil (213-215). Existing evidence has also indicated that the insufficiency of metabolic conversion in marine fish was likely due to either a deficiency in Δ5 desaturase (20:4 n-3 to EPA) or C18 to C20 elongase activity (191). In contrast, fresh water fish such as zebra fish and tilapia have been reported to have increased tissue EPA and DHA levels when feed with diets based on n-3 rich vegetable oil (216, 217). Barramundi is a popular Australian diadromous fish that have been studied extensively in regard to potential for the aquaculture industry. The overall objective of the project was to determine the optimum LA:ALA ratio from vegetable oils on DHA accumulation in the fish, with a view to improving the n-3 LCPUFA status of their flesh, and to examine nutritional

regulation of ALA diets on gene expression of desaturase and elongase mRNA. The objective of these studies was, ultimately, to identify a strategy for developing a cost-effective process for improving n-3 LCPUFA status in humans, which could also be incorporated into the feeds of animals used for human food, such as fish feeds.

1.8 Outline of this thesis

This thesis addresses the relationship between dietary PUFA status, expression of FADS and ELOVL genes and tissue LCPUFA levels. One study in mammals and one study in farmed fish describe the relationship between hepatic desaturase and elongase gene expression and tissue LCPUFA levels, whilst a third study describes the identification and cloning of barramundi desaturase and elongase genes and investigation of their functions and activity. **Chapter 1 and 2** presents an introduction to key background literature, general material and methods used in these studies. **Chapter 3** reports the association between dietary PUFA levels, LCPUFA synthetic gene expression levels (Δ5 and Δ6 desaturases, elongase 2 and 5, PPARα and SREBP-1c) and tissue n-3 LCPUFA composition in rats. **Chapter 4** presents data on the relationship between dietary n-3 PUFA status with desaturase and elongase gene expression as well as tissue n-3 LCPUFA accumulation in juvenile barramundi. **Chapter 5** reports the results of studies in which I cloned and expressed a desaturase gene and an elongase gene from barramundi for investigating enzyme function and possible activities. Finally, the main results, methodological considerations, the strength of the associations between different n-3 PUFA and a general discussion of the implications of the key findings of this thesis are presented in **Chapter 6**.

Chapter 2
Material and Methods

The reagents, materials and methods used in the experiments which from part of this thesis will be described in detail in this chapter. The methods used in this thesis can be divided into three major sections, namely fatty acid analysis, quantitative real-time PCR (qRT-PCR) analysis of hepatic mRNA expression and heterologous expression of enzymes in yeast *Saccharomyces cerevisiae (S. cerevisiae)*. Changes or modifications to these methods in the different studies in this thesis are described in each of the following chapters as appropriate.

2.1 Fatty acid analysis

In the separate studies described in this thesis, fatty acid analyses were carried out on animal feeds, liver and muscle samples in both rat and barramundi. Fatty acid profiles were also analysed in other rat tissues including plasma, erythrocyte, brain, heart and kidney and the composition of the lipids extracted from the yeast cells and media in the *in vitro* experiments were also determined.

2.1.1 Chemicals and reagents

Organic solvents including chloroform, methanol, isopropanol and *n*-heptane were all analytical grade and obtained from Ajax Finechem Pty Ltd (Auckland, New Zealand) or Chem-Supply (SA, Australia). Butylated hydroxyanisol (BHA) and anhydrous sodium sulphate (Na_2SO_4) were purchased from Sigma-Aldrich (Missouri, USA). Sulphuric acid (H_2SO_4) was from APS Finechem (NSW, Australia) and isotonic saline was from Baxter Healthcare (NSW, Australia). Gases for gas chromatography (GC) analysis and sample preparation including ultra-high purity helium and high purity hydrogen, medical air and high purity nitrogen were supplied by Coregas (SA, Australia). Fatty acid methyl ester (FAME) standards were purchased from Nuchek Prep Inc (Elysian, USA) unless specified otherwise.

2.1.2 Lipid extraction and fatty acid methylation

Total lipids were extracted from animal feeds, plasma and tissues with chloroform/methanol (2:1, v/v) (218) and from erythrocytes and yeast cells with chloroform/isopropanol (2:1, v/v) (219). BHA (0.005%, w/v) was added to all organic solvents except *n*-heptane to prevent oxidation.

2.1.2.1 Total lipid analysis of animal diets

A sample of the experimental pelleted diet was ground to a fine powder and 1 g of the sample placed into a glass scintillation vial. The diet was dried at 105°C for 3 hr and then cooled to room temperature in a desiccator to remove moisture. This process was repeated until the sample weight remained constant. The dried and ground sample (0.5 g) was then transferred into a Kimble glass tube containing 2 mL of cold isotonic saline (0.9% sodium chloride) and vortexed vigorously. Chloroform/methanol (2:1, v/v) was added to the tube to extract total lipids. Samples were mixed thoroughly with methanol (3 mL) and allowed to stand for 5 min at room temperature. Chloroform (6 mL) was then added and the tubes were shaken vigorously and allowed to stand for 5 min at room temperature. The tubes were then centrifuged at 3000 rpm (1559 x g) (Heraeus Sepatech, Hanau, Germany) for 10 min to separate the aqueous and organic phases. The chloroform (bottom layer) was transferred into a scintillation vial and evaporated to dryness under a nitrogen stream. The weight of the lipid extracts was recorded. Samples were then methylated by the addition of 5 mL of 1% H_2SO_4 (v/v) in methanol at 70°C for 3 hr. After cooling, the FAME were extracted into 750 µL of distilled water and 2 mL *n*-heptane and then transferred to GC vials containing anhydrous sodium sulphate (Na_2SO_4) as the dehydrating agent.

2.1.2.2 Fatty acid analysis of blood and animal tissues

Whole blood was spun at 4000 rpm (2772 x g) for 5 min to separate erythrocytes and plasma. Plasma was then transferred into glass tubes containing 0.5 mL cold saline and extracted using chloroform/methanol (2:1, v/v). The remaining erythrocytes were washed 3 times in cold saline and 1 mL of the sample transferred into a separate tube. Cold saline (0.5 mL) was added into the tube to make up the total volume to 1.5 mL. Chloroform and isopropanol at a 2:1 (v/v) ratio was added into the tube containing the erythrocytes to extract total lipid. Tissue samples from animals including liver (0.3 g), heart (0.2 g), kidney (0.2 g), muscle (0.3 g) and brain (0.1 g) were finely chopped and homogenized (IKA T25 digital Ultra-Turrax, Germany). Chloroform/methanol (2:1, v/v) was added to the homogenate for total lipid extraction. All the samples listed above were shaken vigorously and allowed to stand for 5 min at room temperature, after addition of the chloroform/methanol or chloroform/isopropanol. The samples were then centrifuged at 3000 rpm (1559 x g) for 10 min to separate the aqueous and solvent phases. The chloroform layer (bottom phase) was transferred into a scintillation vial and evaporated to dryness under a nitrogen stream. The

total lipid weight was recorded and the lipid extracts were then resuspended in 150 µL chloroform/methanol (9:1, v/v) for further lipid class separation.

2.1.2.3 Fatty acid analysis of yeast cells

Yeast cells were harvested by centrifugation at 2800 rpm (1358 x g) for 5 min at 4°C and the wet weight of the cells recorded. The cells were then washed and resuspended in 1.5 mL of ddH_2O. Total lipids were extracted using the chloroform/isopropanol (2:1, v/v) solvent system and heptadecanoic acid (17:0) was added to the harvested cells as an internal standard (ISD). Samples were shaken vigorously and allowed to stand for 5 min at room temperature. After 5 min, samples were centrifuged at 3000 rpm (1559 x g) for 10 min to separate the aqueous and solvent phases. The lipids were obtained by evaporating the solvent layer and then transferred into a vial containing 1% H_2SO_4 (v/v) in methanol at 70°C for 3 hr for transmethylation. After the samples were cooled, the resulting FAME were extracted with *n*-heptane and transferred into vials containing anhydrous sodium sulphate in preparation for GC analysis.

2.1.2.4 Lipid classes separation by thin layer chromatography

The phospholipid and triglyceride fractions were separated from total lipid extracts by thin layer chromatography (TLC) on silica gel plates (Silica gel 60H; Merck, Darmstadt, Germany). A TLC standard 18-5 (Nu-Chek Prep Inc, MN, USA) was run on the plates for lipid identification. The mobile phase for TLC was petroleum spirit/ethyl ether/glacial acetic aicd (180/30/2, v/v). The TLC plates were sprayed with fluorescein 5-isothiocyanate in methanol, and the lipid classes present were visualized under UV light. Cholesteryl ester (CE) (fraction 1) migrated to the solvent front, followed by triglyceride (fraction 2), free fatty acid (FFA) (fraction 3), cholesterol (fraction 4) and then phospholipid (fraction 5) (Figure 2.1). Triglyceride (fraction 2) and phospholipid (fraction 5) fractions were scraped and transferred individually into vials containing 1% (v/v) H_2SO_4 in methanol for transmethylation at 70°C for 3 hr. After cooling, distilled water (250 µL) was added to the methyl esters and extracted into 0.5 mL of *n*-heptane. Samples were then sealed in GC vials with anhydrous sodium sulphate for GC analysis.

2.1.2.5 Gas chromatography analysis of fatty acid methyl esters

FAME in samples were separated and quantified by GC (Hewlett-Packard 6890, CA, USA) equipped with a capillary column (50 m x 0.32 mm id) coated with 0.25 µm film thickness 70% cyanopropyl polysilphenylene-siloxanes (BPX-70; SGC Pty Ltd, Victoria, Australia) and

a flame ionisation detector (FID). The injector temperature was set at 250°C and the FID temperature at 300°C. The oven temperature at injection was initially set at 140°C and was programmed to increase to 220°C at a rate of 5°C per minute and held for 2 min. The temperature was then increased by 20°C per min to 260°C and held for 8 min. Helium gas was utilized as a carrier at a flow rate of 35 cm per second in the column and the inlet split ratio was set at 20:1. The identification and quantification of FAME was achieved by comparing the retention times and peak area (%) values of unknown samples to those of FAME lipid standards 463 (Nu-Chek Prep Inc, MN, USA) with known concentrations (3 mg/mL) using the Hewlett-Packard Chemstation data system. FAME standards for 24:5 n-3 and 24:6 n-3 were purchased from Larodan Fine Chemicals (Malmö, Sweden).

2.2 qRT-PCR analysis of hepatic mRNA level

Gene expression levels in rat and barramundi liver samples were determined by one-step qRT-PCR. qRT-PCR and data analysis were performed in a total volume of 10 µL/reaction using a Rotor-Gene 6000 real-time rotary analyser (Corbett Life Science, NSW, Australia). Total RNA (10 ng) from liver was reverse transcribed and directly amplified in the same microtube. The SYBR reaction mixture consisted of HotStarTaq® Plus DNA polymerase, optimized RT-PCR Buffer, dNTP Mix and SYBR Green I dye. The qRT-PCR products were identified by generating a melting curve. The size of qRT-PCR products were confirmed by gel electrophoresis on an agarose gel stained with ethidium bromide (EtBr) and visualized by exposure to ultraviolet (UV) light. The threshold cycle (Ct) value was determined for each transcript. The Ct value represents the cycle number at which a fluorescent signal increases beyond a specific value (threshold fluorescence). The determination of normalised expression of the target genes was determined using the Microsoft Excel-based Q-Gene software (220-222) to obtain mean normalised expression (MNE).

2.2.1 Chemicals and reagents

RNAlater® (RNA stabilization reagent), RNeasy® Mini Kit and the QuantiFast® SYBR Green RT-PCR Kit were obtained from Qiagen (GmbH, Hilden, Germany). The QuantiFast® SYBR Green RT-PCR Kit contained 2x QuantiFast® SYBR Green RT-PCR Master Mix (consisting of HotStarTaq® Plus DNA Polymerase; QuantiFast® SYBR Green RT-PCR Buffer; dNTP mix and ROX passive reference dye), and QuantiFast® RT Mix (a mixture of the Qiagen Omniscript® Reverse Transcriptase and Sensiscript® Reverse Transcriptase). Ethanol, β-mercaptoethanol (β-ME) and EtBr were purchased from Sigma-Aldrich (MS, USA). DNA

grade agarose was obtained from Progen Industries Ltd (Queensland, Australia), RQ1 RNase-free DNase was obtained from Promega (WI, USA). DNA markers/ladders (1 kb and 100 bp) were purchased from New England Biolabs (MA, USA).

2.2.2 RNA isolation and quality determination

Total RNA was isolated from 10 mg of liver tissue using a Qiagen RNeasy® kit following the protocol provided by the manufacturer with the tissue initially disrupted using a Tissue Lyser (Mixer MM 300, F. Kurt Retsch GmbH & Co. KG, Haan, Germany). Briefly, the liver sample was removed from RNAlater® and 10 mg of the sample placed in a tube containing guanidinium isothiocyanate (GITC) buffer and stainless steel beads (5 mm) (Qiagen). The Mixer MM 300 was applied to homogenize the tissues (1-2 min at 20Hz). After homogenization, the sample was centrifuged. The supernatant was transferred to a fresh tube, mixed with 70% ethanol and then applied to an RNeasy spin column for adsorption of RNA to the membrane. The bound RNA was eluted with 50 µL RNase-free water. Isolated total RNA was treated with RQ1 RNase-free DNase to eliminate genomic DNA contamination. The purified and DNase treated RNA samples were diluted to a final concentration of 5 ng/µL in RNase-free water prior to qRT-PCR to allow dilution of the magnesium concentration in the sample to one which was compatible with the Qiagen QuantiFast® SYBR Green RT-PCR Kit. The quality and concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm (NanoDrop Technologies, Wilmington, DE), and RNA integrity was confirmed by agarose gel electrophoresis and OD 260/280 and 260/230 ratios. Both ratios of less than 1.8 indicate contamination with proteins or chemicals used in the extraction procedure. This is because nucleic acid is detected at 260 nm, whereas protein, salt and solvents are detected at 230 and 280 nm. Both the 260/280 and 260/230 ratios were in the range of 1.9-2.2 for all RNA samples used in this thesis.

2.2.3 Optimization and validation of qRT-PCR assay

To evaluate the efficiency of amplification, a standard curve was constructed using a serial dilution (0.005 ng/µL to 50 ng/µL) of one total RNA sample from a rat and a fish liver per dietary group for each target gene. In the rat study, primers included QuantiTect® Primer Assays, from Qiagen β-Actin (Rn-Actb-1-SG, QT00193473), FADS1 (Rn-Fads1-1-SG, QT00188664), FADS2 (Rn-Fads2-1-SG, QT00186739), ELOVL2 (Rn-Elovl2-predicted-1-SG, QT01683899), ELOVL5 (Rn-Elovl5-1-SG, QT00178059) and primers kindly provided by Dr. Beverly Mühlhaüsler (University of Adelaide) for PPARα (NM_013196) (Fwd 5'-

CCTGTGAACACGATCTGAAAG-3'/Rev 5'-ACAAAAGGCGGATTGTTG-3') and SREBP-1c (CGU09103) (Fwd 5'-GCGCCATGGAGGAGCTGCCCTTCG-3'/Rev 5'-GTCACTGTCTTGGTTGTTGATG-3'). The QuantiTect® Primer Assays amplified PCR products had an expected amplicon size of 145 bp for β-Actin, 117 bp for FADS1, 87 bp for FADS2, 124 bp for ELOVL2 and 79 bp for ELOVL5. The expected amplicon size for PCR fragments amplified by the PPARα and SREBP-1c primers were 152 bp and 131 bp respectively. Amplicons from qRT-PCR were sequenced and their size checked by running the PCR products on a 1.5% agarose gel and aligning the bands with a DNA ladder of known fragment size (Figure 2.2). Primers for qRT-PCR analysis of barramundi hepatic FADS2, ELOVL and 5 potential housekeeping genes (β-Actin, ribosomal protein LP1α (RPLP1α), 18s ribosomal RNA (rRNA), elongation factor 1α (EF1α) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were designed using Primer3Plus (http://www.bioinformatics.nl/primer3plus) (223). The expected amplicon size was 249 bp for FADS2, 154 bp for ELOVL, 175 bp for β-Actin, 224 bp for RPLP1α, 150 bp for 18S rRNA, 154 for EF1α and 238 bp for GAPDH. Single amplicons from the primers were sequenced and the PCR products run on a 1.5% agarose gel with DNA ladder to confirm that they were of the correct size (Figure 2.3). Analyses of dissociation/melt curves were performed at the end of 40 cycles of amplification to confirm the presence of a single amplicon with the same melting temperature (Tm) (Figures 2.4, 2.5, 2.6 and 2.7). No template and no reverse transcriptase (RT) controls were also performed to confirm the absence of contamination in the reagents. PCR amplification efficiency of the reaction for each primer set was computed using the Ct values obtained over the 4-log dilution range (which were from a standard curve which was constructed from a serial dilution of total RNA at five concentrations) were plotted against RNA concentration with the following formular (220):

Exponential amplification=$10^{(-1/slope)}$

Efficiency=$[10^{(-1/slope)}]$-1

2.2.4 qRT-PCR analysis

The qRT-PCR experiment and data analysis were carried out in a total volume of 10 µL/reaction on a Rotor-Gene 6000 real-time rotary analyser (Corbett Life Science, NSW, Australia) with QuantiFast® SYBR Green RT-PCR Kit according to the manufacturer's instructions. Each 10 µL reaction included 2 µL of total RNA (10 ng/reaction), 5 µL of 2x QuantiFast® SYBR Green RT-PCR Master Mix, 1 µL QuantiTect® Primer or 1 µL of 5 µM (0.5 µM in final concentration) self designed primers, 0.1 µL QuantiFast® RT Mix and 1.9 µL

RNase-free molecular grade water to 10 μL. At least three technical replicates were performed for each sample. The qRT-PCR conditions were as follows: reverse transcription at 50°C for 10 min, PCR initial activation step at 95°C for 5 min, two step cycling for 40 cycles in denaturation at 94°C for 10 sec and combined annealing/extension at 60°C for 30 sec. At the end of the amplification, the melting temperature was carried out by a gradually increasing the temperature (0.2°C/sec) from 60°C to 95°C. Amplification was quantified by determining the Ct value for each reaction. Baseline and thresholds for Ct calculation were set manually with the Rotor-Gene 6000 series software and were the same for all experiments. The gene expression level of target genes relative to the housekeeping genes was determined by the relative quantitation method using the Microsoft Excel-based Q-Gene software (220-222). The Q-Gene tool was developed to calculate the MNE with standard errors by averaging the Ct values from replicates and subsequent calculation of the mean normalized expression and correcting for variation in amplification efficiency of the target genes and housekeeping genes. The calculation is based on the equation as follows:

$MNE=(E_{ref})^{Ct\ ref} / (E_{target})^{Ct\ target}$

In this thesis, β-actin was used as a housekeeping gene for both rat and barramundi in the calculation of normalized expression.

2.3 Heterologous expression of enzymes in yeast *S. cerevisiae*

The yeast strain *S. cerevisiae* INVSc1 was used as the host for heterologous expression of barramundi Δ6 desaturase and elongase genes. The yeast and *Escherichia coli* (*E. coli*) shuttle vector pYES2™ is a 5.9 kb vector for GAL-1 promoter inducible expression of foreign proteins in *S. cerevisiae*. The features of the system include the ability to induce protein expression in yeast by galactose and repress gene expression by glucose (224) and a 5' end of CYC1 transcriptional terminator for termination of mRNA, URA3 gene for selection of transformants in yeast host strains with a ura3 genotype and an ampicillin resistance gene for selection in *E. coli*.

2.3.1 Chemicals and reagents

Fatty acid substrates ALA, LA, 18:4 n-3, 18:3 n-6, 20:3 n-3, 20:2 n-6, 20:3 n-6, EPA, AA and DPA (all >98-99% purity) were purchased from Cayman Chemicals (Michigan, USA). Fatty acid substrate 24:5 n-3 was purchased from Larodan Fine Chemicals (Malmö, Sweden). All ingredients for the preparation of media were purchased from Sigma-Aldrich (Missouri, USA) including yeast nitrogen base (YNB), yeast peptone dextrose (YPD) broth, YPD agar, glucose

(>99% purity), galactose (>99% purity), yeast synthetic drop-out medium supplements (Y1501, Uracil$^-$) and bacteriological agar. Yeast host strain INVSc1, pYES2™ vector, MAX Efficiency® DH5α™ competent cells and the *S. c.* EasyComp™ Transformation Kit were purchased from Invitrogen (Invitrogen, CA, USA). Ampicilin (100 mg/mL), prepared luria broth (LB), Tris/Borate/EDTA (TBE) buffer and LB agar plates containing ampicillin were purchased from the Media Preparation Unit (MPU) in the University of Adelaide.

2.3.2 Preparation of *S. cerevisiae* INVSc1 medium

The preparation of the media for both wild-type and recombinant colonies was carried out based on recipes included in the pYES2™ manual (version K) from Invitrogen or reconstitution instructions from Sigma.

2.3.2.1 YPD broth

a) 50 g pre-mixed YPD broth powder added to 1 L of deionized water.

b) Solution autoclaved for 15 min at 121°C.

c) If making plates, 20 g of agar was then added.

2.3.2.2 YPD agar

a) 65 g pre-mixed YPD agar powder added to 1 L of deionized water.

b) Solution heated to boiling while stirring to dissolve all ingredients completely.

c) Solution autoclaved for 15 min at 121 °C.

d) Solution cooled to 50°C, poured into plates and allowed to harden.

e) Plates were inverted and stored at 4°C.

2.3.2.3 SC selective medium^{-U} and plate

a) The medium composed of 0.67% (w/v) YNB (without amino acids), 2% (w/v) carbon source (glucose or galactose) and 0.192% (w/v) amino acid (yeast synthetic drop-out medium supplements, Uracil$^-$) and 2% agar (if making plates).

b) 6.7 g YNB and 1.92 g amino acid was dissolved in 780 mL deionized water, 20 g of agar added and stirred until well dissolved.

c) Solution autoclaved for 15 min at 121 °C.

d) Solution cooled to 50°C and 200 ml of 0.22 μm filter-sterilized 10% (w/v) glucose or 10% (w/v) galactose added.

e) Plates poured and allowed to harden.

f) Plates were inverted and stored at 4°C.

2.3.3 Cloning into pYES2™ vector

Cloning of barramundi Δ6 desaturase and elongase genes will be described in detail in chapter 5. Briefly, a ligation mixture containing the target genes and the pYES2™ vector was heat-shock transformed into competent MAX Efficiency® DH5α™ competent cells. Cells were plated on LB plates containing 100 μg/mL ampicillin for antibiotic selection of transformed cells. The plates were allowed to dry and then incubated overnight at 37°C. Single colonies were selected from LB plates (6 clones per plate) and inoculated into 1 mL of LB liquid media followed by a 2 hr culturing period. The cells were spun down at 8000 x g for 2 min and 10 uL of 0.25% sodium dodecyl sulfate (SDS) solution added to the cell pellet. The samples were then vortexed for 30 sec to crude extract the *E. coli* genomic DNA. The SDS treated cells were then spun at 8000 x g for 2 min and the supernatant collected for further analysis. Successful transformants were analysed by restriction enzyme (RE) digest and sequencing to confirm the presence and orientation of the target inserts.

2.3.4 Glycerol stocks preparation of *E. coli*

Bacterial glycerol stocks of *E. coli* DH5α were prepared from overnight cultures of the *E. coli* DH5α. Stocks were stored in 2 mL labeled cryovial tubes which contained 800 μL of overnight culture and 200 μL of sterile 80% (v/v) glycerol to make glycerol to 20% (v/v) as final concentration. These stocks were stored at -80°C. To recover the strain from the glycerol stock, a sterile toothpick was used to scrape a small amount of the frozen stock, and this was then streaked out onto a LB plate.

2.3.5 Preparation and transformation of *S. cerevisiae* INVSc1 competent cells

The protocol followed the instructions included in the *S. c.* EasyComp™ Transformation Kit (Invitrogen). Briefly, a small amount of INVSc1 strain provided with the kit was streaked on a YPD plate and incubated at 30°C for 2 days. A single colony of an isolated *S. cerevisiae* strain was selected and added to 10 mL of YPD. The cells were grown overnight at 27°C in a shaking incubator (190 rpm) and, on the following day, yeast cells from the overnight culture were diluted to an OD_{600nm} of 0.4 in a total volume of 10 ml of YPD. The cells were grown under the same conditions until the OD_{600nm} reached 0.6-1.0. The cells were then collected by centrifugation at 1500 rpm (389 x g) for 5 min at room temperature, the supernatant discarded and the cell pellet resuspended in 10 ml of Solution I (provided in the kit). The cells were then

centrifuged again at 1500 rpm (389 x g) for 5 min at room temperature, the supernatant discarded and the cell pellet resuspended in 1 mL of Solution II (provided in the kit). 50 µL of the competent cells were aliquoted into labelled 2 mL sterile cryovials, placed in a Styrofoam box and stored at -80°C. 50 µL of yeast competent cells was used for each transformation. One tube of 50 µL competent cells was thawed and 5 µL of target plasmid (including empty pYES2™ vector, pYES2/BarraFADS (barramundi FADS gene was inserted into pYES2™) and pYES2/BarraELOVL (barramundi ELOVL gene was inserted into pYES2™)) added. 500 µL of Solution III (provided in the kit) was then added to the plasmid/cell mixture and the transformation reactions incubated for 2 hr at 30°C, with mixing by vigorous vortexing every 15 min. 200 µL of the transformation reaction was then plated onto selection plates (SC selective medium^{-U} plate), spread evenly and the plates placed in a 30°C incubator and incubated for 3-5 days. Single colonies from the plate were used for further expression assays.

2.3.6 Glycerol stocks preparation of *S. cerevisiae* INVSc1 cells

The protocol was modified from the methods described by Sherman (225). Glycerol stocks of transformed (empty pYES2™ vector, pYES2/BarraFADS or pYES2/BarraELOVL) *S. cerevisiae* INVSc1 cells were prepared from cells which were streak-plated on SC selective plates^{-U}. The cells were scraped from the plate and resuspended in 1 mL of sterile deionized water and 225 µL of sterile 80% (v/v) glycerol (15% as final concentration) in 2 mL labeled cryovial tubes. The vials were capped and vortexed to suspend the cells evenly within the 15% (v/v) glycerol solution. The stocks were immediately transfered into the -80°C freezer. To recover the strain from the glycerol stock, a sterile toothpick was used to scrape some of the frozen stock, and the cells then streaked out on a plate.

2.3.7 Yeast *S. cerevisiae* INVSc1 culture and galactose induction

A single yeast colony transformed with empty pYES2™ vector, pYES2/BarraFADS or pYES2/BarraELOVL from a SC selective plate^{-U}, was inoculated into 25 mL SC selective medium^{-U} (liquid form, 2% glucose, w/v) in a 125 mL Erlenmeyer flask. The yeast cells were grown at 27°C overnight with shaking (190 rpm). The cells were collected by spinning at 2800 rpm (1358 x g) for 5 min after a 24 hr incubation. The supernatant was discarded and the cell pellet was washed with induction medium (SC selective medium^{-U}, liquid form, 2% galactose, w/v) and then suspended in induction medium containing 0.25% (v/v) tergitol® with a cell density of OD_{600nm}=0.4. 10 mL of this cell suspension was added to each 100 mL Erlenmeyer flask with or without addition of either 0.1, 0.25 or 0.5 mM concentration of fatty

acid for desaturase or elongase enzymes, and incubated at 27°C with shaking (190 rpm) for 24-48 hr (24 hr for elongase and 48 hr for desaturase gene transformed INVSc1 yeast). The induced cells were harvested and lipids extracted for further analysis.

2.3.8 Fatty acid supplementation and lipid extraction of *S. cerevisiae* INVSc1 cells

The amplified PCR products containing barramundi putative desaturase and elongase gene were purified, restriction digested and inserted into EcoRI and XhoI digested pYES2™ vector to yield the constructed plasmid pYES2/BarraFADS and pYES2/BarraELOVL. An INVSc1 transformant colony containing the pYES2/BarraFADS or pYES2/BarraELOVL constructs was inoculated into a 125 ml Erlenmeyer flask containing 25 mL SC selective medium^{-U} containing 2% (w/v) glucose and grown overnight at 27°C in an orbital incubator with vigorous shaking at 190 rpm. Expression of heterologous barramundi desaturase and elongase were induced by transferring log-phase yeast cells (OD_{600nm}=0.4) into SC selective medium^{-U} containing 2% (w/v) galactose and 0.25% (v/v) tergitol. Cultures were then supplemented with PUFA substrates from among the following: ALA, LA, 20:3 n-3, 20:2 n-6, 24:5 n-3, EPA and DPA for yeast containing pYES2/BarraFADS plasmid; fatty acid substrates ALA, LA, 18:4 n-3, 18:3 n-6, EPA, AA and DPA were used for yeast containing pYES2/BarraELOVL plasmid. In the same batch of experiments, yeast cells transformed with pYES2/BarraFADS or pYES2/BarraELOVL plasmid without galactose induction (maintained in glucose media) and host cells transformed with an empty pYES2™ vector were used as negative controls. After 48 hr of incubation, yeast cells were harvested by centrifugation at 2800 rpm (1358 x g) for 5 min at 4°C. The cells were washed twice with ddH_2O to eliminate the carryover of culture media. Total lipids were extracted and analysed as described previously in this chapter section 2.1.2.3. At least three independent replicates of the yeast cultures with fatty acid substrate supplementation were performed.

2.4 Statistical analysis

GraphPad InSat 3.10 software (GraphPad Software, Inc., CA, USA) and SPSS 15.0 version for Windows (SPSS Inc, USA) were used for the statistical analyses in this thesis. If there were 3 or more groups, a one-way analysis of variance (ANOVA) test if data were sampled from populations with identical SDs (calculated by the method of Bartlett) and follow Gaussian distributions (calculated by the Kolmogorov and Smirnov test). When the *P* value for the one-way ANOVA was less than 0.05, the Tukey-Kramer multiple comparisons test

was used as post-hoc to identify significant differences between groups. Kruskal-Wallis test with Dunn's multiple comparison test was applied for non-parametric analyses of groups with N<3 or data which did not pass the normality test. For experiments/variables with 2 groups, an unpaired t-test (two tailed) was used to examine differences between groups. When the data were not normally distributed, the non-parametric Mann-Whitney test (two tailed) was performed for data that failed the normality test. A probability level of 0.05 (P<0.05) was used in all tests.

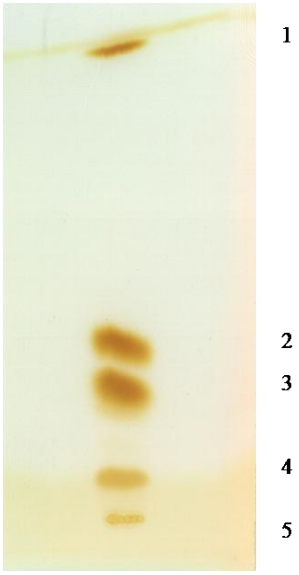


Figure 2. 1 Schematic TLC separation of simple lipids with silica gel 60H layer as the stationary phase and the petroleum spirit/ethyl ether/glacial acetic acid (180:30:2, v/v) as the mobile phase. The plate was visualized by iodine vapour. Each number indicates the following lipids: 1, cholesteryl oleate; 2, triolein; 3, oleic acid; 4, cholesterol and 5, lethithin (1, 2 distearoyl).

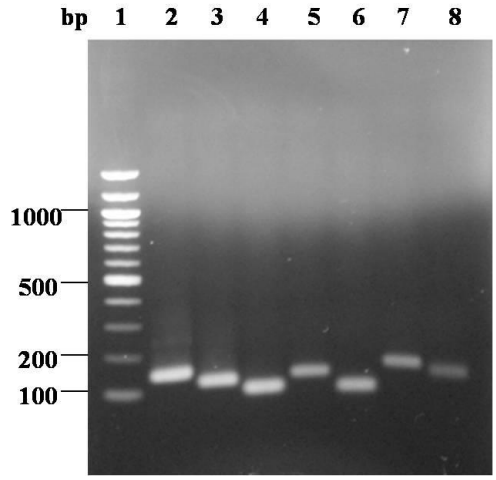


Figure 2. 2 Agarose gel image of qRT-PCR amplified PCR fragments with initial total RNA of 10 ng from rat liver D1-1 (Diet 1, rat 1). The purity and integrity of DNA was examined by electrophoresis on a 1.5% agarose gel staning with EtBr. 100 bp DNA marker (lane 1), PCR amplicon was amplified by rat β-Actin primers (lane 2), FADS1 primers (lane 3), FADS2 primers (lane 4), ELOVL 2 primers (lane 5), ELOVL 5 (lane 6), PPARα (lane 7) and SREBP-1c primers (lane 8).

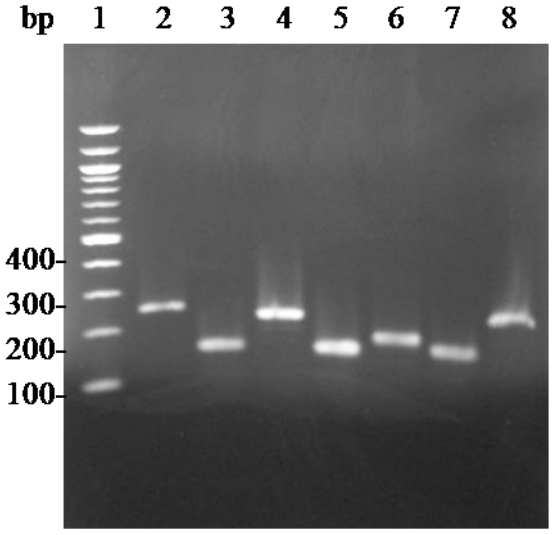
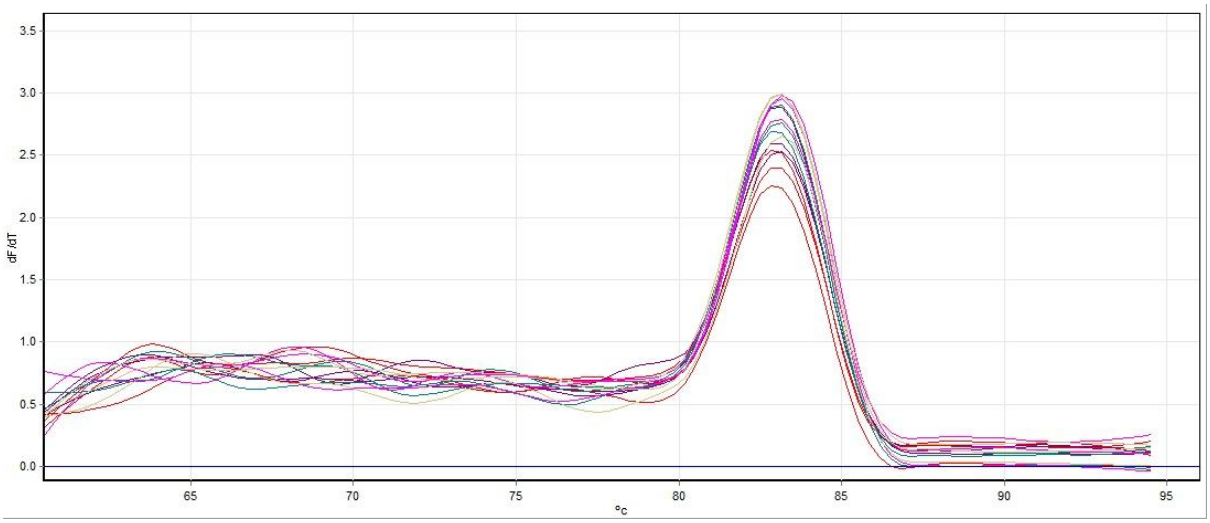


Figure 2. 3 Agarose gel image of qRT-PCR amplified PCR fragments with initial total RNA of 10 ng from barramundi liver D1-1 (Diet 1, fish 1). The purity and integrity of DNA was examined by electrophoresis on a 1.5% agarose gel staning with EtBr. 100 bp DNA marker (lane 1), PCR amplicon was amplified by barramundi FADS2 primers (lane 2), ELOVL primers (lane 3), RPLP1α primers (lane 4), 18S rRNA primers (lane 5), β-Actin (lane 6), EF1α (lane 7) and GAPDH primers (lane 8).

A



B

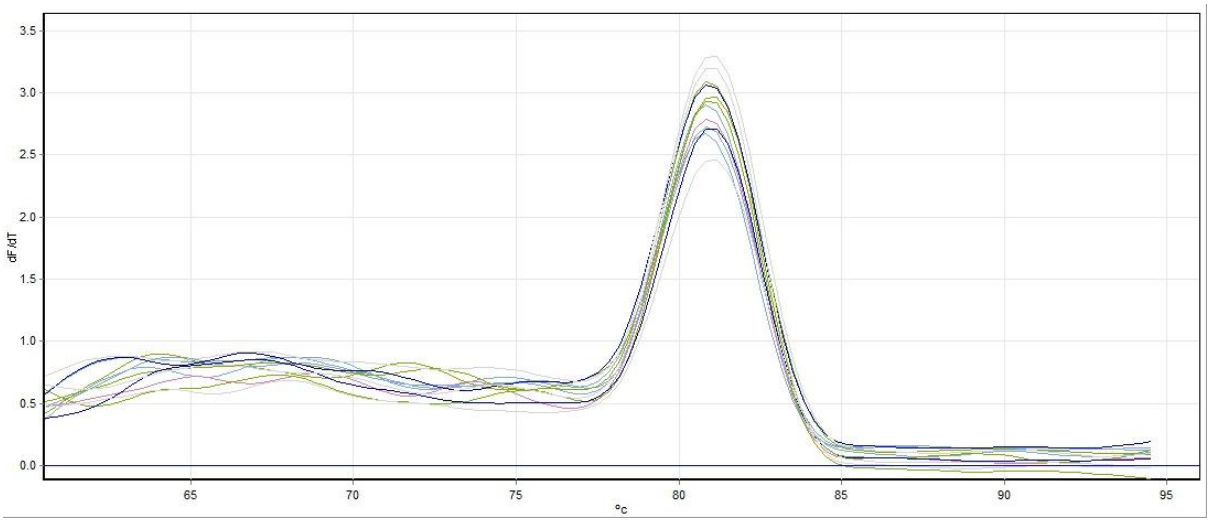
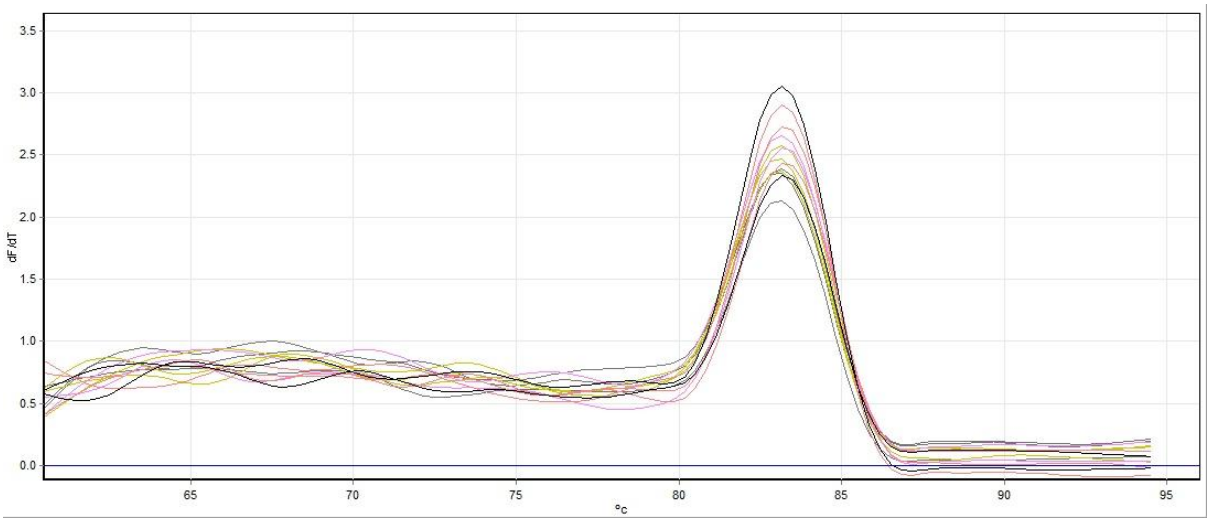


Figure 2. 4 Melt curve analyses of rat FADS1 (A) and FADS2 (B) primer sets.

A



B

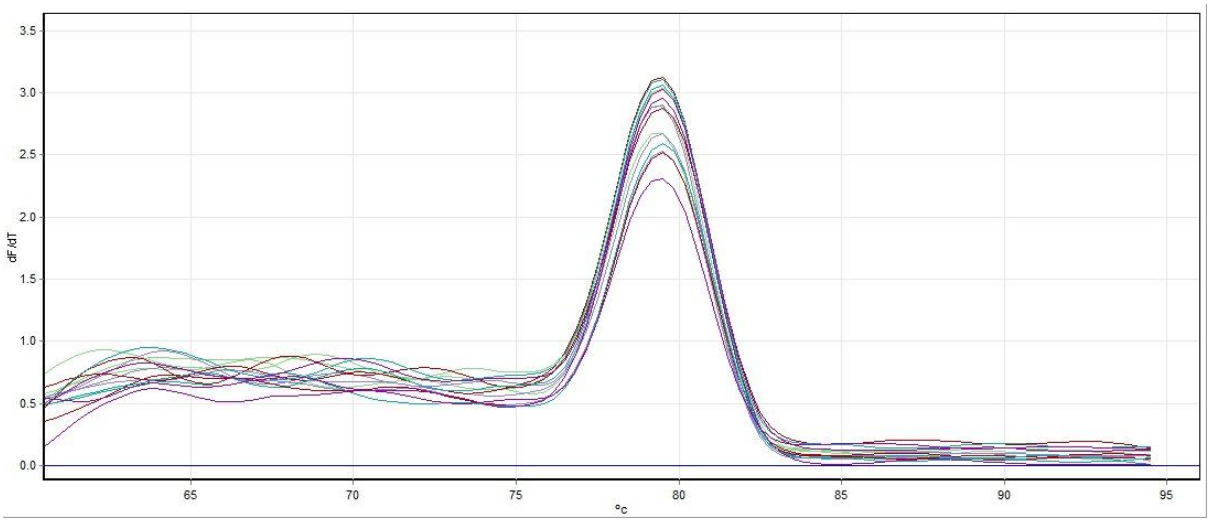
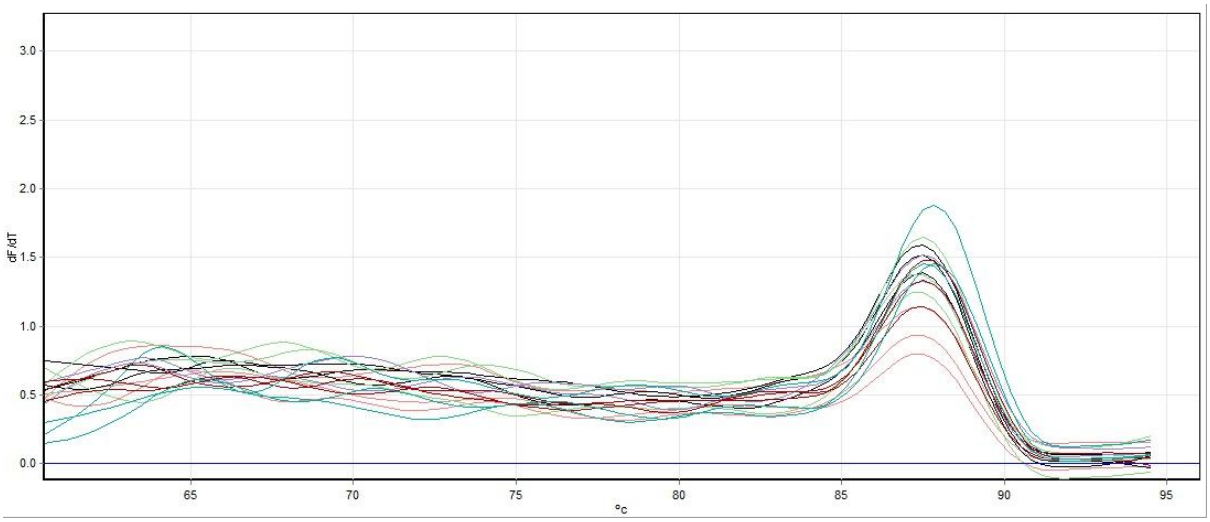


Figure 2. 5 Melt curve analyses of rat ELOVL2 (A) and ELOVL5 (B) primer sets.

A



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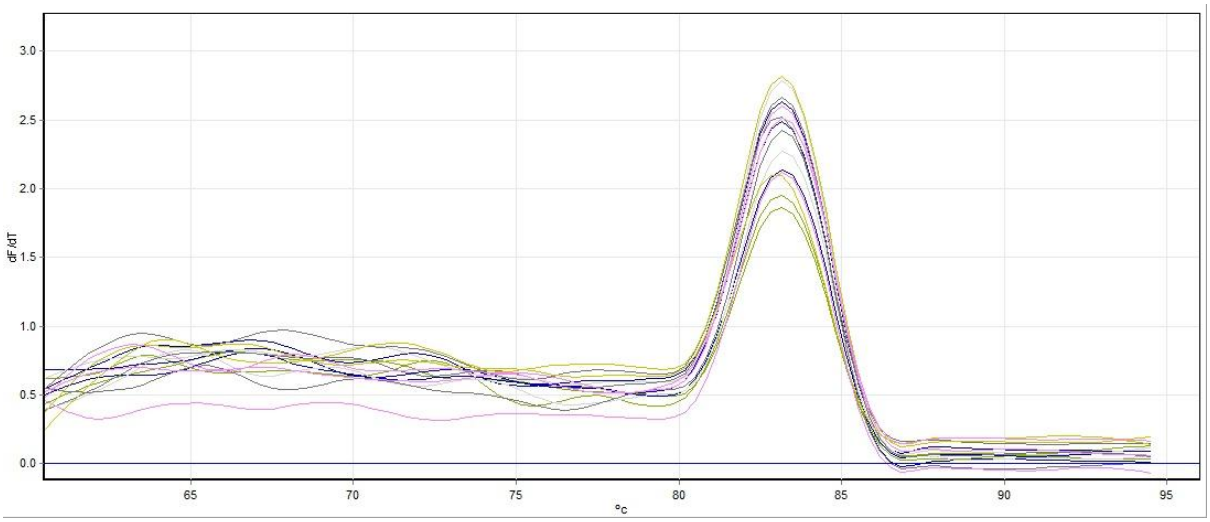


C

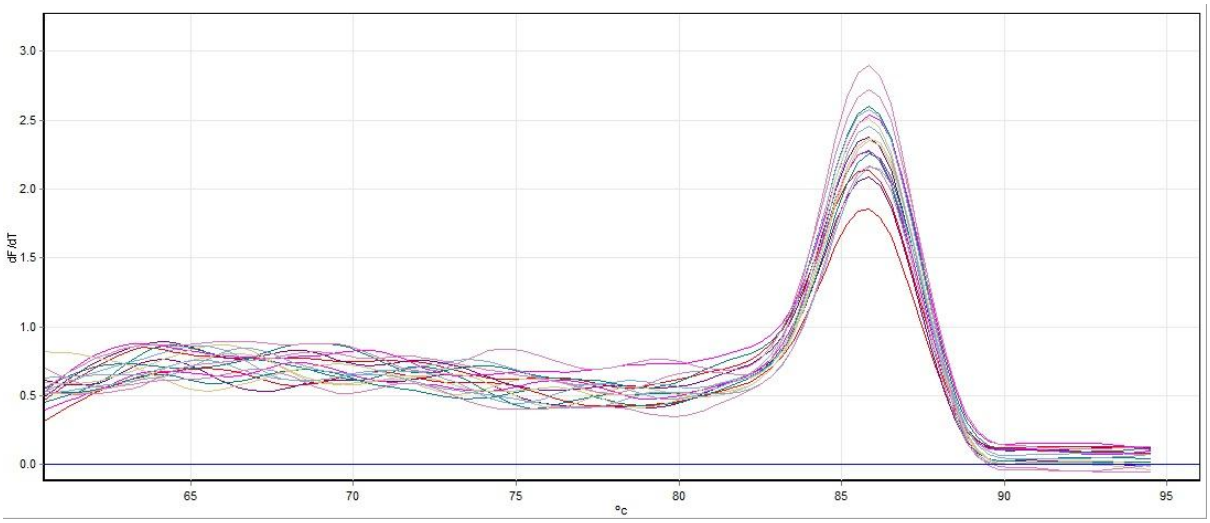
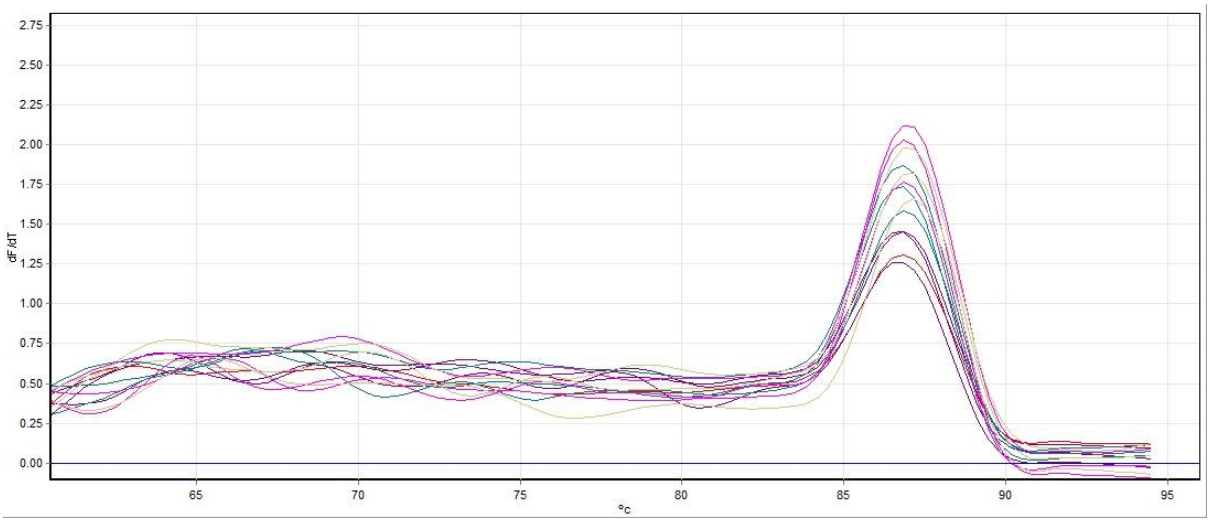
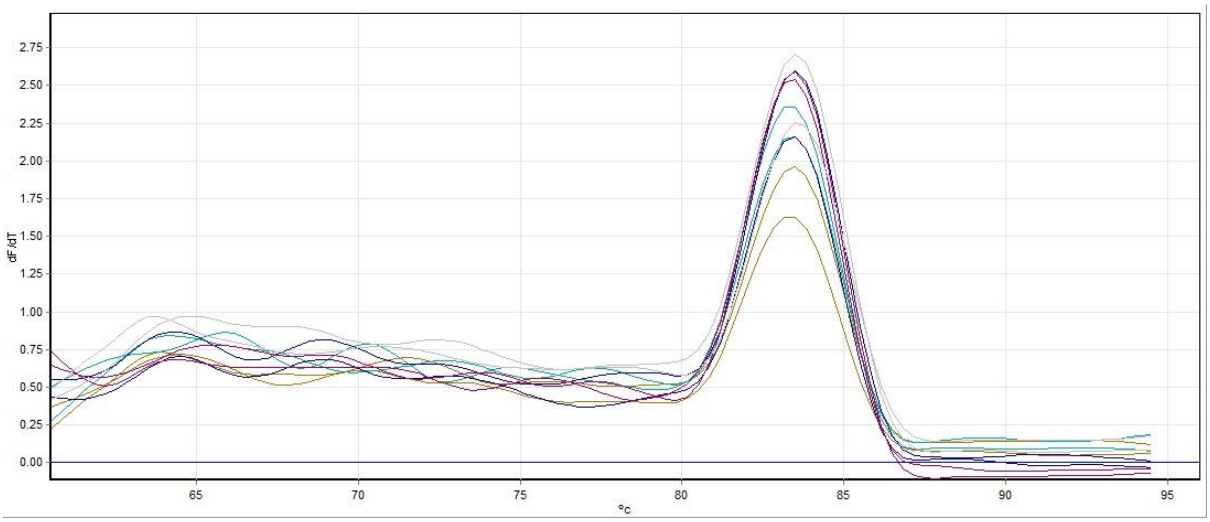


Figure 2. 6 Melt curve analyses of rat SREBP-1c (A) PPARα (B) and β-Actin (C) primer sets.

A



B



C

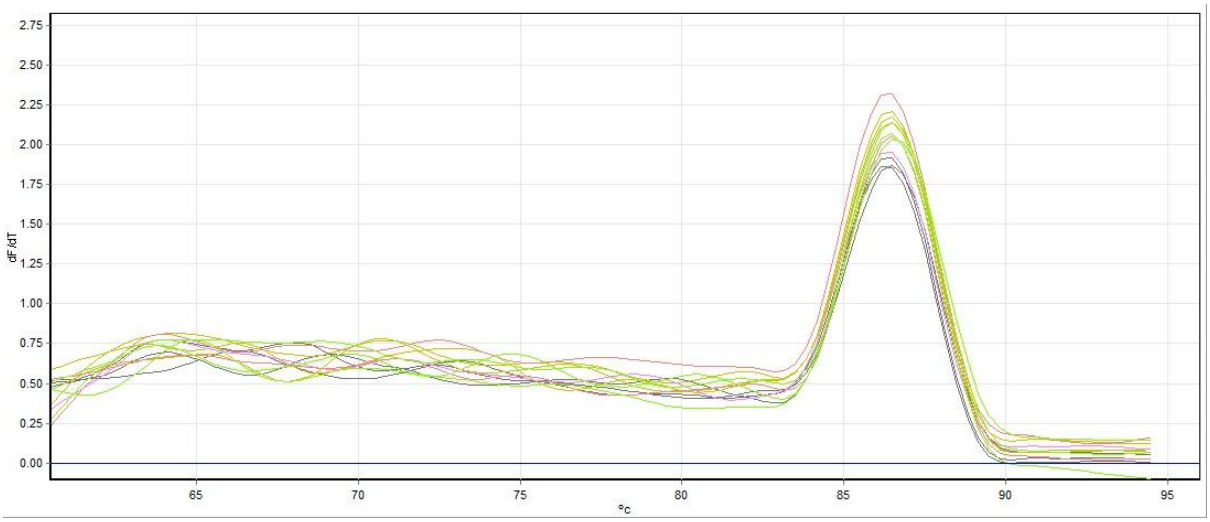


Figure 2. 7 Melt curve analyses of barramundi FADS (A), ELOVL (B) and β-Actin (C).

Chapter 3

The Effects of Dietary ALA Levels on the Synthesis of Omega-3 Fatty Acids and Gene Expression of Desaturases, Elongases and Transcription Factors in the Male Weaning Rat

3.1 Abstract

The conversion of the n-6 PUFA LA and n-3 PUFA ALA to LCPUFA involves a series of desaturation and elongation steps. Although there is evidence that the expression of the enzymes which control these steps can be regulated by extremes of dietary PUFA, the degree to which there is meaningful regulation of desaturase and elongase gene expression within the range of PUFA in normal human diets is unclear. The aim of this study, therefore, was to determine whether the expression of desaturases, elongases and transcription factors known to be involved in the regulation of hepatic lipid metabolism was regulated within the normal physiological range of dietary PUFA and ultimately, whether this may be involved in triggering LCPUFA production. Weaned male Hooded-Wistar rats were fed experimental diets which contained between 0.2 to 2.9%en ALA with a constant LA content of 1%en. A high PUFA (6.6%en as PUFA) diet and a low PUFA (0.4%en as PUFA) diet were used as reference diets. Rats were fed the diets for 3 weeks. Plasma, erythrocytes, livers, kidney, brain, heart and thigh (quadriceps) muscle were collected for fatty acid analysis. Hepatic mRNA expression of desaturases, elongases and related transcription factors were determined by qRT-PCR. Whilst high ALA diets consistently produced higher levels of EPA, DPA and DHA in rat tissues than low n-3 PUFA diets, expression of the Δ6 desaturase and elongase 2 genes were increased only in animals fed the low PUFA (0.4%en) reference diet compared to those fed diets with high PUFA (6.6%en) levels. There was no obvious relationship between the mRNA expression of desaturases, elongases or transcription factors and the levels of EPA, DPA or DHA in rat blood, liver and other tissues as a result of feeding increasing levels of ALA in this study. The results of these studies suggest that, over the range of PUFA encountered in human diets, it is probable that n-3 LCPUFA levels are regulated more by substrate supply than by alterations in the expression of the key genes involved in n-3 LCPUFA synthesis.

3.2 Introduction

The regulation of LCPUFA metabolism remains poorly understood, but is thought to be due to a combination of substrate competition, altered gene expression and nutrient-mediated hormonal regulation (226-228). The pathway from C18 PUFA to C20 and C22 LCPUFA involves the two desaturase enzymes, Δ5 and Δ6 desaturases, which are coded for by the genes FADS1 and FADS2 respectively, and one or more elongase enzymes. Studies of hepatic desaturase activity have suggested that the activities are up-regulated in response to low dietary PUFA levels compared to when n-3 PUFA or n-3 LCPUFA levels in the diet are adequate (57, 229). Elongation of C18, C20 and C22 compounds occurs via elongase enzymes which have also been suggested to have a role in the regulation of LCPUFA synthesis and may be transcribed from one or more genes, including ELOVL2 and ELOVL5 (130). Changes in expression levels of the desaturase and elongase genes is thought to involve transcription factors such as PPARα (230, 231) and SREBP-1c (232).

Early studies by Jump and Clarke (226) and others (233, 234) established that removing PUFA from diets stimulated expression of genes involved in the fatty acid synthetic pathway such as Δ6 desaturase and fatty acid synthase (FAS) while high PUFA and LCPUFA diets were inhibitory. These observations have resulted in the premise that expression of Δ6 desaturase and other enzymes in the fatty acid pathway help regulate the rate of conversion of the C18 fatty acids LA and ALA to the C20 and C22 LCPUFA. Conversely, because the n-6 and n-3 fatty acids compete for a single set of desaturating and elongating enzymes, several *in vivo* studies have highlighted that the relative levels of n-6 and n-3 LCPUFA in animal tissues can be regulated by simply altering the balance of LA and ALA in the diet. Thus, the degree to which expression of the key enzymes in the fatty acid pathway regulates LCPUFA levels compared with simple competition between available substrates is a matter of conjecture. The purpose of this study was to seek evidence that might contribute to our understanding of the relative degree to which the changes in tissue LCPUFA levels, induced by increasing the dietary ALA levels across a range that could reasonably be present in human diets, could be explained by changes in gene expression of key hepatic enzymes involved in fatty acid metabolism.

3.3 Design of the study

The aim of the experiments in this chapter was to precisely evaluate the relationship between dietary ALA level, at a constant background level of LA, on n-3 LCPUFA synthesis and

accumulation, and to examine the relationship between tissue LCPUFA level and hepatic mRNA abundance of major enzymes in the fatty acid pathway. The total amount of fat and dietary LA levels in each diet were held constant and only the level of ALA altered to minimise variables in the study. A total of 7 dietary treatments with total PUFA (LA+ALA) content of 0.4 to 6.6%en were assessed in the experiment.

3.4 Methods and Materials

3.4.1 Animals

All procedures were performed in accordance with institutional guidelines for the use of animals and the Australian code of practice for the care and use of animal for scientific purposes. The protocol was approved by the Animal Ethics Committee, University of Adelaide (Ethics number S-092-2007). Thirty-five weaned male Hooded-Wistar rats (3 weeks of age) were obtained from the Laboratory Animal Service (University of Adelaide, Australia). Five weanling male rats initially weighing approximately 38 g were assigned to each dietary group. The animals were housed in a laboratory animal facility at thermal neutrality (~25°C), a constant relative humidity of 45% and a 12 hr light and 12 hr dark cycle. The rats had free to access to fresh water and to modified rodent chow AIN-93G-based diets. The rats were fed and observed daily for any sign of illness, changes in food or water intake or waste output, and injury or welfare problems. Diets were added to the cages daily to maintain the total amount available at 100 g per cage. The rats were weighed at the start of the experiment, at weekly intervals during the 21 day feeding trial and on the day of sacrifice.

3.4.2 Diets

The animal diets used in the study all contained 5% fat (w/w), and the base diet was comprised of a modified rodent chow formulation of the AIN-93G-based diet (Specialty Feeds Company, GlenForrest, WA, Australia). A modest reduction in fat content from 7% to 5% (w/w) was the only modification made to the basal diet and this resulted in a 2% reduction in calculated energy for all experimental diets compared to the standard rat chow. The diets provided total energy of 3797 kcal per kg (15.9 MJ per kg) composed by 67.8%en of carbohydrate, 11.8%en of fat (vegetable oil) and 20.4%en of protein. The calculated energy content was based on the standard fuel values for protein (19.4%), fat (5%) and carbohydrate (64.4%) of 4, 9 and 4 kcal/g, respectively. The nutritional parameters were provided by the feed company (Speciality feeds) and the macronutrients in all diets were as follows: sucrose 100 g/kg, casein 200 g/kg, fat 50 g/kg, starch (including dextrinised starch) 556 g/kg,

cellulose 50 g/kg, dl methionine 3 g/kg, AIN93 minerals 1.4 g/kg, AIN93 vitamins 10 g/kg and other minerals 29.2 g/kg. Two reference diets were used in this study, namely, a high PUFA diet (6.6%en as PUFA; 5% by weight of total diet as soybean oil) and a low PUFA diet (0.4%en as PUFA; 5% by weight of total diet as macadamia oil). The fat contents of the diets were analysed and the level was in a range of 4.93-5.24% (w/w), and statistical analyses indicated there was no difference between the total fat contents of the diets. Five experimental diets (Diets 1-5) were composed that were designed to provide levels of ALA ranging from 0.2 to 2.9%en while keeping LA constant at 1%en (Table 3.1). Vegetable oils were purchased from the commercial suppliers and the oil blends were prepared in our laboratory by blending varying proportions of macadamia oil (17.83% saturates, 79.37% monoenes, 2.23% LA and 0.18% ALA of total fatty acids; Suncoast Gold Macadamias (Aust) Limited, Australia), flaxseed oil (11.57% saturates, 19.23% monoenes, 15.52% LA and 53.46% ALA of total fatty acids; Melrose Laboratories Pty Ltd, Australia) or sunflower oil (11.80% saturates, 26.16% monoenes, 61.43% LA and 0.09% ALA of total fatty acids; Buona Cucino, Italy). All vegetable oil blends were incorporated into nutritionally complete feed and pellets manufactured by Specialty Feeds to generate a solid feed with pellet size of 12 mm. Soybean oil for the high PUFA reference diet was provided by the feed company. Table 3.1 shows the major fatty acids components in the experimental diets. The estimated ALA%en and LA%en were calculated as: fatty acid (%en)=[(% fatty acid in diets) x fat%en] x 100.

3.4.3 Blood and tissue collection

At the end of the experimental feeding period (21 days), all rats from each dietary group were weighed individually and anaesthetized with 2-5% anaesthetic agent, isoflurane (Veterinary Companies of Australia Pty Ltd, NSW, Australia) by inhalation in an anaesthesia induction box. Upon loss of responsiveness and spontaneous movement, the rats were removed from the induction box and continuously anaesthetized with 0.4-3% isoflurane in air-oxygen mixture with spontaneous respiration. At least 4 mL of blood was collected by intra-cardiac puncture with a 23 gauge disposable needle fitted to a 5 mL disposable syringe and immediately transferred into Lithium heparin coated tubes (Greiner bio-one, Kremsmunster, Austria). The rats were then killed by exsanguination and cervical dislocation whilst still unconscious. A sample of liver, kidney, brain, heart and thigh (quadriceps) muscle was collected from each rat and placed immediately into labelled plastic vials and frozen at -20°C for later fatty acid profiling. An additional sample of around 100 mg of liver from each animal was stored in

labelled tube containing 0.9 mL RNAlater® (Qiagen) for RNA extraction and gene expression analysis.

3.4.4 Fatty acid analyses of blood and tissue

Whole blood (~4 mL) was obtained from intra-cardiac puncture and transferred to tubes containing lithium heparin. Plasma and erythrocytes were separated and total lipid extracted. Total lipid extracts were also obtained from liver, kidney, heart, brain and thigh muscle. Extracted total lipids from plasma, erythrocytes and tissues were separated to isolate the phospholipid fraction, and this fraction was methylated and the fatty acid composition analysed by capillary GC following procedures previously described (Chapter 2).

3.4.5 RNA isolation

Total RNA was isolated from 10 mg of rat liver disrupted by a tissue lyser. RNA quality was determined by measuring the ratio of OD 260/280 and 260/230. The concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm and RNA integrity was confirmed by 1.5% agarose gel electrophoresis. Procedures for liver tissue disruption, RNA isolation, RNA quality determination were as described earlier (Chapter 2).

3.4.6 qRT-PCR analysis

The relative gene expression levels (mRNA abundance) of all genes were determined by one-step qRT-PCR using SYBR green fluorescence. Each qRT-PCR reaction was consisted of 10 ng of total hepatic RNA, 5 μL of 2x QuantiFast® SYBR Green RT-PCR Master Mix, 1 μL QuantiTect® Primer or 1 μL of 5 μM (0.5 μM in final concentration) self designed primers (PPARα and SREBP-1c), 0.1 μL QuantiFast® RT Mix and then supplemented with RNase-free water to achieve a total volume of 10 μL. The primers used in this chapter are reported in Table 3.2. The qRT-PCR conditions, primer validation and detection procedures were as previously described (Chapter 2).

3.5 Statistical analysis

All fatty acid composition data and mean normalised expression data between different diets fed rats were tested using one-way ANOVA followed by Tukey-Kramer multiple comparisons test. An unpaired t-test was used to examine differences between expression between high PUFA and low PUFA diets fed rats. A probability level of 0.05 ($P<0.05$) was accepted as statistically significant in all analyses. Analysis was performed using GraphPad

InSat 3.10 software for the t-test and SPSS 15.0 version for Windows ANOVA analysis. Detailed selection criteria for statistical methods were previously outlined in chapter 2.

Table 3. 1 Major fatty acid composition of experimental diets

	Reference		**Experimental**				
Diets	**H-PUFA**[1]	**L-PUFA**[2]	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
ALA%en	0.6	0.03	0.2	0.5	1	1.9	2.9
LA%en	6	0.4	1	1	1	1	1
LA:ALA Ratio	11	14	7.4	2.4	1.2	0.6	0.4
Fatty acids[3]							
Total saturates	18.2±0.4	19.2±0.9	19.0±0.8	18.3±0.8	17.8±0.7	17.6±0.6	16.4±0.5
Total monoenes	25.8±1.3	76.4±1.1	69.4±1.3	67.1±1.3	63.5±1.3	55.3±1.3	48.0±1.2
18:2 n-6 (LA)	50.4±0.9	3.7±0.2	9.8±0.4	10.0±0.5	10.1±0.4	10.5±0.4	10.8±0.4
Total n-6	50.7±0.9	3.7±0.2	9.9±0.4	10.1±0.6	10.2±0.4	10.6±0.4	10.9±0.4
18:3 n-3 (ALA)	4.8±0.1	0.3±0.0	1.3±0.0	4.2±0.1	8.2±0.2	16.2±0.3	24.5±0.4
Total n-3	4.8±0.1	0.6±0.1	1.7±0.1	4.5±0.2	8.5±0.2	16.5±0.4	24.7±0.4

[1]High PUFA.
[2]Low PUFA.
[3]Data represent percent, relative to total fatty acids. Data are means ± SEM of n=3 per group.

Table 3. 2 Primers for qRT-PCR analysis

Genes	F/R	Sequence 5'-3'	Accession. No.	Amplicon (bp)	E[1]
β-Actin[2]		Rn-Actb-1-SG, QT00193473	NM_031144	145	1.06
FADS1[2]		Rn-Fads1-1-SG, QT00188664	NM_053445	117	1.09
FADS2[2]		Rn-Fads2-1-SG, QT00186739	NM_031344	87	1.09
ELOVL2[2]		Rn-Elovl2-predicted-1-SG, QT01683899	NM_001109118	124	1.1
ELOVL5[2]		Rn-Elovl5-1-SG, QT00178059	NM_134382	79	1.08
PPARα	F	CCTGTGAACACGATCTGAAAG	NM_013196	152	1.05
	R	ACAAAAGGCGGATTGTTG			
SREBP-1c	F	GCGCCATGGAGGAGCTGCCCTTCG	GU09103	131	1.09
	R	GTCACTGTCTTGGTTGTTGATG			

[1]Amplification efficiency.
[2]The commercial primers were purchased from Qiagen and the sequence information is confidential.

3.6 Results

3.6.1 Animal body weight and fat content in tissues

There was no difference in body weight of the animals in any of the groups at either the start of the experiment or on the day of sacrifice (Figure 3.1). There was no significant difference in total fat content (%, w/w) of liver and thigh muscle between the 7 diets however the fat content of the kidney was significantly lower ($P<0.05$) in rats fed diets 3, 4 and 5 compared to all other dietary treatments (Figure 3.2). There was no significant difference in the total fat content of brain and heart in diet 1-5 fed rats. The group of rats fed the high PUFA reference diet had a lower fat content in the brain compared to that in rats fed on diets 1 ($P<0.01$), 2 ($P<0.01$), 3 ($P<0.05$) or 5 ($P<0.01$) and a higher total fat content in heart compared with diet 2 ($P<0.05$), 4 ($P<0.01$) and 5 ($P<0.05$).

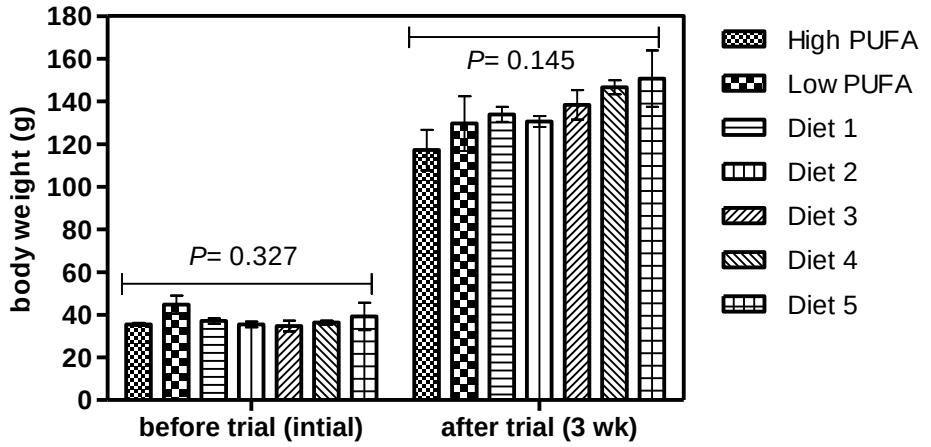


Figure 3. 1 Rat body weight at the beginning and the end of the feeding trial. No significant difference was observed between dietary groups either before or after the feeding trial as determined by one-way ANOVA with Tukey-Kramer multiple comparisons test. Data are means ± SEM of n=5 per group.

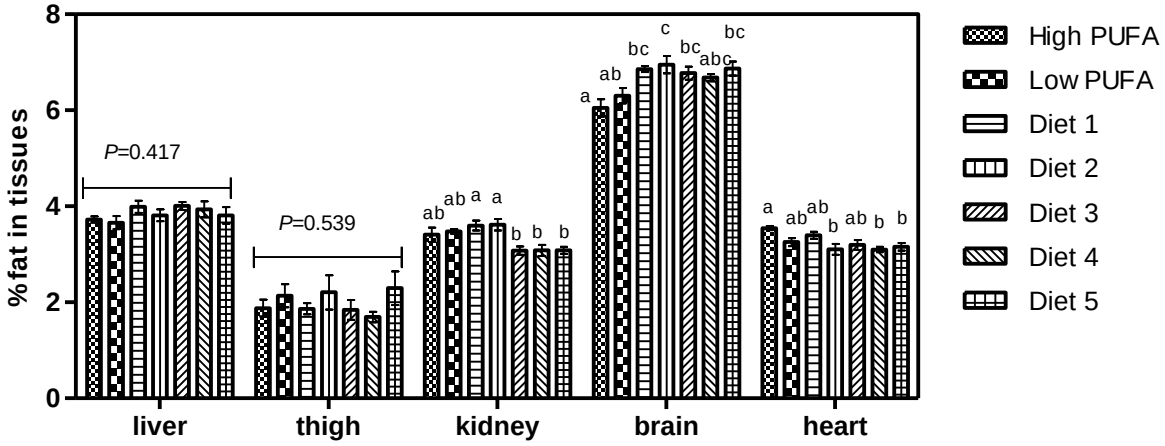


Figure 3. 2 Total fat contents in tissue (expressed as % tissue weight). Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer multiple comparisons test. Data are means ± SEM of n=5 per group.

3.6.2 Blood fatty acid analysis

The effect of feeding diets with high PUFA, low PUFA and increasing levels of ALA from 0.2-2.9%en while holding the level of dietary LA constant at 1%en on the fatty acid profiles of plasma and erythrocyte phospholipids are shown in Table 3.3 and 3.4, respectively. In the plasma phospholipid fraction, the two reference diets showed the expected differences in n-3 LCPUFA levels, with DPA and DHA being 4.5 and 2.4 fold higher respectively in rats fed the high PUFA diet relative to the low PUFA diet. No obvious differences were observed in total saturates but total monoenes decreased with increasing dietary ALA content for the experimental diets (Diets 1-5). Total monoene content in plasma phospholipids in the low PUFA group were significantly greater than in the high PUFA group. Levels of 20:3 n-3 increased with increasing dietary ALA content, indicating that elongation had occurred, however the increase as a proportion of total fatty acids was very low. Despite the fact that the dietary LA level was held constant across all diets, plasma phospholipid LA content gradually increased (from 9.5 to 15.1% of total fatty acids) with increasing dietary ALA content. At the same time, plasma phospholipid AA levels decreased from 13.4% down to 6.3% of total fatty acids in response to increasing dietary ALA (Figure 3.3). The plasma phospholipid ALA content (as % total fatty acids) was positively related to dietary ALA level but was always less than 1% total fatty acids. The accumulation of EPA and DPA into plasma phospholipid was linearly related to dietary ALA and the levels of AA appeared to be the inverse of EPA levels. DHA levels increased in response to dietary ALA but only up to 1%en dietary ALA, after which they plateaued and then declined (Figure 3.3).

In erythrocyte phospholipids, the expected differences in n-3 LCPUFA levels were observed for rats fed the 2 reference diets, with DPA and DHA being 1.9 and 1.4 fold higher respectively in rats fed the high PUFA diet relative to the low PUFA diet (Table 3.4). The fatty acid composition of the erythrocyte phospholipid fraction in rats fed the experimental diets 1-5 showed a significant increase in total saturates from 46% up to 47.7% and decrease in total monoenes from 18.9 to 16% total fatty acids with increasing dietary ALA content. As expected, the total monoenes in rats fed the low PUFA diet was 1.9 fold higher than that of high PUFA fed rats. Similar to plasma phospholipid LA, the LA in erythrocyte phospholipid fraction gradually increased from 4.7 to 6.4% total fatty acids as the level of ALA in the diets increased, however the effect was not as marked as that observed in plasma phospholipids. In erythrocyte phospholipids, total n-6 fatty acids (AA, 22:4 n-6 and 22:5 n-6) levels decreased from 29.2 to 22.8% of total fatty acids in response to increasing dietary ALA (Table 3.4).

Similar to the for the plasma phospholipid ALA content, erythrocyte ALA levels were positively related to dietary ALA content, but ALA always made up less than 1% total fatty acids. The accumulation of EPA and DPA into erythrocyte phospholipid was also linearly related to dietary ALA, and the levels of AA appeared to be the inverse of EPA and DPA levels. DHA in erythrocyte phospholipid reached maximum level when dietary ALA was at 1%en (Figure 3.4).

3.6.3 Tissue fatty acid analysis

The effects of dietary PUFA on liver phospholipids (Table 3.5) were similar to the effects seen in plasma and erythrocytes. The reference diets showed the expected differences in n-3 LCPUFA levels with DPA and DHA being 6.2 and 2.1 fold higher respectively in rats fed the high PUFA diet relative to the low PUFA diet (Table 3.5). Total saturates did not differ between the experimental diets and total monoenes decreased with increasing dietary ALA content. Moreover, the total hepatic monoene content for rats in the low PUFA reference diet was 2.3 fold greater than the level in the high PUFA reference group. There was a linear relationship between increasing dietary ALA and liver 20:3 n-3, EPA and DPA content. Liver LA initially increased with increasing dietary ALA, however the effect was not significant and there were no further increases in hepatic LA content beyond the ALA 0.5%en diet. The hepatic levels of other n-6 PUFA decreased with increasing dietary ALA content. Liver DHA content reached a maximum level at a dietary ALA level of 1%en (Table 3.5 and Figure 3.5).

The fatty acid composition of the kidney, heart, brain and thigh muscle of rats fed high PUFA and low PUFA and each of the 5 experimental diets are shown in Table 3.6-3.9. There was no effect of dietary ALA content on total monoene levels in these tissues (Table 3.6). There was no effect of dietary treatment on the saturated fatty acid content in brain, heart and thigh muscle phospholipids between the 5 experimental diets. Total saturates in kidney phospholipids in the low PUFA reference group, however, were significantly lower than those in the high PUFA reference group (Table 3.6). In the brain, this effect was reversed, and saturated fat content was increased in the low PUFA reference group (Table 3.7). The total monoene levels were not different in kidney and brain between the 5 experimental diets, but there was a decrease in the monoene levels in heart (Table 3.8) and thigh muscle (Table 3.9) phospholipids with increasing dietary ALA.

All tissue phospholipids, with the exception of the brain, showed a greater level of total monoenes in the low PUFA reference group compared to the high PUFA reference group. The fatty acid changes in tissues followed the same general pattern as seen in plasma, erythrocyte and liver phospholipids (Table 3.3-3.5). The diet-induced shifts in the DHA level were calculated as the fold change in phospholipid DHA content between the highest and lowest phospholipid DHA. Based on this calculation, the accumulation of DHA was greatest in plasma (1.98 fold), followed by thigh muscle (1.91 fold), heart (1.77 fold), liver (1.76 fold), kidney (1.45 fold), erythrocytes (1.44 fold) and then brain (1.16 fold). Heart and thigh muscle tissues showed gradual increases in phospholipid DHA with increasing dietary ALA levels (Table 3.8 and 3.9). Although the overall change in the DHA content of the brain was small, the proportion of DHA was reflective of DHA concentrations seen in blood and tissues including liver and kidney (Figure 3.10). The effects of diet on individual LCPUFA EPA, DPA, DHA and AA in the plasma, erythrocyte and tissue phospholipid fractions are highlighted in Figure 3.3-3.9.

Table 3. 3 Fatty acid composition in plasma

Plasma phospholipid	**Reference**		**Experimental**				
Diets	**H-PUFA**[1]	**L-PUFA**[2]	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
ALA%en	0.6	0.03	0.2	0.5	1	1.9	2.9
LA%en	6	0.4	1	1	1	1	1
LA:ALA Ratio	11	14	7.4	2.4	1.2	0.6	0.4
Fatty acids[3]							
Total saturates	49.8±0.6^{b}	46.0±0.8^{a}	49.0±0.7^{b}	47.7±0.6^{ab}	48.3±0.6^{ab}	49.2±0.3^{b}	50.2±0.5^{b}
18:1 n-9	5.5±0.4^{a}	15.7±0.6^{c}	11.2±0.4^{b}	11.4±0.4^{b}	11.2±0.3^{b}	11.4±0.4^{b}	9.9±0.5^{b}
18:1 n-7	2.8±0.1^{a}	5.5±0.2^{c}	4.9±0.2^{bc}	4.8±0.3^{bc}	4.4±0.1^{b}	3.4±0.1^{a}	2.8±0.1^{a}
Total monoenes	10.0±0.6^{a}	24.7±0.8^{d}	18.5±0.7^{c}	18.9±0.8^{c}	18.2±0.4^{c}	17.4±0.5^{bc}	15.0±0.3^{b}
18:2 n-6 (LA)	17.3±0.9^{e}	8.0±0.3^{a}	9.5±0.6^{ab}	11.2±0.6^{bc}	13.2±0.4^{cd}	13.8±0.3^{d}	15.1±0.5^{de}
18:3 n-6	0.1±0.0^{c}	0.1±0.1^{c}	0.1±0.0^{bc}	0.1±0.0^{bc}	0.1±0.0^{ab}	< 0.05^{a}	< 0.05^{a}
20:3 n-6	0.6±0.1^{a}	1.0±0.1^{b}	1.0±0.0^{b}	1.2±0.0^{c}	1.2±0.0^{c}	1.0±0.1^{b}	0.9±0.1^{b}
20:4 n-6 (AA)	16.0±0.9^{e}	10.1±0.2^{bc}	13.4±0.7^{d}	10.7±0.6^{c}	7.9±0.3^{ab}	7.1±0.3^{a}	6.3±0.3^{a}
22:4 n-6	0.4±0.0^{d}	0.2±0.0^{c}	0.2±0.0^{c}	0.2±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{a}	0.1±0.0^{a}
22:5 n-6	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
Total n-6	34.8±0.2^{e}	19.5±0.3^{a}	24.4±0.1^{d}	23.5±0.1^{cd}	22.7±0.3^{bc}	22.1±0.3^{b}	22.5±0.2^{bc}
18:3 n-3 (ALA)	0.1±0.0^{a}	< 0.05^{a}	< 0.05^{a}	0.1±0.0^{ab}	0.2±0.0^{b}	0.4±0.0^{c}	0.8±0.1^{d}
18:4 n-3	N. D.	0.1±0.0^{c}	< 0.05^{b}	< 0.05^{a}	N. D.	N. D.	N. D.
20:3 n-3	0.1±0.0^{a}	0.1±0.0^{b}	N. D.	N. D.	0.1±0.0^{b}	0.10±0.0^{b}	0.13±0.0^{c}
20:5 n-3 (EPA)	0.1±0.0^{a}	0.1±0.0^{a}	0.2±0.0^{a}	0.9±0.1^{b}	1.5±0.1^{c}	2.9±0.1^{d}	3.3±0.2^{d}
22:5 n-3 (DPA)	0.5±0.0^{c}	0.1±0.0^{a}	0.16±0.01^{a}	0.4±0.040^{b}	0.7±0.0^{d}	1.0±0.1^{e}	1.4±0.0^{f}
22:6 n-3 (DHA)	4.4±0.3^{b}	1.8±0.1^{a}	3.4±0.1^{b}	5.7±0.3^{c}	6.8±0.1^{c}	6.1±0.3^{c}	6.1±0.4^{c}
Total n-3	5.1±0.2^{c}	2.2±0.1^{a}	3.9±0.1^{b}	7.1±0.3^{d}	9.3±0.2^{e}	10.5±0.3^{f}	11.7±0.2^{g}

[1]High PUFA.
[2]Low PUFA.
[3]Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer multiple comparisons test. Data are means ± SEM of n=5 per group.

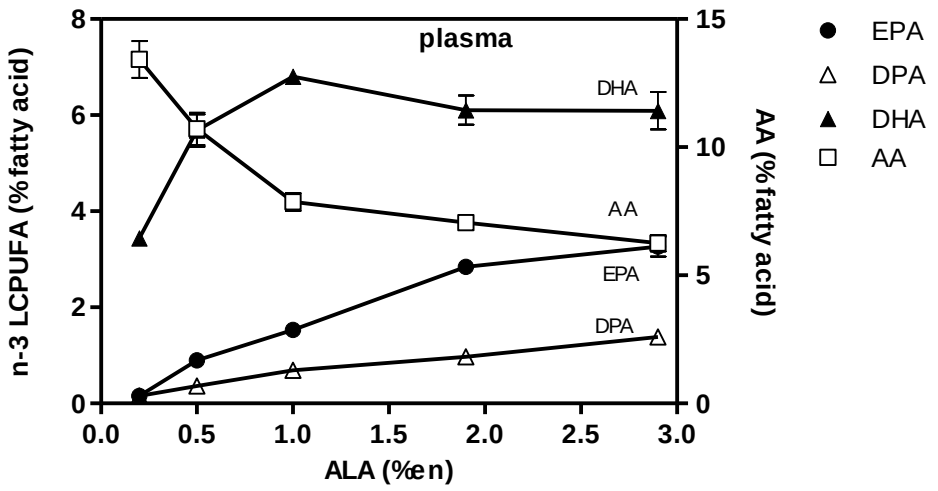


Figure 3. 3 Proportions of phospholipid EPA, DPA, DHA and AA in plasma of rats fed a range of diets with varying ALA levels ranging from 0.2-2.9%en. Data points are means ± SEM, n=5 per group.

Table 3. 4 Fatty acid composition in erythrocytes

Erythrocyte phospholipid	**Reference**		**Experimental**				
Diets	**H-PUFA**[1]	**L-PUFA**[2]	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
ALA%en	0.6	0.03	0.2	0.5	1	1.9	2.9
LA%en	6	0.4	1	1	1	1	1
LA:ALA Ratio	11	14	7.4	2.4	1.2	0.6	0.4
Fatty acids[3]							
Total saturates	48.0±0.3^{c}	45.7±0.3^{a}	46.0±0.2^{ab}	46.0±0.4^{ab}	47.0±0.1^{bc}	47.2±0.3^{bc}	47.7±0.4^{c}
18:1 n-9	6.3±0.1^{a}	12.2±0.2^{d}	11.0±0.1^{c}	10.9±0.1^{c}	10.6±0.1^{c}	10.4±0.1^{bc}	9.9±0.2^{b}
18:1 n-7	3.2±0.0^{a}	5.3±0.1^{d}	5.4±0.0^{d}	5.2±0.1^{d}	4.8±0.1^{c}	4.3±0.0^{b}	4.0±0.0^{b}
Total monoenes	10.7±0.1^{a}	20.5±0.3^{f}	18.9±0.2^{e}	18.8±0.3^{de}	17.9±0.2^{d}	17.0±0.1^{c}	16.0±0.3^{b}
18:2 n-6 (LA)	9.1±0.4^{e}	3.6±0.1^{a}	4.7±0.2^{b}	5.5±0.1^{bc}	5.9±0.1^{cd}	6.3±0.2^{cd}	6.4±0.2^{d}
18:3 n-6	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{ab}	0.1±0.0^{ab}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{ab}
20:3 n-6	0.4±0.0^{a}	0.6±0.0^{bc}	0.6±0.0^{bc}	0.7±0.0^{d}	0.7±0.0^{d}	0.6±0.0^{c}	0.5±0.0^{b}
20:4 n-6 (AA)	23.2±0.3^{e}	21.5±0.3^{d}	22.1±0.2^{de}	19.8±0.3^{c}	18.0±0.2^{b}	16.7±0.3^{b}	15.1±0.3^{a}
22:4 n-6	2.6±0.0^{f}	1.9±0.1^{e}	1.6±0.0^{d}	1.0±0.0^{c}	0.8±0.0^{bc}	0.6±0.0^{ab}	0.5±0.0^{a}
22:5 n-6	< 0.05^{bc}	< 0.05^{ab}	N.D.	N.D.	N.D.	< 0.05^{abc}	< 0.05^{c}
Total n-6	35.9±0.2^{f}	27.9±0.4^{d}	29.2±0.1^{e}	27.2±0.2^{d}	25.6±0.2^{c}	24.0±0.2^{b}	22.8±0.2^{a}
18:3 n-3 (ALA)	0.1±0.0^{a}	< 0.05^{a}	< 0.05^{a}	0.1±0.0^{a}	0.2±0.0^{b}	0.4±0.0^{c}	0.6±0.0^{d}
18:4 n-3	N.D.	< 0.05^{b}	< 0.05^{ab}	< 0.05^{a}	N.D.	N.D.	N.D.
20:3 n-3	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
20:5 n-3 (EPA)	0.2±0.0^{a}	0.2±0.0^{a}	0.3±0.0^{a}	1.1±0.1^{b}	2.0±0.1^{c}	3.5±0.1^{d}	4.9±0.4^{e}
22:5 n-3 (DPA)	1.7±0.1^{c}	0.9±0.0^{a}	1.2±0.0^{b}	2.0±0.1^{d}	2.7±0.0^{e}	3.4±0.0^{f}	4.0±0.1^{g}
22:6 n-3 (DHA)	3.0±0.1^{bc}	2.2±0.1^{a}	2.5±0.0^{ab}	3.4±0.2^{cd}	3.7±0.1^{d}	3.5±0.2^{cd}	3.5±0.1^{cd}
Total n-3	5.1±0.1^{b}	3.4±0.1^{a}	4.1±0.1^{a}	6.7±0.2^{c}	8.7±0.1^{d}	10.9±0.2^{e}	13.1±0.3^{f}

[1]High PUFA.
[2]Low PUFA.
[3]Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer multiple comparisons test. Data are means ± SEM of n=5 per group.

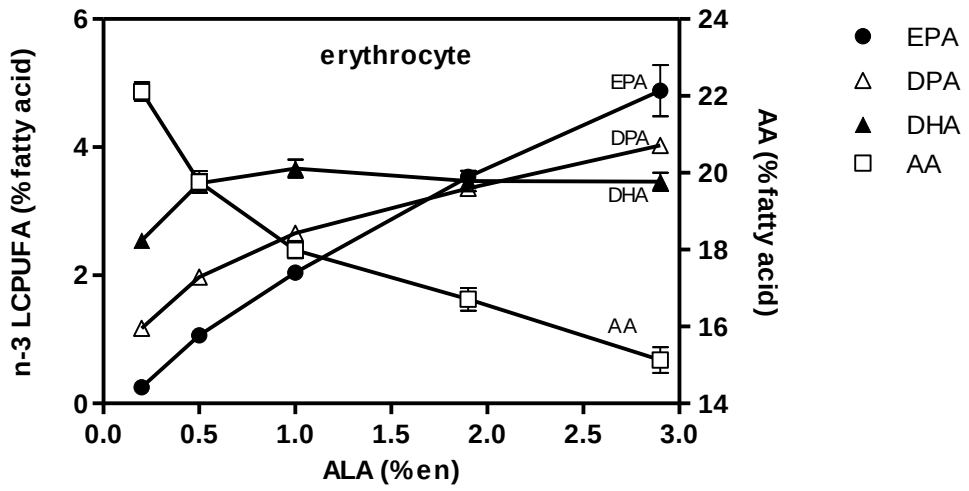


Figure 3. 4 Proportions of phospholipid EPA, DPA, DHA and AA in erythrocyte of rats fed a range of diets with varying ALA levels ranging from 0.2-2.9%en. Data points are means ± SEM, n=5 per group.

Table 3. 5 Fatty acid composition in liver

Liver phospholipid	**Reference**		**Experimental**				
Diets	**H-PUFA**[1]	**L-PUFA**[2]	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
ALA%en	0.6	0.03	0.2	0.5	1	1.9	2.9
LA%en	6	0.4	1	1	1	1	1
LA:ALA Ratio	11	14	7.4	2.4	1.2	0.6	0.4
Fatty acids[3]							
Total saturates	42.0±0.4^{ab}	40.9±0.5^{a}	42.2±0.5^{ab}	41.0±0.5^{a}	40.8±0.2^{a}	42.1±0.3^{ab}	43.3±0.1^{b}
18:1 n-9	4.7±0.3^{a}	11.8±0.4^{c}	8.2±0.3^{b}	8.5±0.3^{b}	8.5±0.3^{b}	8.4±0.2^{b}	7.9±0.2^{b}
18:1 n-7	3.1±0.1^{a}	5.9±0.2^{c}	5.4±0.2^{bc}	5.1±0.3^{bc}	4.8±0.1^{b}	4.0±0.1^{a}	3.3±0.0^{a}
Total monoenes	9.6±0.4^{a}	21.9±0.4^{d}	16.3±0.6^{c}	16.6±0.8^{c}	16.2±0.3^{c}	15.1±0.4^{bc}	13.7±0.3^{b}
18:2 n-6 (LA)	12.4±0.5^{d}	5.2±0.2^{a}	6.5±0.3^{b}	7.8±0.2^{c}	8.9±0.2^{c}	8.6±0.1^{c}	8.4±0.1^{c}
18:3 n-6	0.2±0.0^{cd}	0.3±0.0^{d}	0.2±0.0^{bc}	0.2±0.0^{abc}	0.1±0.0^{ab}	0.1±0.0^{a}	0.1±0.0^{a}
20:3 n-6	0.6±0.1^{a}	1.0±0.0^{bc}	1.1±0.0^{cd}	1.2±0.0^{d}	1.2±0.0^{cd}	1.0±0.1^{bc}	0.9±0.0^{b}
20:4 n-6 (AA)	25.2±0.5^{e}	18.7±0.4^{c}	21.6±0.6^{d}	17.1±0.5^{c}	13.9±0.3^{b}	11.6±0.3^{a}	9.9±0.4^{a}
22:4 n-6	0.4±0.0^{e}	0.2±0.0^{d}	0.2±0.0^{d}	0.1±0.0^{c}	0.1±0.0^{bc}	0.1±0.0^{ab}	0.1±0.0^{a}
22:5 n-6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total n-6	39.2±0.4^{f}	25.5±0.5^{cd}	29.7±0.4^{e}	26.5±0.3^{d}	24.3±0.4^{c}	21.5±0.3^{b}	19.5±0.3^{a}
18:3 n-3 (ALA)	0.1±0.0^{ab}	< 0.05^{a}	< 0.05^{a}	0.1±0.0^{a}	0.1±0.0^{b}	0.3±0.0^{c}	0.5±0.0^{d}
18:4 n-3	N.D.	0.1±0.0^{d}	0.1±0.0^{c}	< 0.05^{b}	< 0.05^{a}	N.D.	N.D.
20:3 n-3	0.1±0.0^{ab}	0.1±0.0^{b}	N.D.	0.1±0.0^{a}	0.1±0.0^{ab}	0.1±0.0^{bc}	0.2±0.0^{c}
20:5 n-3 (EPA)	0.2±0.0^{a}	0.2±0.0^{a}	0.4±0.0^{a}	2.1±0.1^{b}	4.3±0.1^{c}	7.2±0.2^{d}	9.0±0.4^{e}
22:5 n-3 (DPA)	0.7±0.1^{c}	0.1±0.0^{a}	0.2±0.0^{a}	0.5±0.0^{b}	0.9±0.0^{c}	1.3±0.1^{d}	1.8±0.0^{e}
22:6 n-3 (DHA)	7.7±0.3^{b}	3.7±0.1^{a}	6.7±0.2^{b}	10.4±0.4^{c}	11.7±0.2^{d}	11.3±0.3^{cd}	11.3±0.3^{cd}
Total n-3	8.8±0.2^{c}	4.4±0.1^{a}	7.4±0.2^{b}	13.2±0.2^{d}	17.1±0.3^{e}	20.3±0.2^{f}	22.9±0.3^{g}

[1]High PUFA.
[2]Low PUFA.
[3]Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer multiple comparisons test. Data are means ± SEM of n=5 per group.

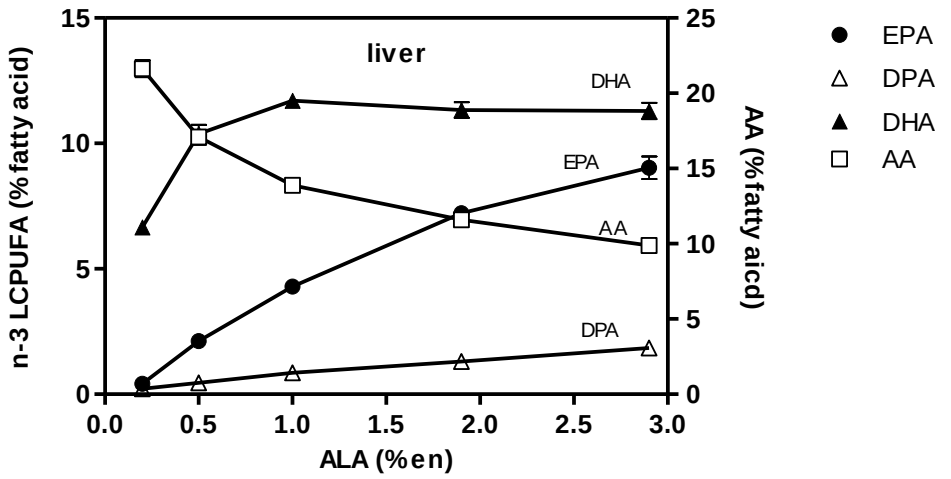


Figure 3. 5 Proportions of phospholipid EPA, DPA, DHA and AA in liver of rats fed a range of diets with varying ALA levels ranging from 0.2-2.9%en. Data points are means ± SEM, n=5 per group.

Table 3. 6 Fatty acid composition in kidney

Kidney phospholipid	**Reference**		**Experimental**				
Diets	**H-PUFA**[1]	**L-PUFA**[2]	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
ALA%en	0.6	0.03	0.2	0.5	1	1.9	2.9
LA%en	6	0.4	1	1	1	1	1
LA:ALA Ratio	11	14	7.4	2.4	1.2	0.6	0.4
Fatty acids[3]							
Total saturates	44.8±0.2^{c}	43.4±0.3^{ab}	42.9±0.1^{a}	43.2±0.3^{ab}	43.3±0.2^{ab}	42.7±0.1^{a}	44.1±0.2^{bc}
18:1 n-9	7.0±0.2^{a}	11.8±0.2^{d}	9.7±0.1^{b}	10.7±0.3^{c}	10.8±0.2^{c}	10.7±0.1^{c}	10.7±0.2^{c}
18:1 n-7	2.4±0.0^{a}	4.2±0.1^{f}	3.9±0.0^{e}	3.9±0.1^{e}	3.4±0.0^{d}	3.1±0.0^{c}	2.8±0.0^{b}
Total monoenes	10.6±0.3^{a}	19.3±0.4^{d}	16.4±0.1^{b}	17.6±0.3^{c}	17.0±0.3^{bc}	16.5±0.1^{b}	16.1±0.3^{b}
18:2 n-6 (LA)	9.9±0.2^{de}	4.9±0.2^{a}	7.1±0.3^{b}	8.2±0.3^{c}	9.5±0.2^{d}	10.8±0.2^{e}	10.6±0.2^{e}
18:3 n-6	0.1±0.0^{bc}	0.1±0.011^{c}	0.1±0.0^{ab}	0.1±0.0^{ab}	0.1±0.0^{ab}	0.1±0.0^{a}	0.1±0.0^{a}
20:3 n-6	0.8±0.1^{ab}	0.8±0.0^{abc}	0.9±0.0^{c}	0.9±0.0^{c}	0.8±0.0^{abc}	0.9±0.0^{bc}	0.7±0.0^{a}
20:4 n-6 (AA)	29.4±0.4^{f}	27.1±0.4^{e}	28.1±0.2^{ef}	24.2±0.5^{d}	22.1±0.5^{c}	19.9±0.3^{b}	17.1±0.8^{a}
22:4 n-6	0.7±0.0^{e}	0.5±0.0^{d}	0.5±0.0^{d}	0.3±0.0^{c}	0.3±0.0^{bc}	0.2±0.0^{ab}	0.2±0.0^{a}
22:5 n-6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total n-6	41.2±0.3^{d}	33.4±0.4^{b}	36.8±0.1^{c}	33.8±0.7^{b}	32.8±0.5^{b}	32.0±0.2^{b}	28.8±0.7^{a}
18:3 n-3 (ALA)	0.1±0.0^{b}	< 0.05^{a}	< 0.05^{a}	0.1±0.0^{b}	0.2±0.0^{c}	0.4±0.0^{d}	0.6±0.0^{e}
18:4 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:3 n-3	0.2±0.0^{c}	0.2±0.0^{bc}	0.1±0.0^{a}	0.1±0.0^{ab}	0.2±0.0^{abc}	0.2±0.0^{d}	0.2±0.0^{e}
20:5 n-3 (EPA)	0.1±0.0^{a}	0.1±0.0^{a}	0.2±0.0^{a}	1.1±0.^{b}	2.5±0.1^{c}	4.1±0.1^{d}	6.0±0.3^{e}
22:5 n-3 (DPA)	0.3±0.0^{c}	0.1±0.0^{a}	0.2±0.0^{b}	0.4±0.0^{d}	0.6±0.0^{e}	0.9±0.0^{f}	1.0±0.0^{g}
22:6 n-3 (DHA)	2.6±0.0^{c}	1.6±0.1^{a}	2.1±0.0^{b}	2.9±0.1^{d}	3.1±0.1^{d}	2.9±0.1^{d}	2.9±0.0^{d}
Total n-3	3.3±0.0^{b}	2.0±0.1^{a}	2.7±0.0^{ab}	4.6±0.2^{c}	6.5±0.2^{d}	8.5±0.2^{e}	10.8±0.4^{f}

[1]High PUFA.
[2]Low PUFA.
[3]Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer multiple comparisons test. Data are means ± SEM of n=5 per group.

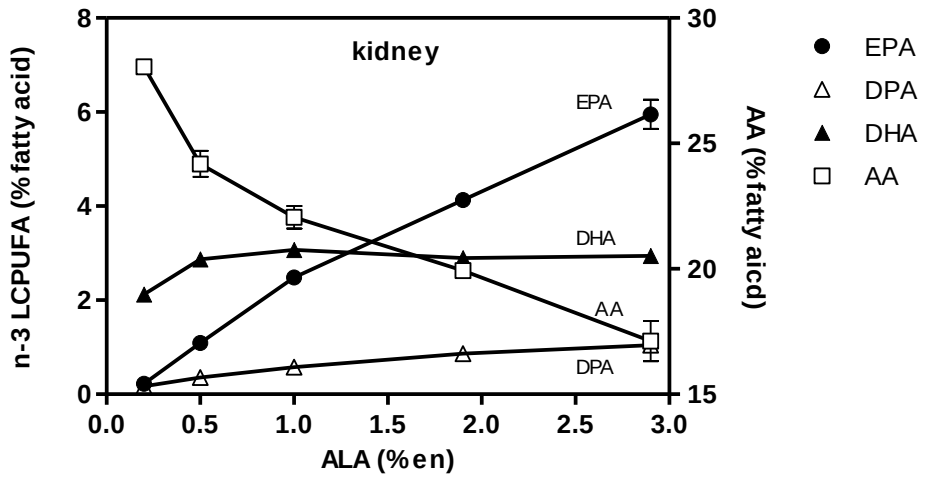


Figure 3. 6 Proportions of phospholipid EPA, DPA, DHA and AA in kidney of rats fed a range of diets with varying ALA levels ranging from 0.2-2.9%en. Data points are means ± SEM, n=5 per group.

Table 3. 7 Fatty acid composition in brain

Brain phospholipid	**Reference**		**Experimental**				
Diets	**H-PUFA**[1]	**L-PUFA**[2]	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
ALA%en	0.6	0.03	0.2	0.5	1	1.9	2.9
LA%en	6	0.4	1	1	1	1	1
LA:ALA Ratio	11	14	7.4	2.4	1.2	0.6	0.4
Fatty acids[3]							
Total saturates	52.2±0.2^{a}	54.3±0.3^{d}	54.2±0.1^{cd}	53.1±0.2^{b}	53.2±0.2^{b}	53.3±0.2^{bc}	53.2±0.2^{bc}
18:1 n-9	12.6±0.1	12.7±0.4	12.8±0.1	13.1±0.3	13.0±0.4	21.9±0.1	13.8±0.3
18:1 n-7	2.9±0.1^{a}	3.5±0.1^{b}	3.7±0.1^{b}	3.7±0.1^{b}	3.7±0.1^{b}	3.5±0.1^{b}	3.6±0.1^{b}
Total monoenes	17.0±0.2^{a}	17.7±0.4^{ab}	18.1±0.2^{ab}	18.5±0.5^{ab}	18.4±0.5^{ab}	18.0±0.2^{ab}	19.1±0.4^{b}
18:2 n-6 (LA)	0.7±0.0^{d}	0.3±0.0^{a}	0.4±0.0^{b}	0.5±0.0^{bc}	0.5±0.0^{c}	0.5±0.0^{c}	0.6±0.0^{c}
18:3 n-6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:3 n-6	0.3±0.0^{bc}	0.3±0.1^{a}	0.3±0.0^{b}	0.3±0.0^{bcd}	0.3±0.0^{bcd}	0.3±0.0^{cd}	0.4±0.0^{d}
20:4 n-6 (AA)	11.6±0.4^{d}	10.7±0.5^{cd}	9.6±0.2^{bc}	8.9±0.2^{ab}	8.6±0.2^{ab}	8.7±0.1^{ab}	8.2±0.0^{a}
22:4 n-6	3.1±0.1^{b}	3.1±0.0^{b}	2.9±0.1^{b}	2.5±0.1^{a}	2.3±0.1^{a}	2.4±0.1^{a}	2.3±0.2^{a}
22:5 n-6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total n-6	15.9±0.5^{d}	14.3±0.5^{c}	13.3±0.3^{bc}	12.3±0.3^{ab}	11.8±0.2^{a}	12.1±0.2^{ab}	11.5±0.1^{a}
18:3 n-3 (ALA)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18:4 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:3 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:5 n-3 (EPA)	< 0.05^{c}	< 0.05^{a}	< 0.05^{ab}	< 0.05^{bc}	< 0.05^{d}	< 0.05^{e}	0.1±0.0^{f}
22:5 n-3 (DPA)	0.2±0.0^{c}	0.1±0.0^{a}	0.1±0.0^{ab}	0.2±0.0^{bc}	0.2±0.0^{d}	0.3±0.0^{e}	0.4±0.0^{f}
22:6 n-3 (DHA)	14.7±0.4^{abc}	13.2±0.5^{a}	14.0±0.3^{ab}	15.6±0.2^{cd}	16.3±0.3^{d}	16.1±0.3^{cd}	15.5±0.3^{bcd}
Total n-3	14.8±0.4^{bc}	13.3±0.5^{a}	14.2±0.3^{ab}	15.8±0.2^{cd}	16.5±0.3^{d}	16.5±0.3^{d}	16.0±0.3^{cd}

[1]High PUFA.
[2]Low PUFA.
[3]Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer multiple comparisons test. Data are means ± SEM of n=5 per group.

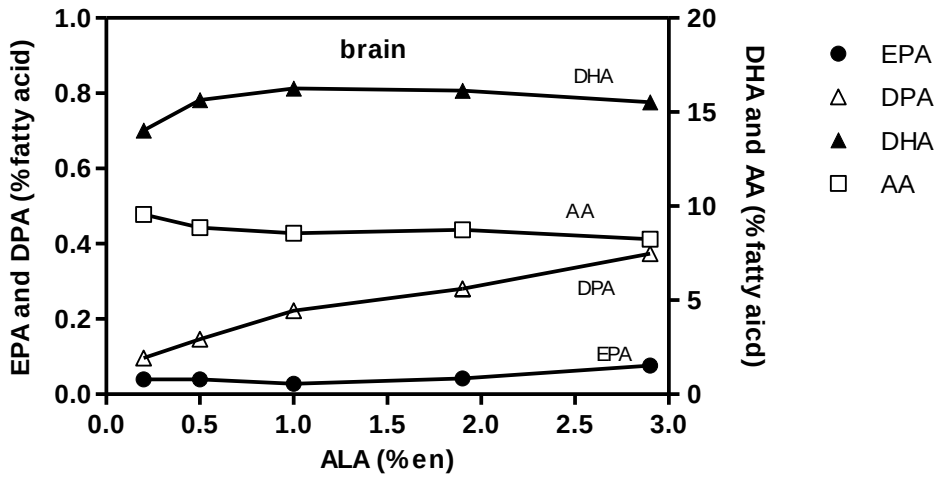


Figure 3. 7 Proportions of phospholipid EPA, DPA, DHA and AA in brain of rats fed a range of diets with varying ALA levels ranging from 0.2-2.9%en. Data points are means ± SEM, n=5 per group.

Table 3. 8 Fatty acid composition in heart

Heart phospholipid	**Reference**		**Experimental**				
Diets	**H-PUFA**[1]	**L-PUFA**[2]	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
ALA%en	0.6	0.03	0.2	0.5	1	1.9	2.9
LA%en	6	0.4	1	1	1	1	1
LA:ALA Ratio	11	14	7.4	2.4	1.2	0.6	0.4
Fatty acids[3]							
Total saturates	40.5±0.3^{b}	38.4±0.7^{ab}	37.2±0.4^{a}	36.8±0.3^{a}	36.6±0.6^{a}	37.0±0.6^{a}	38.6±0.5^{ab}
18:1 n-9	4.0±0.2^{a}	11.5±0.25^{f}	9.1±0.2^{e}	8.9±0.3^{de}	8.3±0.1^{cd}	7.6±0.2^{bc}	6.9±0.3^{b}
18:1 n-7	3.8±0.1^{a}	6.0±0.1^{d}	5.5±0.0^{c}	5.3±0.0^{c}	5.2±0.1^{c}	4.6±0.0^{b}	4.3±0.1^{b}
Total monoenes	8.2±0.4^{a}	19.6±0.2^{e}	16.3±0.2^{d}	15.8±0.3^{cd}	14.9±0.1^{c}	13.4±0.2^{b}	12.4±0.3^{b}
18:2 n-6 (LA)	19.0±0.7^{c}	10.4±0.4^{a}	14.8±0.5^{b}	16.8±0.7^{bc}	16.9±0.5^{bc}	17.5±0.3^{bc}	16.7±1.0^{bc}
18:3 n-6	< 0.05^{ab}	< 0.05^{b}	< 0.05^{b}	< 0.05^{b}	< 0.05^{a}	< 0.05^{a}	< 0.05^{a}
20:3 n-6	0.3±0.0^{a}	0.6±0.0^{bc}	0.6±0.0^{bc}	0.6±0.0^{c}	0.6±0.0^{bc}	0.6±0.0^{bc}	0.5±0.0^{b}
20:4 n-6 (AA)	21.7±0.5^{d}	24.5±0.3^{e}	23.5±0.7^{de}	19.4±0.4^{c}	18.0±0.4^{bc}	17.1±0.3^{ab}	15.9±0.6^{a}
22:4 n-6	1.4±0.0^{e}	1.1±0.1^{d}	0.8±0.0^{c}	0.6±0.0^{b}	0.4±0.0^{ab}	0.3±0.0^{a}	0.3±0.0^{a}
22:5 n-6	< 0.05^{a}	N.D.	N.D.	N.D.	< 0.05^{a}	< 0.05^{a}	< 0.05^{a}
Total n-6	42.8±0.4^{a}	36.7±0.6^{a}	39.7±0.3^{a}	37.4±0.4^{b}	36.0±0.5^{c}	35.7±0.2^{d}	33.4±0.5^{e}
18:3 n-3 (ALA)	< 0.05^{a}	N.D.	N.D.	0.1±0.0^{b}	0.3±0.0^{c}	0.5±0.0^{d}	0.7±0.0^{e}
18:4 n-3	N.D.	< 0.05^{b}	< 0.05^{a}	N.D.	N.D.	N.D.	N.D.
20:3 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.1±0.0
20:5 n-3 (EPA)	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{ab}	0.4±0.0^{b}	0.87±0.0^{c}	1.3±0.1^{d}	1.6±0.2^{e}
22:5 n-3 (DPA)	1.2±0.1^{c}	0.6±0.0^{a}	0.6±0.0^{a}	1.0±0.0^{b}	1.6±0.0^{d}	2.2±0.1^{e}	2.7±0.1^{f}
22:6 n-3 (DHA)	6.9±0.4^{bc}	4.4±0.2^{a}	5.8±0.3^{ab}	8.3±0.4^{cd}	9.7±0.2^{de}	9.9±0.5^{de}	10.3±0.5^{e}
Total n-3	8.2±0.4^{a}	5.1±0.2^{bc}	6.6±0.3^{ab}	9.8±0.4^{c}	12.3±0.3^{d}	13.9±0.6^{de}	15.5±0.4^{e}

[1]High PUFA.
[2]Low PUFA.
[3]Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer multiple comparisons test. Data are means ± SEM of n=5 per group.

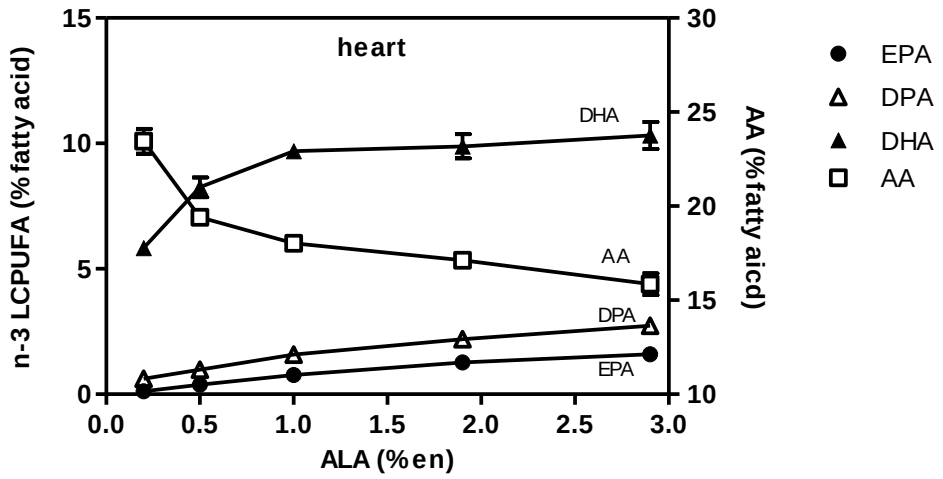


Figure 3. 8 Proportions of phospholipid EPA, DPA, DHA and AA in heart of rats fed a range of diets with varying ALA levels ranging from 0.2-2.9%en. Data points are means ± SEM, n=5 per group.

Table 3. 9 Fatty acid composition in thigh muscle

Thigh phospholipid	**Reference**		**Experimental**				
Diets	**H-PUFA**[1]	**L-PUFA**[2]	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**
ALA%en	0.6	0.03	0.2	0.5	1	1.9	2.9
LA%en	6	0.4	1	1	1	1	1
LA:ALA Ratio	11	14	7.4	2.4	1.2	0.6	0.4
Fatty acids[3]							
Total saturates	38.7±0.4	37.7±0.5	38.6±0.3	37.9±0.1	38.1±0.4	38.1±0.4	38.7±0.4
18:1 n-9	5.0±0.2^{a}	15.6±0.4^{e}	13.7±0.2^{d}	13.3±0.4^{d}	11.1±0.3^{c}	10.2±0.1^{bc}	9.4±0.3^{b}
18:1 n-7	3.1±0.1^{a}	4.1±0.1^{bc}	3.8±0.1^{b}	3.8±0.1^{b}	4.2±0.1^{c}	3.9±0.1^{bc}	3.8±0.0^{b}
Total monoenes	9.6±0.2^{a}	23.9±0.4^{e}	21.4±0.2^{d}	20.9±0.5^{d}	18.7±0.4^{c}	17.2±0.1^{bc}	16.1±0.5^{b}
18:2 n-6 (LA)	20.4±0.4^{c}	13.0±0.5^{a}	16.4±0.3^{b}	17.1±0.2^{b}	17.0±0.2^{b}	16.9±0.3^{b}	16.4±0.3^{b}
18:3 n-6	0.1±0.0^{ab}	0.1±0.0^{c}	0.1±0.0^{c}	0.1±0.0^{b}	0.1±0.0^{ab}	0.1±0.0^{a}	0.1±0.0^{a}
20:3 n-6	0.9±0.0^{bc}	0.9±0.0^{c}	0.9±0.0^{c}	0.9±0.0^{bc}	0.8±0.0^{bc}	0.8±0.0^{ab}	0.7±0.0^{a}
20:4 n-6 (AA)	16.7±0.4^{e}	13.9±0.4^{d}	13.2±0.3^{d}	10.9±0.4^{c}	10.3±0.3^{bc}	9.1±0.3^{ab}	8.0±0.3^{a}
22:4 n-6	1.8±0.1^{e}	1.5±0.1^{d}	1.1±0.0^{c}	0.8±0.0^{b}	0.6±0.0^{ab}	0.59±0.0^{a}	0.4±0.0^{a}
22:5 n-6	1.6±0.0^{c}	1.7±0.1^{c}	1.1±0.1^{b}	0.5±0.0^{a}	0.4±0.0^{a}	0.4±0.0^{a}	0.3±0.0^{a}
Total n-6	41.8±0.4^{e}	31.3±1.0^{cd}	32.9±0.3^{d}	30.3±0.3^{c}	29.3±0.5^{bc}	27.8±0.3^{ab}	26.1±0.2^{a}
18:3 n-3 (ALA)	0.2±0.0^{a}	< 0.05^{a}	0.2±0.0^{a}	0.4±0.0^{b}	0.7±0.0^{c}	1.1±0.0^{d}	1.5±0.1^{e}
18:4 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:3 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	0.1±0.0^{a}	0.1±0.0^{b}
20:5 n-3 (EPA)	0.1±0.0^{a}	0.1±0.0^{a}	0.2±0.0^{a}	0.8±0.0^{b}	1.6±0.1^{c}	2.4±0.1^{d}	3.0±0.3^{e}
22:5 n-3 (DPA)	2.0±0.1^{c}	1.1±0.1^{a}	1.1±0.0^{a}	1.6±0.0^{b}	2.5±0.1^{d}	3.5±0.0^{e}	4.4±0.1^{f}
22:6 n-3 (DHA)	7.4±0.2^{b}	5.5±0.1^{a}	5.2±0.2^{a}	7.7±0.3^{bc}	8.7±0.3^{cd}	9.5±0.3^{d}	9.9±0.4^{d}
Total n-3	9.7±0.2^{b}	6.8±0.2^{a}	6.7±0.2^{a}	10.6±0.3^{b}	13.6±0.3^{c}	16.7±0.3^{d}	19.0±0.4^{e}

[1]High PUFA.
[2]Low PUFA.
[3]Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer multiple comparisons test. Data are means ± SEM of n=5 per group.

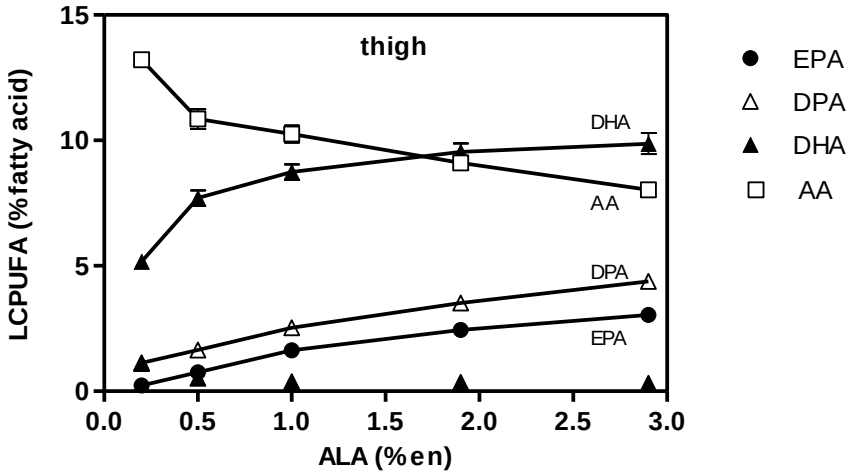


Figure 3. 9 Proportions of phospholipid EPA, DPA, DHA and AA in thigh of rats fed a range of diets with varying ALA levels ranging from 0.2-2.9%en. Data points are means ± SEM, n=5 per group.

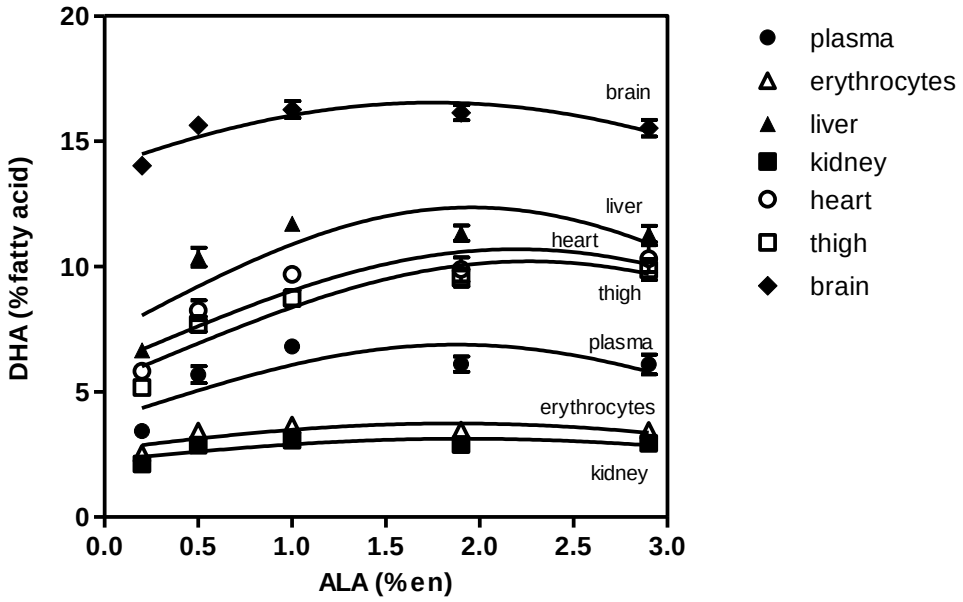


Figure 3. 10 Proportions of phospholipid DHA in plasma, erythrocyte, liver, kidney, heart, thigh muscle and brain of rats fed a range of diets with varying ALA levels ranging from 0.2-2.9%en. Data points are means ± SEM, n=5 per group.

3.6.4 Relationship between plasma and tissue phospholipid n-3 LCPUFA

To evaluate whether plasma and erythrocyte n-3 LCPUFA were valid markers to estimate tissue n-3 LCPUFA level, simple linear regression was performed to evaluate the strength of relationships between values for phospholipid EPA, DPA and DHA fatty acid content in plasma and in peripheral tissues.

There were strong correlations between blood (plasma and erythrocyte) n-3 LCPUFA and liver n-3 LCPUFA (R^2=0.81-0.99, P<0.0001) (Figure 3.11). Similar strong and positive correlations were also present between blood phospholipid EPA and DPA and the content of these same fatty acids in kidney, heart and thigh muscle phospholipid (R^2=0.93-0.99, P<0.0001) (Figure 3.12, 3.13 and 3.15 A-D). Whilst there was a significant relationship between DHA content in blood and other tissues, this was not as strong as relationships observed for the other fatty acids (kidney, R^2=0.70-0.79, P<0.0001, heart R^2=0.64-0.79, P<0.0001) and thigh (R^2=0.57-0.72, P<0.0001) (Figure 3.12, 3.13 and 3.15 E and F).

The correlation between blood phospholipid DPA and brain phospholipid DPA was strong (R^2=0.90-0.91, P<0.0001) (Figure 3.14 C and D), however there was only a weak correlation between the proportion of blood phospholipid DHA and the DHA in brain (R^2=0.43-0.57, P=0.0004) (Figure 3.14 E and F) and an even weaker relationship between blood phospholipid EPA and brain phospholipid EPA (R^2=0.27-0.28, P<0.01) (Figure 3.14 A and B). A strong correlation for the total n-3 LCPUFA levels in phospholipids between plasma and erythrocytes was also identified (R^2=0.80-0.97, P<0.0001) (Figure 3.16 A-C).

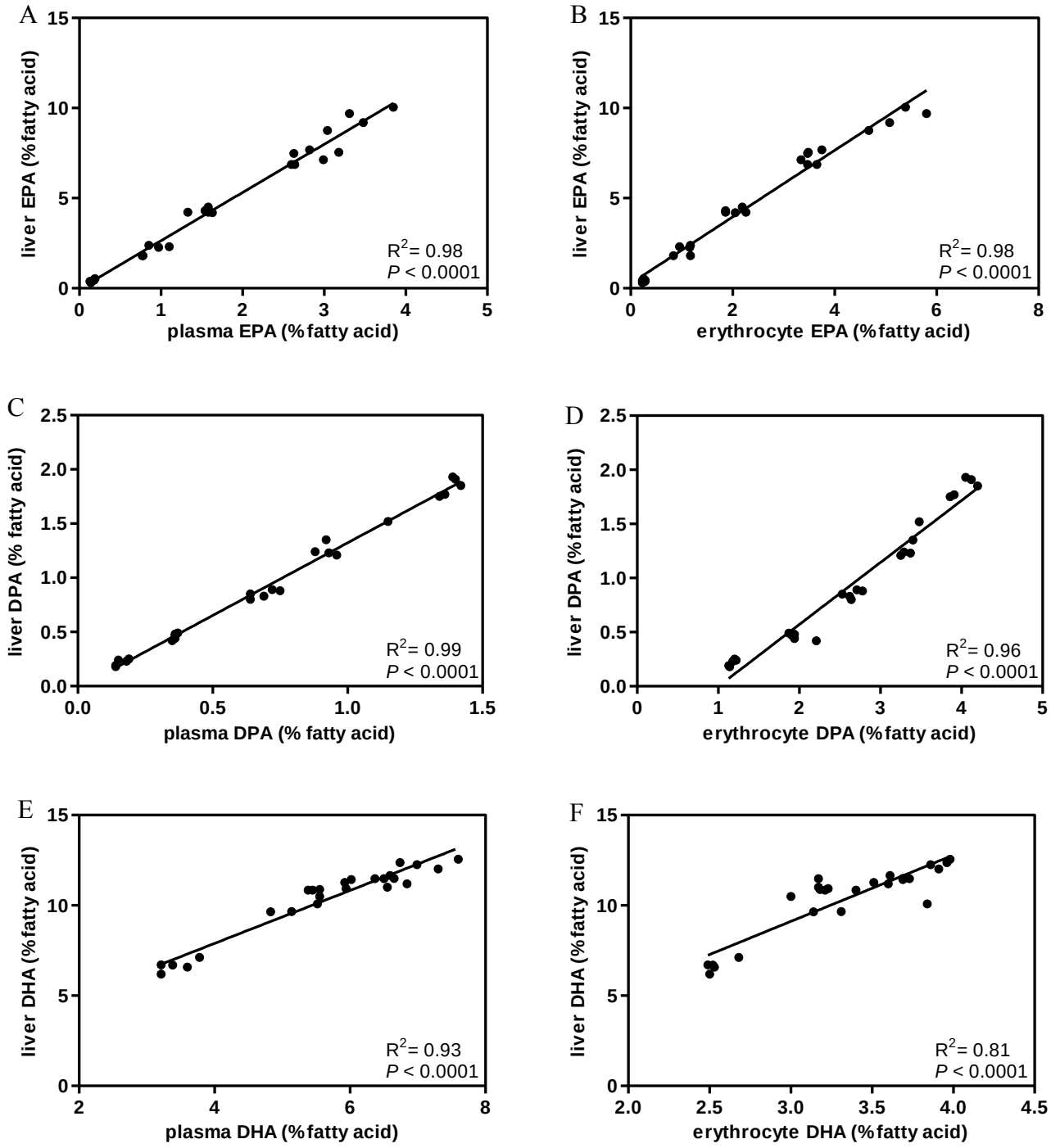


Figure 3. 11 Correlation between plasma and liver phospholipid EPA (A), DPA (C), DHA (E). The correlation between erythrocyte and liver phospholipid EPA (B), DPA (D), DHA (F) are shown on right panel.

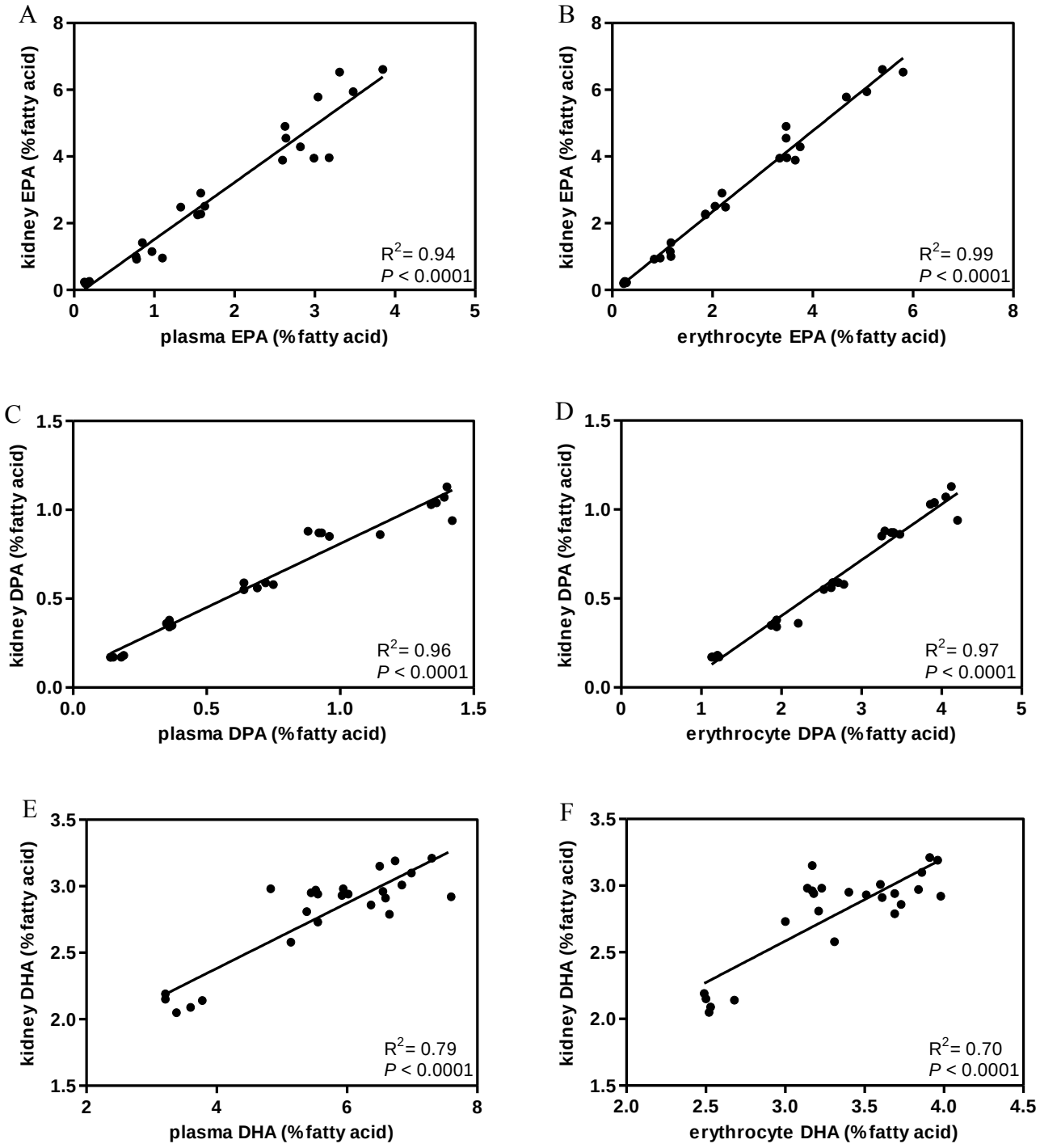


Figure 3. 12 Correlation between plasma and kidney phospholipid EPA (A), DPA (C), DHA (E). The correlation between erythrocyte and kidney phospholipid EPA (B), DPA (D), DHA (F) are shown on right panel.

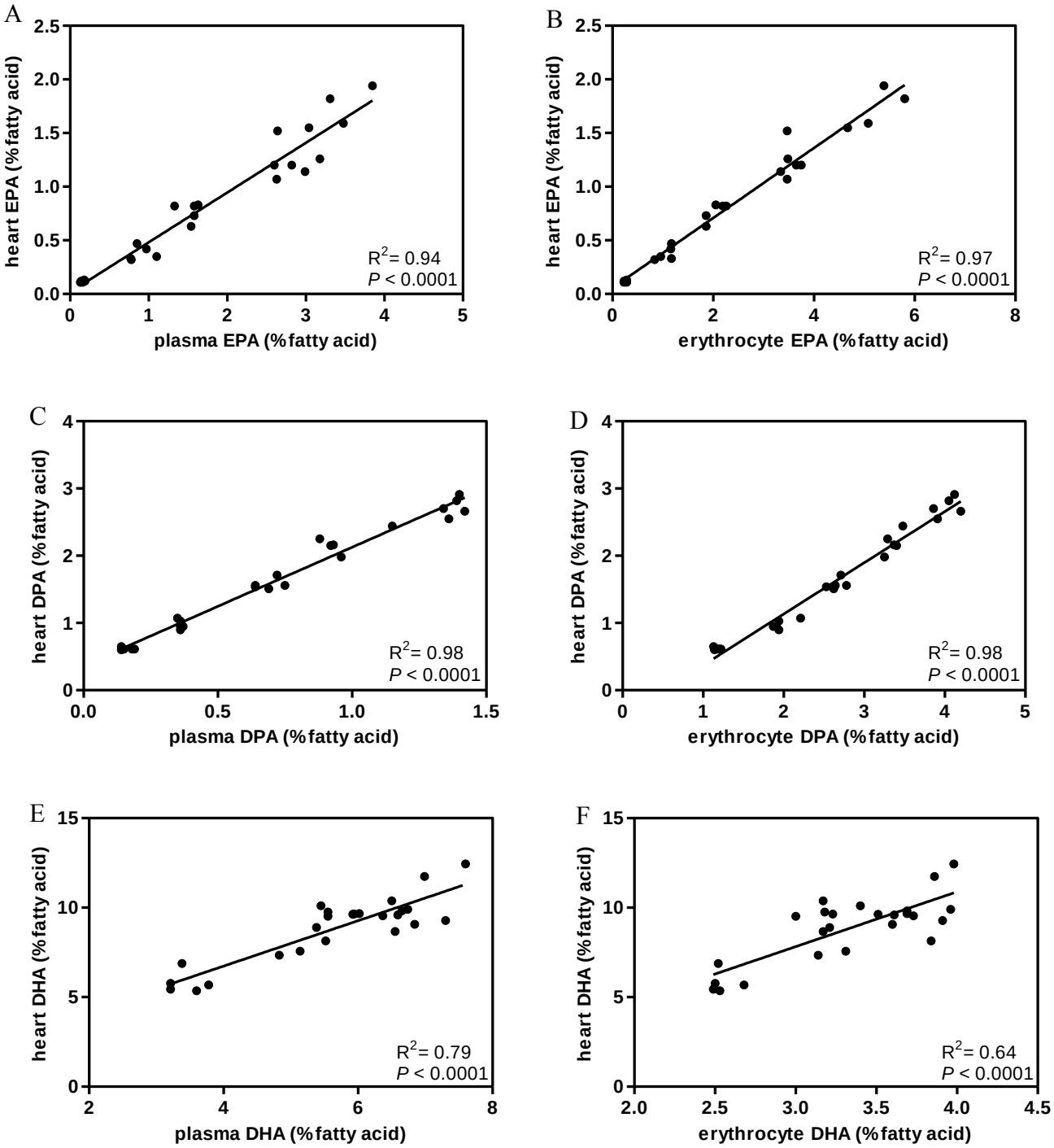


Figure 3. 13 Correlation between plasma and heart phospholipid EPA (A), DPA (C), DHA (E). The correlation between erythrocyte and heart phospholipid EPA (B), DPA (D), DHA (F) are shown on right panel.

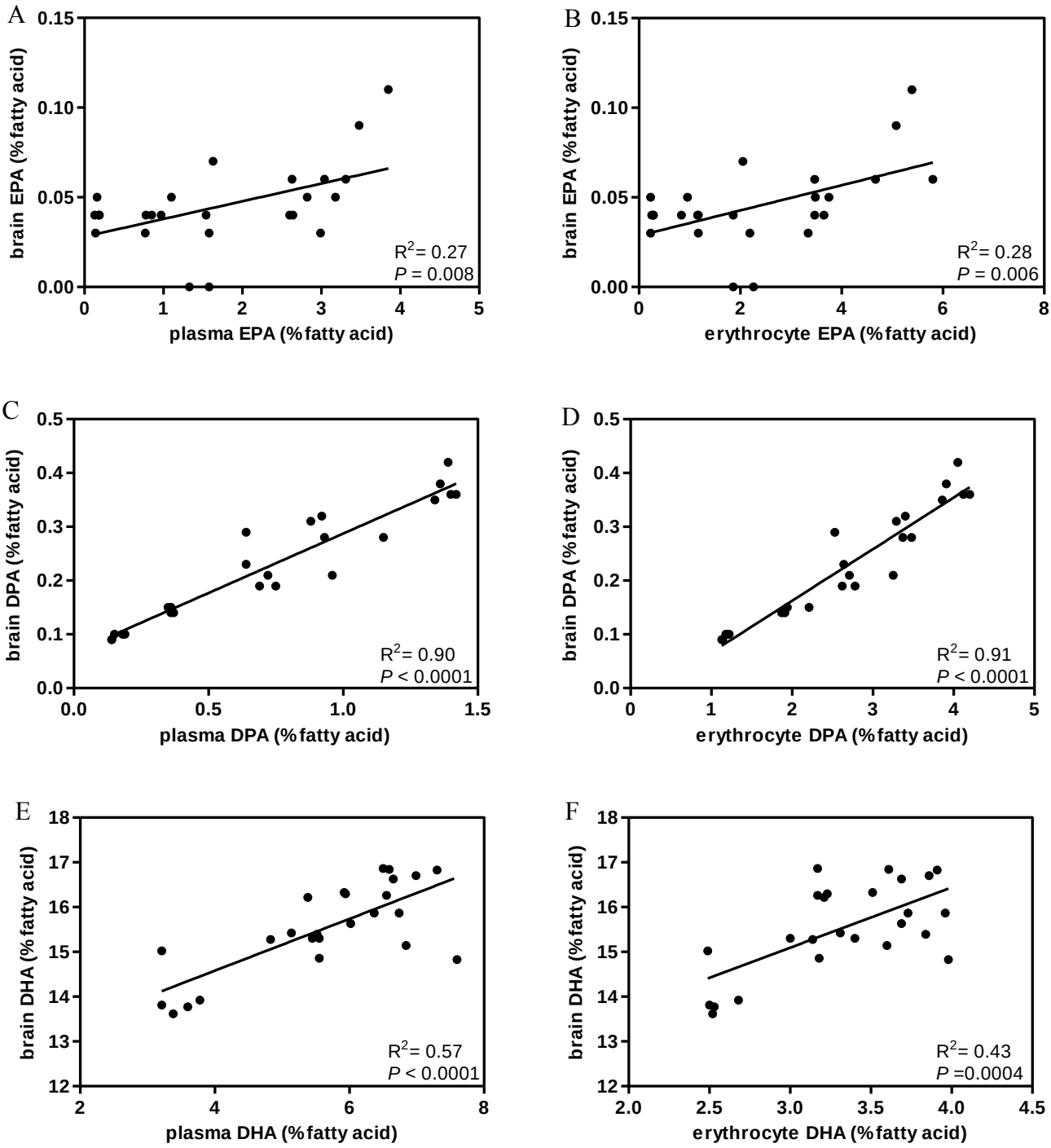


Figure 3. 14 Correlation between plasma and brain phospholipid EPA (A), DPA (C), DHA (E). The correlation between erythrocyte and brain phospholipid EPA (B), DPA (D), DHA (F) are shown on right panel.

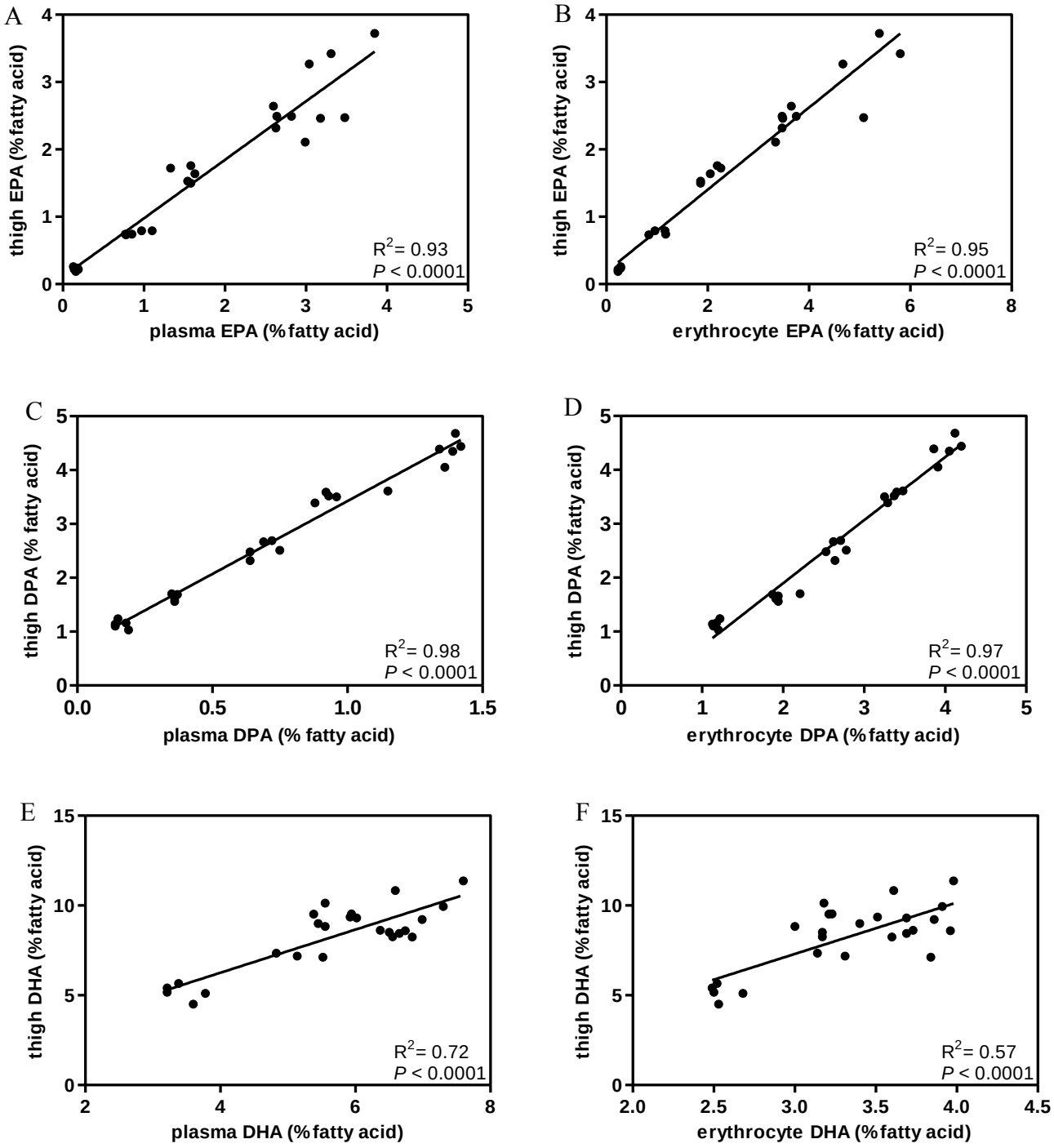


Figure 3. 15 Correlation between plasma and thigh muscle phospholipid EPA (A), DPA (C), DHA (E). The correlation between erythrocyte and thigh muscle phospholipid EPA (B), DPA (D), DHA (F) are shown on right panel.

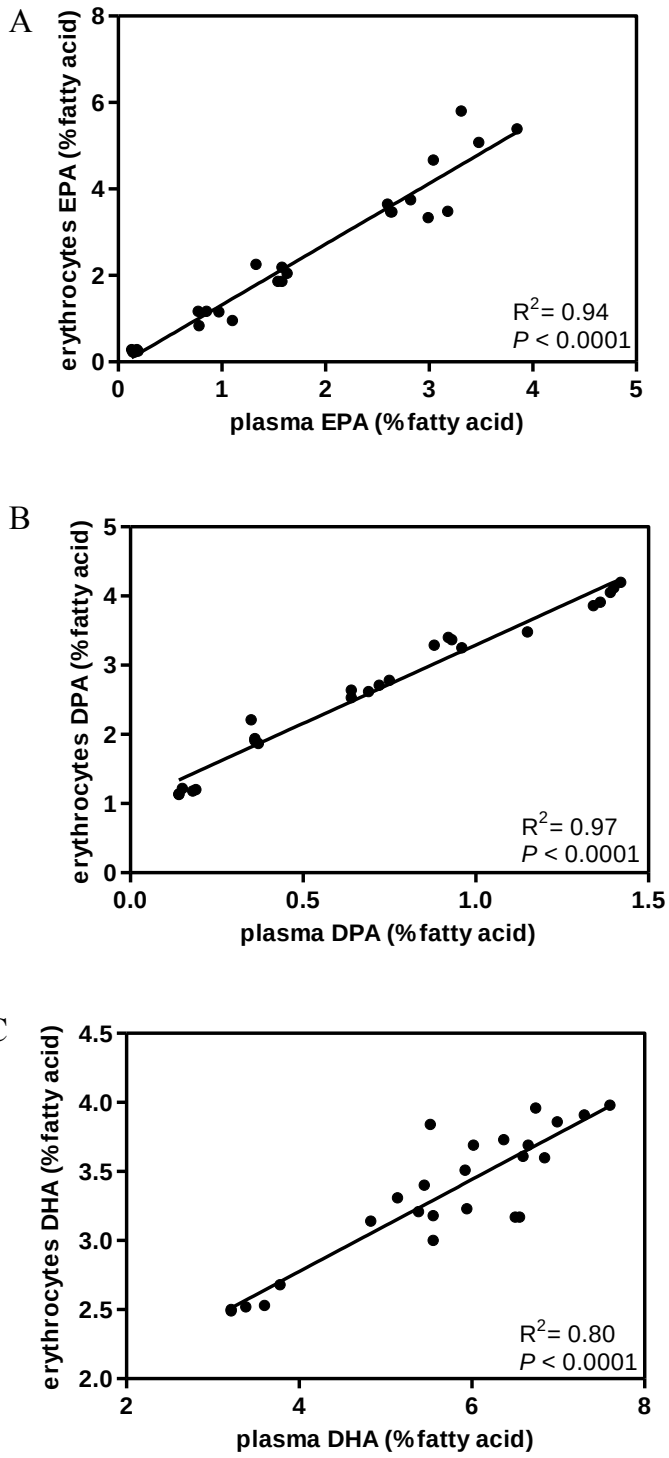


Figure 3. 16 Correlation between plasma and erythrocyte phospholipid EPA (A), DPA (B), DHA (C).

3.6.5 The effect of dietary ALA content on hepatic mRNA expression of PUFA pathway genes

The effect of dietary ALA content on the mRNA expression of the four key enzymes in the LCPUFA synthetic pathway, Δ5 desaturase (FADS1), Δ6 desaturase (FADS2), elongase 2 (ELOVL2) and elongase 5 (ELOVL5) is shown in Figure 3.17 to 3.19. In the low PUFA reference diet (total PUFA 0.4%en) fed rats, the relative gene expression level of FADS2 was 2.3 fold and ELOVL2 was 1.8 fold higher than in the high PUFA reference diet (6.6%en) group (Figure 3.17 A). However, the mRNA expression of FADS1 and ELOVL5 was not different between the two reference groups. For the experimental diets, none of the fatty acid genes (FADS1, FADS2, ELOVL2 and ELOVL5) exhibited differential expression at differing levels of dietary ALA from 0.2 to 2.9%en (Figure 3.18 and 3.19).

3.6.6 The effect of dietary ALA content on hepatic mRNA expression of PPARα and SREBP-1c

No significant difference was found between high PUFA and low PUFA reference diets in mRNA levels of the transcription factors PPARα and SREBP-1c (Figure 3.17 B). The relationship between dietary ALA levels and mRNA abundance of the transcription factors PPARα and SREBP-1c is shown in Figure 3.20. There was no difference in the mRNA expression of SREBP-1c and PPARα between rats fed diets containing ALA over the range from 0.2 to 2.9%en.

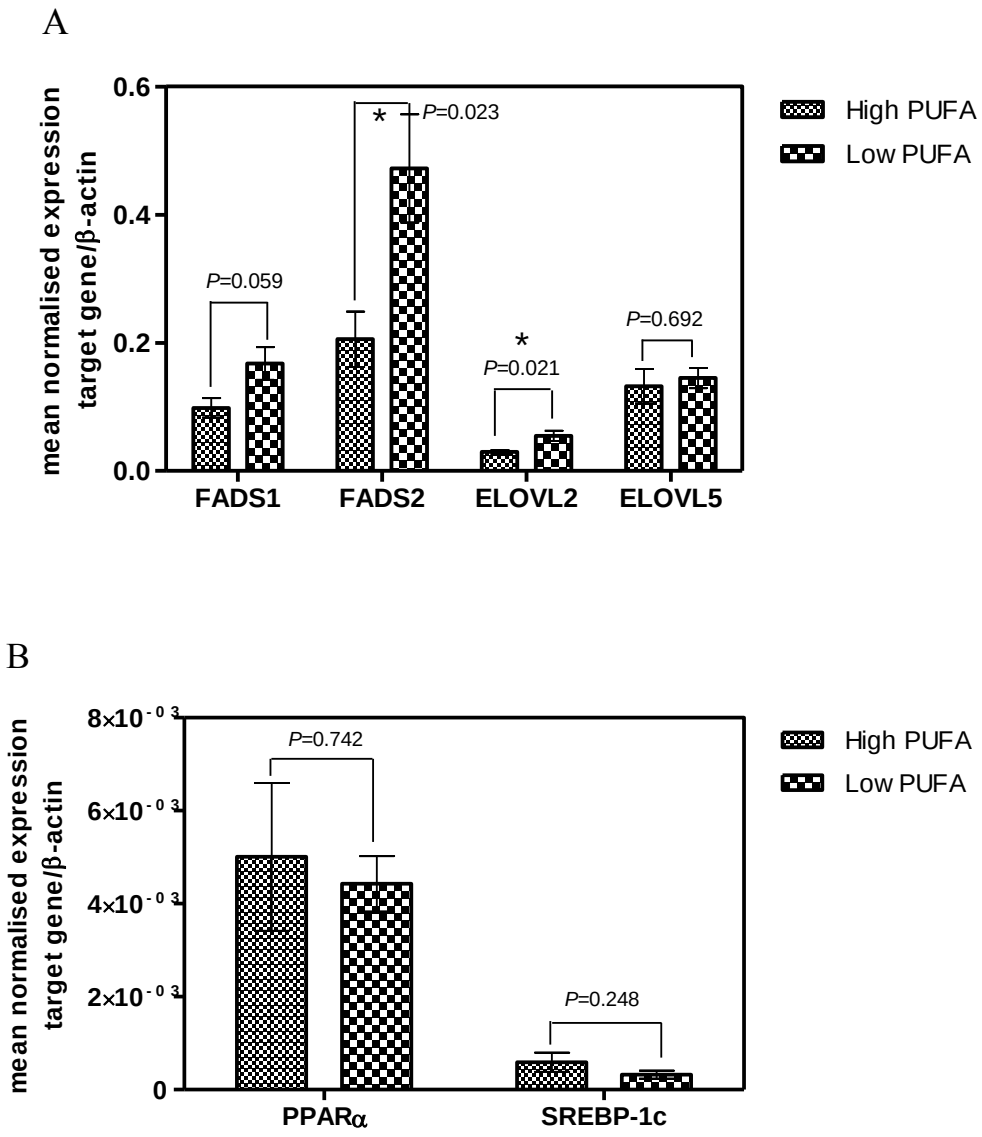


Figure 3. 17 The relative gene expression of hepatic Δ5 desaturase (FADS1), Δ6 desaturase (FADS2), elongase 2 (ELOVL2), elongase 5 (ELOVL5) (A) and PPARα and SREBP-1c (B) in rats fed with low PUFA (0.4%en as PUFA) and high PUFA (6.6%en as PUFA) diets. The expression of FADS1, FADS2, ELOVL2, ELOL5, PPARα and SREBP-1c mRNA was normalised to the housekeeping gene β-Actin. Abundance of mRNA is expressed as the mean normalised expression value. Asterisks indicate values that are significantly different between the high PUFA and low PUFA diets ($P<0.05$). Data are mean ± SEM, n=5 per group.

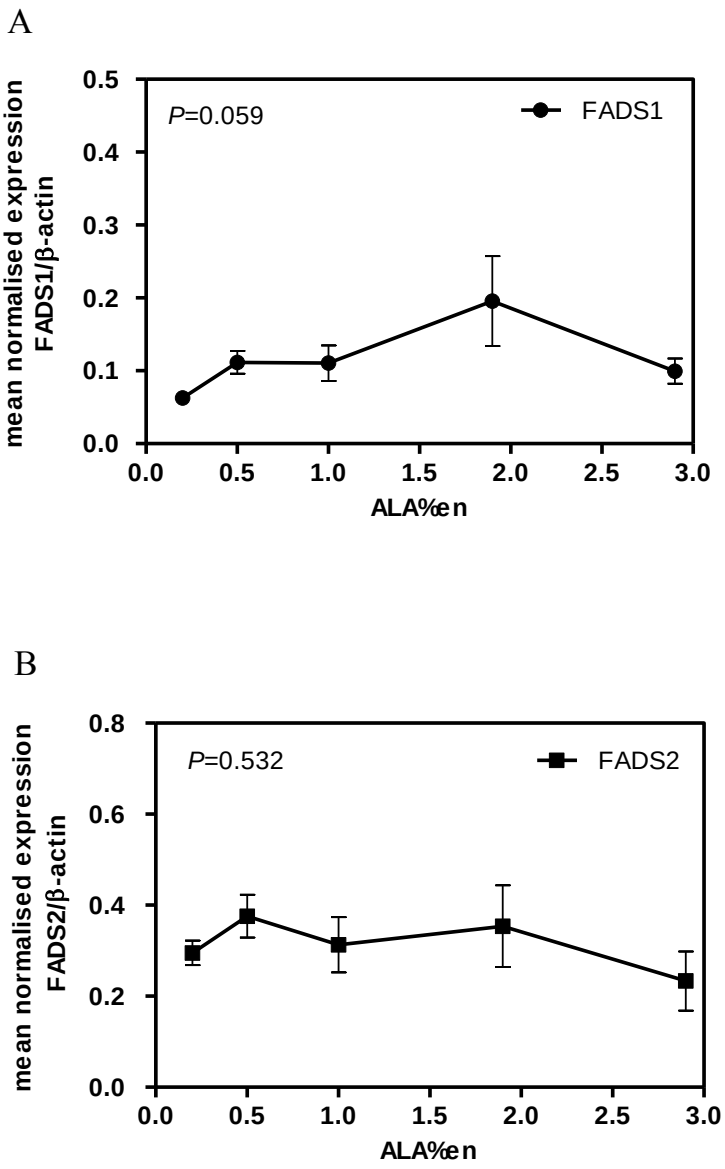


Figure 3. 18 The relative gene expression of FADS1 (A) and FADS2 (B) in rats fed diets containing various levels of ALA. The expression of FADS1 and FADS2 mRNA was normalised to the housekeeping gene β-Actin. Abundance of mRNA is expressed as the mean normalised expression value. Data are mean ± SEM, n=5 per group.

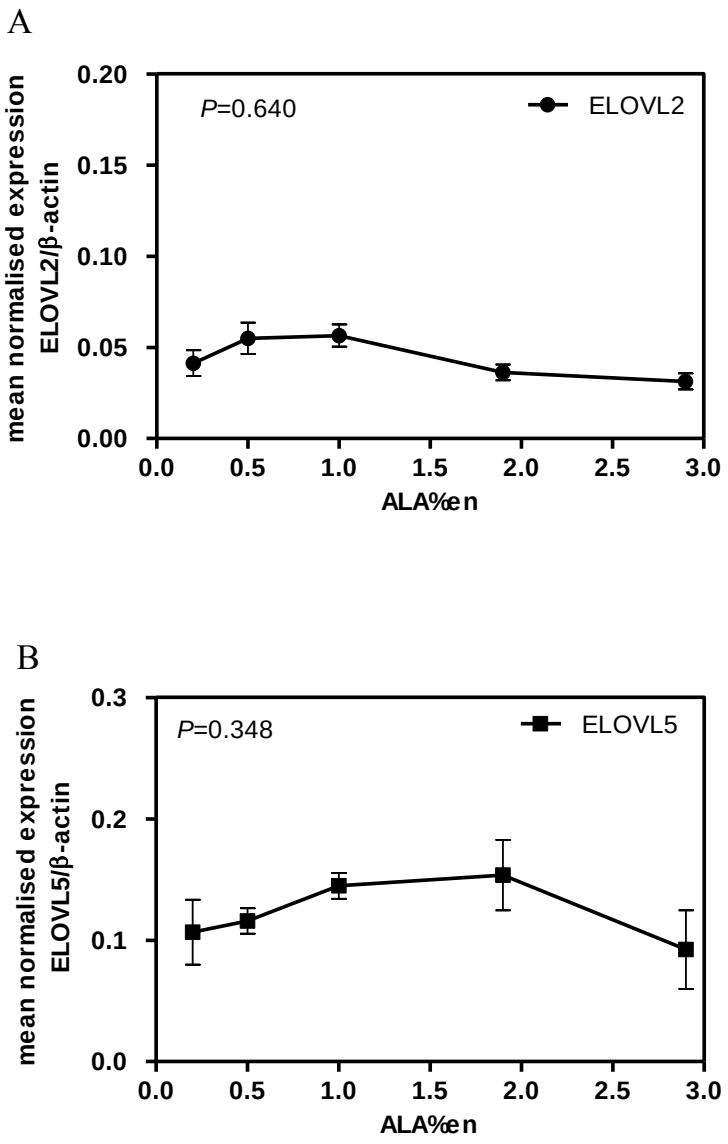


Figure 3. 19 The relative gene expression of ELOVL2 (A) and ELOVL5 (B) in rats fed diets containing various levels of ALA. The expression of ELOVL2 and ELOVL5 mRNA was normalised to the housekeeping gene β-Actin. Abundance of mRNA is expressed as the mean normalised expression value. Data are mean ± SEM, n=5 per group.

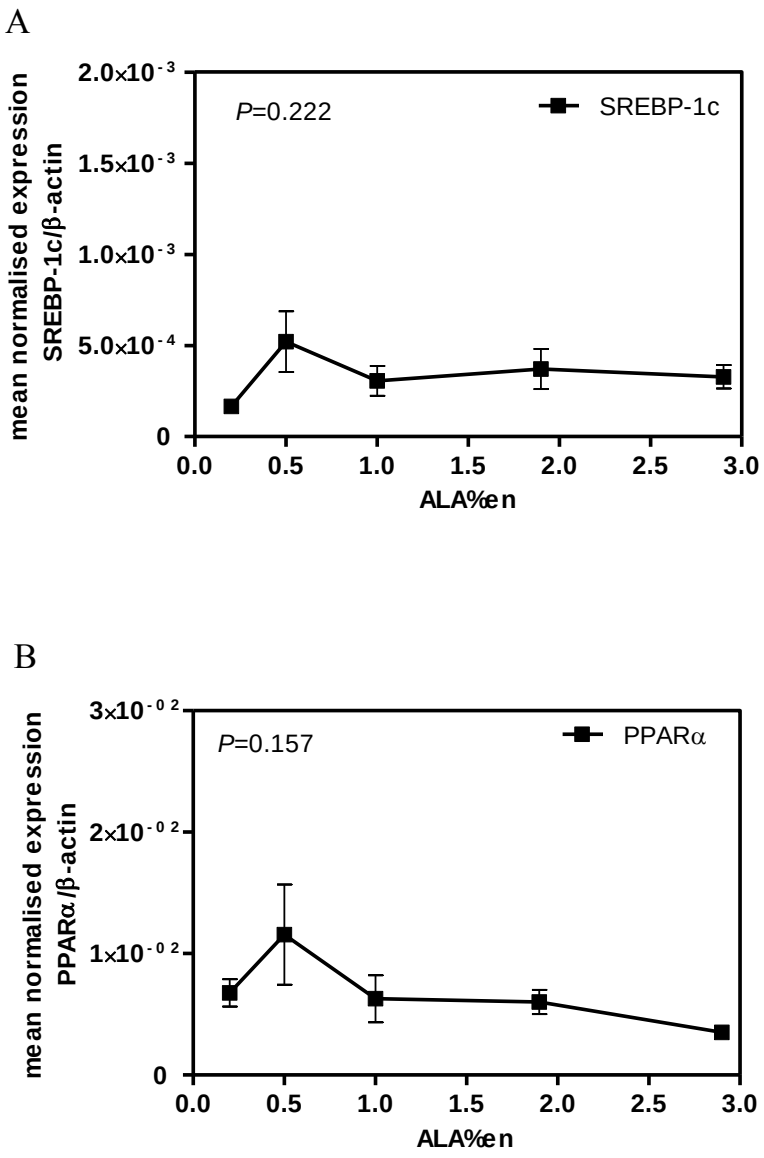


Figure 3. 20 The relative gene expression of transcription factors SREBP-1c (A) and PPARα (B) in livers of rats fed diets containing various levels of ALA. The expression of SREBP-1c and PPARα mRNA was normalised to the housekeeping gene β-Actin. Abundance of mRNA is expressed as the mean normalised expression value. Data are mean ± SEM, n=5 per group.

3.7 Discussion

This study was designed to test the effect of increasing the level of dietary ALA over a range that could conceivably be incorporated into human diets in order to enhance n-3 LCPUFA status (74). One of the most important findings of this work is the observation that the different n-3 LCPUFA (EPA, DPA, DHA) exhibited different responses to an increase in dietary ALA content, and that this varied between the phospholipids of different tissues. Specifically, the content of EPA and DPA in blood and most tissues increased linearly with increasing dietary ALA content, whereas DHA level did not. Increasing the ALA level from 0.2 to 1%en resulted in an increase in the proportion of DHA in all tissues examined, however further increases in dietary ALA%en content did not result in further increases, and in some cases actually resulted in decreases in the DHA content of the phospholipid at the same time as the proportion of other n-3 LCPUFA (20:3 n-3, EPA and DPA) increased continuously. These findings are consistent with the results of a similar dietary study in piglets (4), in which the authors found a linear response of tissue phospholipid content of EPA and DPA to increases in dietary ALA content, but at the same time found a curvilinear response of tissue DHA content to the increased ALA intake. In the studies reported in this chapter, the total dietary PUFA (n-3 and n-6 PUFA) content varied from ~11 to 35% of the total fatty acids. Thus, it could be that some of the results here might be explained in terms of total PUFA rather than ALA level only. Moreover, although the dietary LA levels were kept constant, the blood and tissue LA levels were increased continuously with increasing ALA intakes. The reason for this elevation is unclear but might be due to that LA competes with ALA for intestinal absorption (235) or reduced conversion of LA to AA because of the increasing proportion of ALA intake.

The second important observation in this work is the strong correlation between blood phospholipid n-3 LCPUFA levels, particularly levels of EPA and DPA, and the level of these same fatty acids in peripheral tissues, which indicates that blood fatty acid profile can be used to reliably represent tissue fatty acid compositions.

The third important finding of this study is the fact that the gene expression levels of key enzymes in the LCPUFA synthetic pathway did not appear to be related to dietary ALA levels in the range that could reasonably be expected to be present in normal animal and human diets. In agreement with previous studies, very low PUFA diets (0.4%en) resulted in increased expression of FADS2 and ELOVL2 genes relative to higher PUFA (6.6%en) diets. The

degree of change in FADS2 gene expression was similar to the changes seen in the report of Tang *et. al.* (233) who compared fat free diets with safflower based diet and the report of Li *et. al.* (236) who demonstrated clear inhibition of Δ6 desaturase gene expression in rodents fed high PUFA diets compared with those fed very low PUFA diets. It has been recommended that for the rats, the ALA and LA intake in rats should make up 0.4% and 2.4%en, respectively, for the maximal production of DHA in brain cells because beyond this point the brain DHA level reached plateau (12). It has also been suggested that intake of LA (~1%en) and ALA (~0.2%en) from diets are adequate to prevent adverse effects on growth and development in infants (13). In the present study, the range of dietary ALA levels tested, in conjunction with a sufficient amount of LA to avoid EFA deficiency (1%en) (237, 238), resulted in the expected alterations in the level of the n-3 LCPUFA in plasma and tissue phospholipids. These findings are consistent with previous reports by others (239, 240) that PUFA in the diet directly influence the fatty acid profile of plasma and liver phospholipid in rats. In our study, tissue and plasma fatty acid profiles indicated that there was endogenous conversion of dietary ALA into EPA, DPA and DHA. This is based on the fact that the animals were placed on the experimental diets immediately after weaning, and the body weight of animals increased from an average of 38 to 135 g during the experimental period in all dietary groups. Thus, any LCPUFA in the cells of these animals must have come either from pre-formed LCPUFA already deposited in tissues at the end of weaning or from endogenous synthesis. Given that the LCPUFA content of tissues was altered by the dietary treatments, and that there was a direct relationship between dietary ALA and the level of n-3 LCPUFA in cells, it appears most likely that endogenous conversion of ALA to LCPUFA is responsible for the observed changes in tissue LCPUFA content.

Although the relationship between dietary ALA content and the amount of product which accumulated in tissue phospholipids was essentially linear for EPA and DPA, for DHA there was a curvilinear relationship with a maximum at 1%en dietary ALA (2%en PUFA) , after which DHA levels plateaued or declined (Figures 3.3-3.9). A similar result has previously been reported in piglets but in that case the optimum level of DHA accumulation in a variety of tissues was when the LA:ALA ratio was 2:1 (4) rather than at the 1:1 ratio reported here. The discrepancy could be due to the fact that piglets were fed diets higher in LA (~7%en), or due to differences in fatty acid metabolism in pigs compared to rodents. In an *in vitro* study, Portolesi *et. al.* reported a limited accumulation of 24:6 n-3 and DHA in response to increasing concentrations of supplemented ALA, suggesting that competition between 24:5 n-

3 and ALA for Δ6 desaturase may limit DHA synthesis, and subsequent accumulation in cell membranes, in the presence of high dietary ALA (53). Therefore it is probable that the nonlinear relationship between dietary ALA and tissue DHA was because of competition between substrates for enzymes and, potentially, inhibition of parts of the LCPUFA biosynthetic pathway or accumulation in response to increases in the level of LCPUFA in the tissue.

Despite the clear effect of the dietary interventions on LCPUFA content of plasma and tissues, these same diets resulted in little or no change in the expression of the key genes FADS1, FADS2, ELOVL2 or ELOVL5 in the fatty acid pathway (Figure 3.18 and 3.19). Furthermore, I was unable to find any relationship between the mRNA expression of these genes and the increase in levels of EPA and DPA, or the curvilinear pattern of DHA in plasma, liver and other tissue phospholipids induced by increasing dietary ALA%en.

Similar to the gene expression results in desaturase and elongase enzymes, the mRNA levels of transcription factors PPARα and SREBP-1c within the liver had no direct correlation to either the dietary ALA level or the levels of any of the n-3 LCPUFA in tissues. Our results contrast with the increasing evidence for the involvement of transcription factors in the regulation of fatty acid synthesis (241-245). There have been previous reports that the expression of mRNA for the Δ5 and Δ6 desaturase in mouse liver were regulated by SREBP-1c and PPARα during fasting and refeeding treatments (96), that these two transcription factors are involved in the regulation of Δ6 desaturase gene and that the action of these transcription factors is necessary for the induction of LCPUFA synthesis (236). The expression level of both SREBP-1 and PPARα in the rodent liver has also been shown to be altered by fish oil feeding (246). However, in this study, where the dietary treatments did not induce changes in fat level and only minor changes in fatty acid concentrations, the results suggest that the production of LCPUFA in plasma and liver was due more to the increased availability of ALA, rather than any induction of gene expression of genes in the fatty acid pathway. Whilst PPARα and SREBP-1c have been reported to be important transcription factors involved in the regulation of fatty acid metabolism, there is no evidence for a direct effect of either PPARα or SREBP-1c on the promoter regions of the desaturase and elongase genes. Whether this control involves direct interaction of these transcription factors with regulatory elements in the promoters of the desaturases and elongases or indirect control through other mechanisms is still unclear. Knight *et. al.* (247) reported unchanged SREBP-1c

mRNA expression following fibrate feeding, and suggested that the observed increases in fatty acid synthesis in their experiment was due to the effect of proteolytic cleavage of the SREBP-1c precursor to the mature form. In a dietary deprivation study by Igarashi *et. al.* (248), the liver mRNA level of desaturases and elongases were upregulated by a n-3 PUFA deficient diet, but the PPARα and SREBP-1c remained unchanged. It therefore appears that these genes may not be sensitive to changes in dietary PUFA content within the range of PUFA used in this experiment or, increases in gene activity could be due to increased protein synthesis or post-translational modification or activation of the enzyme, rather than changes in mRNA expression.

3.8 Summary

This study confirms previous studies showing a direct relationship between dietary ALA and elevated EPA and DPA accumulation in rat blood and tissues. Also in line with previous studies, the alterations in DHA levels were more complex, with increasing dietary ALA elevating DHA but only up to a maximum at 1%en ALA. The rats fed diets with the highest ALA%en (the lowest LA:ALA ratio) elevated the incorporation of total n-3 PUFA into liver phospholipid by 3.08 fold (22.88 to 7.42% total fatty acid) higher than rats fed the lowest ALA%en (the highest LA:ALA ratio) diets. In blood and other tissues, the increase in the degree of n-3 LCPUFA incorporation between the dietary ALA with 2.9%en and 0.2%en treatments was as follows: plasma (3.03 fold), erythrocytes (3.19 fold), kidney (4.03 fold), brain (1.13 fold), heart (2.35 fold) and thigh muscle (2.82 fold). However, when considering the diet-induced shifts in the DHA level which was calculated by the changing rate from lowest to highest phospholipid DHA, the order as follows: plasma (1.98 fold), followed by thigh muscle (1.91 fold), heart (1.77 fold), liver (1.76 fold), kidney (1.45 fold), erythrocytes (1.44 fold) and then brain (1.16 fold).

A strong correlation between n-3 LCPUFA in blood phospholipid and the n-3 LCPUFA in liver phospholipid was identified. Similar strong and positive correlations were also found between blood phospholipid EPA and DPA and the levels in other tissue phospholipid (except brain EPA). The correlations between blood phospholipid DHA and the DHA level in other tissues were from modest (kidney, heart and thigh muscle) to weak (brain). The correlation between blood phospholipid EPA and brain phospholipid EPA was weaker than the correlation between blood and brain DHA levels. This difference could be the result of a

higher incorporating rate of DHA than other fatty acids in brain due to its important functions to nervous tissues.

While high n-3 PUFA diets consistently induced higher levels of EPA and DPA in rat tissues than low n-3 PUFA diets, the FADS2 and ELOVL2 genes appeared to be stimulated only in animals fed the very low PUFA reference diet; experimental diets exceeded 1.2%en PUFA and when ALA was in the range of 0.2 to 2.9%en in the diet there had no influence on the mRNA expression of desaturase and elongase genes or transcription factors involved in hepatic lipid metabolism. Furthermore there was no relationship between the mRNA expression of these hepatic genes and the concentration of PUFA or LCPUFA in either blood or tissue phospholipids. Therefore, at the levels of dietary PUFA used in this experiment, n-3 LCPUFA levels would seem to be regulated more by competitive interaction between distinct substrates at different stages of LCPUFA biosynthesis and esterification into glycerolipids than alterations in the expression of key components of the lipid metabolism and LCPUFA biosynthetic pathway.

Chapter 4

The Effects of Dietary ALA Levels on the Regulation of Omega-3 Fatty Acids and Gene Expression of Desaturase and Elongase in Barramundi (*Lates calcarifer*) Fingerlings

4.1 Abstract

World supplies of marine fish oils and fish meal are limited and the continued growth of the aquaculture industry has aggravated the situation, since this industry relies on wild fish stocks to produce the feed farmed fish. Substitution of marine-derived oils and protein with alternative sources without compromising fish health and product quality is urgently needed to eliminate the heavy dependence on marine resources. The experiments in this chapter were carried out to determine the effects of substituting fish oil and fish meal in the diet with a blend of vegetable oils and defatted poultry meal on fillet and liver fatty acid profiles in barramundi fingerlings. After a 3-week washout period during which fish were fed on very low PUFA macadamia-based diets (LA 1.4%en, ALA 0.1%en, LA:ALA ratio of 15.7), a total of 270 fish (15 fish/tank in triplicate) with similar body weight were allocated to one of 6 dietary treatments. The experimental dietary treatments consisted of 5 vegetable oil-based diets with an ALA content ranging from 0.1 to 3.2%en with the LA content held constant at 2.4%en. The diet 6, a commercial diet which contained fish-derived EPA and DHA, was used as a reference diet. Results showed that the fatty acid composition of barramundi liver and fillet reflected the dietary lipid source; as the ALA content of the diet increased, the ALA level in the liver and fillet increased in a dose-dependent manner. There was, however, no corresponding increase in the tissue levels of the n-3 LCPUFA EPA, DPA and DHA. Increasing levels of dietary ALA has no effect on hepatic mRNA expression of desaturase (FADS2) and elongase (ELOVL) genes. Hepatic gene expression levels of FADS2 and ELOVL were increased by approximately 10 fold and 3 fold, respectively, in all vegetable oil-based dietary groups relative to the fish oil reference diet. These data demonstrate that barramundi have the capacity for fatty acid desaturation and elongation which can be regulated through food intake, however, increased intake of ALA showed no correlation to tissue LCPUFA levels, in particular DHA. In line with other studies in marine or carnivorous species, it appears that the enzyme activity of barramundi Δ6 desaturase is low, which may explain the limited production of EPA and DHA from dietary ALA in this species. It was also

noted, however, that there was a large amount of variation between individual fish in their tissue n-3 LCPUFA content, suggesting that some fish are more efficient at conversion than others. This suggests that it may be possible to produce barramundi which have a greater capacity for DHA production through selective breeding programs.

4.2 Introduction

Fish have long been considered as a major source of high quality protein and physiologically essential LCPUFA for human consumption and feeds for both farmed fish and other farmed animals such as pigs and chickens (163, 249, 250). It has been estimated that aquaculture is the largest overall consumer of fish meal and fish oil, accounting for 60% and 85%, respectively, of the global usage in 2008 at a time when the pork, poultry and other livestock industry sectors shared the remaining 40% of fish meal usage (251). Although aquaculture is expected to meet consumer demand for a more sustainable fish supply in the future, the substantial use of fish oils in this industry has raised concerns that the rapid expansion of the aquaculture industry will in fact put yet more pressure on already declining marine resources (252). Unless alternatives for fish meal and fish oil are developed, aquaculture will continue to use up the majority of the fish meal and oil produced to maintain LCPUFA contents in farmed fish (253). It has been predicted that there will be about a 2 fold increase in total production and total feed usage from year 2010 to 2020, according to current rate of expansion of global aquaculture production (163). In this context, replacing fish oil in aquaculture feed with vegetable oils rich in C18 ALA (precursors of EPA and DHA) may help to reduce the dependence of the aquaculture industry on wild fisheries.

Feed for herbivorous and omnivorous farmed fish species such as Murray cod (*Maccullochella peelii*) and tilapia does not need to contain fish meal or fish oil, because these fish have the capacity to convert C18 PUFA to their long-chain derivatives, and are able to maintain a consistent n-3 LCPUFA status when fed on diets high in C18 PUFA (176, 254). In contrast, carnivorous marine species such as gilthead sea bream (*Sparus aurata*) and turbot (*Scophthalmus maximus*) are unable to produce LCPUFA from ALA to any physiologically relevant extent, due to apparent deficiencies in one or more steps of the fatty acid metabolic pathway (191, 255). The barramundi (*Lates calcarifer*) (Bloch, 1790), is one of the most highly regarded sport and table fish in Australia. It inhabits rivers and inshore waters over roughly 8000 km of coastline in Queensland, Northern Territory and Western Australia and is important to commercial and recreational fisheries throughout this range (256). Several

studies have been undertaken to examine the nutritional requirements of farmed barramundi and most of these studies suggest that this species requires a relatively high amount of protein (crude protein 40-50%) and fish and meat-based diets in which fish meal is the predominant protein source, consistent with the carnivorouos/piscivorous nature of the fish (257-259). However, a study in barramundi in which part of the fish oil was replaced with vegetable oils has shown that partial or complete replacement of fish oil in barramundi diets could potentially be used to provide an alternative energy source without compromising growth, feed conversion ratio or body protein and fat contents (260). It has also been shown that fish meal can be replaced by meat meal as the predominant protein source in diets for juvenile barramundi (258). However, whilst both marine derived-protein/-fat were reported to be replaced by other non-marine sources, all of these previous feeding trials included at least some fish meal or fish oil in all the experimental diets, making it difficult to draw clear conclusions as to the ability of these fish to derive n-3 LPCUFAs from C18 PUFA precursors.

The overall process of lipid metabolism is largely influenced by dietary conditions prompting this study to investigate the effect of variations in dietary ALA content, in the absence of any exogenous source of n-3 LCPUFA and at a constant level of LA, on tissue n-3 LCPUFA synthesis and gene expression levels of FADS2 and ELOVL in barramundi.

4.3 Design of the study

To eliminate the interference effects of LCPUFA in any fish by-products on the endogenous LCPUFA synthesis of barramundi fingerlings, non-marine protein sources were used in the experimental fish diets, including poultry meal, soybean protein concentrate and wheat gluten. Vegetable oil blends were used as the only lipid source in the feed and designed to provide an increasing level of ALA as a %en while keeping the %en of LA constant. Fish liver and fillet fatty acid together with hepatic mRNA levels analyses were conducted to determine whether the PUFA level in diets provided by vegetable oil blends was capable of increasing the synthesis of EPA and DHA and/or regulating desaturase and elongase mRNA levels. Prior to the actual feeding trial, a washout diet composed of macadamia oil (low PUFA with high monoenes) was used to dilute the LCPUFA levels in fish which had previously been fed a commercial feed containing marine-derived fat and protein. The 5 experimental diets with varying ALA concentrations and a constant LA level were used to determine the optimum PUFA amounts for fish to synthesize LCPUFA, and to clarify the relationship between the LCPUFA levels and gene expression of desaturase and elongase genes in the barramundi.

4.4 Methods

4.4.1 Animals and feeding trial management

All experimental procedures were performed in accordance with institutional guidelines for the use of animals and the Australian code of practice for the care and use of animal for scientific purpose. The protocol was approved by the Animal Ethics Committee, University of Adelaide (Ethic number S-28-08). Australian barramundi fingerlings (~5 g) were obtained from a commercial supplier (W. B. A. Hatcheries, SA, Australia) and maintained at the South Australia Research Development Institute (SARDI, SA, Australia). After arrival, 400 fish were held in a 1 kilolitre (kL) tank provided with flow through temperature controlled (28°C) seawater and situated within an environmentally controlled aquarium room. The tank was piped with filtered recirculated seawater from Gulf Saint Vincent and the compressed air was provided via a diffuser to the tank for oxygenation of the water. The tank was tightly covered with a screened top to prevent fish from jumping out of the tank and 3 weeks were allowed for acclimation and dilution of tissue LCPUFA levels before initiation of the experiment. The washout diet, containing a very low PUFA level, was fed to fish throughout this 3 week pre-trial period. The experimental set up comprised of 18 conical-based aqua research tanks (70 L) with identical and independent recirculation systems, netting and a bottom filter for uneaten feed collection. After the washout period, the fish were sampled from the holding tank and relocated into the experimental tanks in batches according to their body weight. First, 10 fish with similar body weights were removed from the holding tank and placed in each experimental tank, with a total weight of 60-70 g for the full batch of ten fish. Another 5 fish were then selected from the holding tank, with a weight of 30-40 g per batch. In total, 15 fish were placed in each of the experimental tanks, and the initial mean weight per tank was ~100 g (Table 4.1).

All diets were fed *ad libitum* to apparent satiation by hand twice daily (9:00 am and 3:30 pm) 6 days a week. The experimental trial was performed at a constant warm water temperature which was maintained by lowering ambient temperature in the aquaculture room and heating the water in the reservoirs to 28°C. The water quality in each tank was monitored daily for temperature, pH and dissolved oxygen and weekly for salinity to ensure water quality remained within normal parameters. The diurnal variation in water temperature in each tank was less than 1°C. Photoperiod was held to a constant 12 hr light/12 hr dark cycle. The

seawater had a salinity of 37 g/L and a pH of 8 and the oxygen level was 95% throughout the trial.

4.4.2 Diets

The barramundi basal diets used in the study were designed by Dr David A. J. Stone (SARDI, SA, Australia) based on recommended levels of protein, lipid and micronutrients included in the standard barramundi diet used commercially (259). Diets used in washout and experimental period were all formulated using reduced fat ingredients to achieve a LA content of 2.4%en and an ALA content of between 0.1-3.2%en. The three major protein ingredients of the diets were poultry meal (Poultry BP meal refined grade, Skretting, TAS, Australia), soybean protein concentrates (Inpak foods, SA, Australia) and low fat wheat gluten (Inpak foods, SA, Australia). Lipid content and fatty acid composition of the protein meals were determined in our laboratory prior to the commencement of the feeding trial and are shown in Table 4.2. Other ingredients used for formulating the diets were gelatinised wheat starch (Inpak foods, SA, Australia), choline chloride and vitamin min/premix (SARDI YTK) (Lienert, SA, Auatralia). The barramundi were maintained on the reference or experimental diets for 4 weeks.

4.4.2.1 Poutry meal defatting procedure

The poultry meal was defatted using a modification of the defatting procedure previously described by Smith *et. al.* (261). Briefly, approximately 500 g of the poultry meal were placed in glass beakers, saturated with 1.6 L of 70°C cyclohexane and ethanol (2:1, v/v), mixed thoroughly and allowed to settle. The solvent was then decanted and the ingredients were filtered through a Büchner funnel (1 L capacity) with filter paper attached to a 2 L filter flask under vacuum to remove the excess solvent. The process was repeated twice using 800 mL and 500 mL respectively of the cyclohexane and ethanol (2:1, v/v). The final defatted poultry meal was filtered, dried at ambient temperature overnight and then placed in oven at 60°C for 1 hr and 50°C for a further 3 hr to evaporate the solvent. Lipid extraction was carried out using the chloroform/methanol (2:1, v/v) solvent system (described previously in chapter 2 section 2.1.2.1) and indicated that the total fat content of the defatted poultry meal was reduced to 1.3% (w/w), compared to 15% in the original sample. The crude protein content (%, w/w) was determined by total nitrogen analysis performed by Waite Analytical Service (WAS, University of Adelaide) and multiplied by the conventional conversion factor 6.25

(262). Crude protein contents and lipid composition of poultry meal, defatted poultry meal, soybean protein concentrates, low fat wheat gluten are given in Table 4.2.

4.4.2.2 Vegetable oil blends preparation

Vegetable oils were purchased from the local supermarket and the oil blends were prepared by blending varying proportions of macadamia oil (16.10% saturates, 81.249% monoenes, 2.13% LA and 0.2% ALA of total fatty acids; Suncoast Gold Macadamias Limited, Australia), flaxseed oil (11.09% saturates, 18.46% monoenes, 18.64% LA and 51.61% ALA of total fatty acids; Melrose Laboratories Pty Ltd, Australia) and/or sunflower oil (11.03% saturates, 27.53% monoenes, 60.9% LA and 0.04% ALA of total fatty acids; Buona Cucino, Italy). Fatty acid composition analysis of all vegetable oil blends were performed prior to formulating the feeds.

4.4.2.3 Diet formulating

All powdered ingredients were carefully weighed and pre-mixed prior to the addition of vegetable oil blends. The powdered ingredients were dry-mixed (Hobart A-200T, France) thoroughly for 2 min in a bench-top food mixer before distilled water (600 mL) and vegetable oil was added and mixing continued for a further 10 min. All feeds were made on an experimental extruder fitted with a die-plate with 2 mm diameter holes (La Prestigiosa medium 2500, Italy) which was configured to match a commercial extruder used to make the commercial barramundi feeds used in Australia. The soft feed dough was cold extruded into a 2-5 mm die-size strand and then cut, pelleted and dried at ambient temperature for 3 hr. The feeds were then placed on a sieve and oven-dried at 40°C for approximately 24 hr until the moisture content was below 10% (w/w). The dried feed was then broken into 2 mm pellets, sealed in a plastic container and stored at -20°C until commencement of the feeding trial. All equipment used for making up feeds was washed and dried before the next diet was produced to avoid cross-contamination. The commercial barramundi diet (Grobest Corporation Co., Ltd., Thailand) was purchased from W. B. A. Hatcheries and used as a reference diet.

4.4.2.4 Proximate and fatty acid analysis

The final formulation and proximate composition of the diets used in this study are given in Table 4.3 and 4.4, respectively. The fatty acid composition of the feeds was determined using procedures described in detailed previously (Chapter 2) and are presented in Table 4.5. The ingredient compositions of the diets was analysed by Agrifood Technology Pty Ltd (Victoria,

Australia). The formulated diets each contained an average of 3931 kcal/kg which was comprised of 50.3%en of protein, 28.7%en of fat and 21%en of carbohydrate. The estimated energy values were based on the standard physiological fuel values for protein, fat and carbohydrate of 4, 9 and 4 kcal/g, respectively. The estimated ALA%en and LA%en were calculated as: fatty acid (%en)=[(% fatty acid in diets) x fat%en] x 100. Thus, LA provided 2.4%en and ALA provided 0.1-3.2%en among the 5 vegetable oil-based experimental diets (Diets 1-5) (Table 4.4 and 4.5). The commercial feed provided 3640 kcal/kg which was comprised of 22%en of carbohydrate, 24.5%en of fat and 53.5%en of protein. LA and ALA contributed 3.3%en and 0.4%en, respectively, in the commercial diet (Table 4.4).

Table 4. 1 Tank layout and fish sorting management

Tank	Corresponding Diet[1]	1st sorting 10 fish (g)	2nd sorting 5 fish (g)	Total weight of 15 fish (g)/tank
1	**3**	69.8	30.9	100.7
2	**1**	64.1	35.6	99.7
3	**4**	67.8	32.3	100.1
4	**5**	72.8	28	100.8
5	**2**	68.9	31.2	100.1
6	**1**	67.0	33.4	100.4
7	**6**	70.0	29.4	99.4
8	**4**	63.3	37	100.3
9	**5**	64.9	35	99.9
10	**6**	63.6	37	100.6
11	**4**	69.0	30.6	99.6
12	**2**	67.4	32.5	99.9
13	**3**	70.7	30	100.7
14	**5**	65.5	34	99.5
15	**3**	69.2	30.8	100.0
16	**2**	65.4	34.8	100.2
17	**6**	66.2	33	99.2
18	**1**	64.8	36	100.8

[1]The diets were fed in triplicate tanks. i.e, diet 1 was fed to fish in tank 2, 6 and 18.

Table 4. 2 Lipid contents and fatty acid compositions of protein meals

Protein meal	Poultry meal	Defatted poultry meal	Soybean protein concentrate	Low fat wheat gluten
Crude protein (% dry weight)[1]	63	69	75	75
Fat (% dry weight)[2]	14.8±0.2	1.3±0.2	0.6±0.0	0.3±0.0
Fatty acids[3]				
Total saturates	32.0±0.1	41.7±0.1	19.4±0.1	22.1±0.1
Total monoenes	49.4±0.1	33.2±0.1	24.1±0.1	12.0±0.0
18:2 n-6 (LA)	13.0±0.0	13.0±0.0	49.3±0.1	61.6±0.1
20:4 n-6 (AA)	1.5±0.0	4.8±0.0	N.D.	N.D.
22:4 n-6	0.3±0.0	1.0±0.0	N.D.	N.D.
Total n-6	15.5±0.0	20.3±0.1	49.5±0.1	62.4±0.1
18:3 n-3 (ALA)	1.6±0.0	0.9±0.0	6.6±0.0	3.4±0.0
20:5 n-3 (EPA)	0.2±0.0	0.4±0.0	N.D.	N.D.
22:5 n-3 (DPA)	0.3±0.0	0.7±0.0	N.D.	N.D.
22:6 n-3 (DHA)	0.2±0.0	0.9±0.0	N.D.	N.D.
Total n-3	2.4±0.0	3.1±0.0	6.6±0.0	3.4±0.0

[1]Protein value is presented as % of total nitrogen (N) (w/w), N x 6.25. The nitrogen content of all samples was analysed using an Elementar Instrument by Waite Analytical Service, University of Adelaide.
[2]Fat value is present as % total lipid (w/w).
[3]Fatty acid values are presented as % of total fatty acid analysed by GC. Data are means ± SEM of n=3.

Table 4. 3 Macronutrient content of the diets

	Washout diet	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Commercial diet[1]
Macronutrients[2] (g/100g)							
Defatted poultry meal	34	34	34	34	34	34	Protein Min 43% (w/w)
Soybean protein concentrate	25	25	25	25	25	25	Carbohydrate Max 15% (w/w)
Wheat gluten	12	12	12	12	12	12	Ash Max 13% (w/w)
Gelatinised wheat starch	10	10	10	10	10	10	Fibre Max 2% (w/w)
Vegetable oil	14	14	14	14	14	14	Fat Min 9% (w/w)
Oil proportion (% w/w in 14% fat)							
Macadamia oil	100	94	92	90	83	76	
Flaxseed oil	0	0	3	5.5	15	24	
Sunflower oil	0	6	5	4.5	2	0	
Choline chloride	3	3	3	3	3	3	
Vitamin/mineral premix	2	2	2	2	2	2	

[1]Commercial diet (Grobest Corporation Co., Ltd., Thailand) was purchased from W. B. A. Hatcheries.
[2]Macronutrients of commercial diet were provided by the Grobest Corporation Co., Ltd., Thailand.

Table 4. 4 Ingredient composition of the diets

	Washout diet[1]	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Commercial diet**
Ingredient composition (% dry weight)[2]							
Crude protein (N x 6.25)	50.0	50.6	50.0	50.4	48.6	50.0	48.7
Crude fat (by acid hydrolysis)	12.7	13.7	12.5	13.0	12.7	11.4	9.9
Crude ash	7.6	7.8	7.4	7.6	7. 8	7.4	12.2
Crude carbohydrate (by difference)	20.9	23.1	21.2	20.8	19.3	20.0	20.0
Dry matter (% wet weight)	94.9	95.1	95.5	95.8	94	94.2	95.3
Total energy (Kcal/kg)	3930.8	3976.3	3975.5	4019.9	3856.9	3825.6	3640.3
Energy (%en)[3]							
Carbohydrate	**21.0**	**22.1**	**21.3**	**20.7**	**20.0**	**20.9**	**22.0**
Fat	**28.7**	**29.5**	**28.3**	**29.2**	**29.6**	**26.8**	**24.5**
Protein	**50.3**	**48.4**	**50.4**	**50.1**	**50.4**	**52.3**	**53.5**

[1]The values were estimated by the average proximate composition of the experimental diets (Diets 1-5). The values of energy, carbohydrate, fat and protein were calculated accordingly.
[2]Ingredient composition was analysed by Agrifood Technology Pty Ltd (Victoria, Australia).
[3]The calculate of calorie content was based on the standard physiological fuel values for carbohydrate, fat and protein of 4, 9 and 4, respectively.

Table 4. 5 Fatty acid compositions of the diets

	Washout diet	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Commercial diet**
Fat%en[1]	28.7	29.5	28.3	29.2	29.6	26.8	24.5
LA:ALA Ratio	15.7	21.9	4.7	2.9	1.2	0.7	8.8
LA%en[1]	1.4	2.5	2.3	2.4	2.5	2.3	3.3
ALA%en[1]	0.1	0.1	0.5	0.8	2.2	3.2	0.4
Fatty acids (%)[2]							
Total saturates	17.5±0.0	17.0±0.0	16.9±0.0	16.9±0.0	16.6±0.0	16.1±0.0	33.1±0.4
Total monoenes	76.9±0.1	74.2±0.1	73.2±0.1	71.8±0.0	67.4±0.1	63.4±0.0	25.6±0.2
18:2 n-6 (LA)	5.0±0.0	8.3±0.0	8.0±0.0	8.3±0.0	8.5±0.0	8.6±0.0	13.3±0.2
20:4 n-6 (AA)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.1±0.0
Total n-6	5.0±0.0	8.3±0.0	8.0±0.0	8.3±0.0	8.5±0.0	8.6±0.0	14.7±0.1
18:3 n-3 (ALA)	0.3±0.0	0.4±0.0	1.7±0.0	2.9±0.0	7.4±0.0	11.8±0.0	1.5±0.0
20:5 n-3 (EPA)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	7.3±0.1
22:5 n-3 (DPA)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.3±0.0
22:6 n-3 (DHA)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	14.7±0.2
Total n-3	0.4 ± 0.0	0.4 ± 0.0	1.8 ± 0.0	3.0 ± 0.0	7.4 ± 0.0	11.8±0.0	25.2±0.1

[1]The fat%en of washout diet was estimated by the average fat%en of the experimental diets (Diets 1-5), the LA%en and ALA%en level were calculated accordingly.
[2]Values are presented as % of total fatty acids. Data are means ± SEM of n=3.

4.4.3 Fish sampling

In total, 3 stages of fish sampling were performed, once before the start of the washout diet, once immediately following the washout period and once at the end of the 4 weeks on the experimental diets. Fish were fasted for one day prior to weighing and sampling and were killed with an over-dose of the anaesthetic, benzocaine. The first fish were randomly selected from the 1 kL tank and are referred to as the initial fish (before washout period). Initial fish weight and fork length (the length from the tip of the snout to the end of the middle caudal fin rays) was measured and lipid compositions of fillet and liver were analysed. After the 3-week washout period and at the end of the 4-week experimental period, a further 8-10 fish from washout feeding and 24 fish from each experimental diet feeding (8 fish/tank) were sampled and tissues collected for body weight, fork length, tissue lipid analyses and hepatic RNA extractions.

4.4.4 Growth performance measurements

Initial body weight (g, IBW) was measured on the second day after the fish had arrived from the WBA Hatcheries.

After washout body weight (AWB, g) was measured at the end of 3-week washout period.

Final body weight (FBW, g) was measured at the end of the experimental feeding trial.

Length (cm) was measured from the tip of the snout to the end of the tail.

Width (mm) was measured the distance from the dorsal fin to the ventral.

Weight gain (%) was calculated based on [(FWB - AWB) / ABW] x 100.

Specific growth rate (SGR, %) or percentage of weight gain per day was calculated based on [(log of FWB - log of AWB) / feeding day] x 100.

Feed conversion ratio (FCR) was calculated based on [dry feed intake (g) / (FBW - AWB)].

Feed intake (g/day/fish) was calculated based on (dry feed intake per fish (g) / feeding day).

Hepatosomatic index (HSI, %) was calculated based on [liver weight (g) / FBW (g)] x 100.

Survival (%) was calculated based on [(initial fish number before feeding - dead fish number after feeding) / initial fish number before feeding] x 100.

4.4.5 Fatty acid analyses of fillet and liver tissues

Total lipids were extracted from whole fillet and liver using chloroform/methanol (2:1 v/v) as described in detail in chapter 2. Extracted total lipids were weighed, and phospholipid and triglyceride fractions were separated, methylated and quantified by GC following procedures previously described (Chapter 2).

4.4.6 RNA isolation and quality determination

Total RNA was isolated from 10 mg of fish liver disrupted by a tissue lyser. The quality of the RNA was determined by measuring the ratio of OD 260/280 and 260/230. The RNA concentration was determined by measuring the absorbance at 260 and 280 nm and RNA integrity was confirmed by 1.5% agarose gel electrophoresis. Procedures for liver tissue disruption, RNA isolation, RNA quality determination were as described previously (Chapter 2).

4.4.7 Selection of housekeeping genes

Hepatic RNA samples from juvenile barramundi in each dietary treatment (Diets 1 to 6) after feeding for 4 weeks, were used to determine the most stable housekeeping genes for normalizing the mRNA abundance of the target genes, FADS2 and ELOVL among different diets. Five housekeeping gene candidates, including β-Actin, RPLP1α, 18s rRNA, EF1α and GAPDH were selected from commonly used reference genes (263, 264). To enable species specific PCR primers to be developed for performing qRT-PCR, sequence information of barramundi specific gene was needed. However, no barramundi specific gene sequence information was initially available for the 5 housekeeping genes at the time that I checked GenBank® database. I therefore had to first design degenerate primer pairs for each gene based on cross species pairwise alignment of other teleosts sequences available from GenBank® according to their homologous regions. Those teleosts included Japanese amberjack (*Seriola quinqueradiata*) (AB179839), cobia (*Rachycentron canadum*) (EU266539), Mozambique tilapia (*Oreochromis mossambicus*) (AB037865), rainbow trout (*Oncorhynchus mykiss*) (AF254414), zebra fish (*Danio rerio*) (BC165823) for identifying β-Actin mRNA sequence in barramundi; zebra fish (FJ915075), Atlantic salmon (*Salmon salar*) (AJ427629), rainbow trout (AF243428), cobia (DQ279821), Ishikawa's cherry salmon (*Oncorhynchus masou ishikawae*) (AF243427) for 18s rRNA; zebra fish (NM_131263), Atlantic salmon (AF321836), European seabass (*Dicentrarchus labrax*) (AJ866727) and fathead minnow (*Pimephales promelas*) (AY643400) for EF1α; Senegalese sole (*Solea senegalensis*) (AB374984), European flounder (*Platichthys flesus*) (AJ843879), Atlantic halibut (*Hippoglossus hippoglossus*) (EU412645) and three-spined stickleback (*Gasterosteus aculeatus*) (BT026793) for RPLP1α. Over the period of primer design and sequence identification, De Santis *et. al.* (20) deposited 18s rRNA (GQ507431), EF1α (GQ507427) and GAPDH (GQ507430) partial cDNA sequence into GenBank®. I therefore also included

GAPDH as one of the candidates for the barramundi housekeeping gene selection. Primers used for obtaining partial mRNA sequence were: β-Actin F- 5'-CCGCACTGGTTGTTGACAACGG-3'/R-5-'TCGTACTCCTGCTTGCTGATCC-3'; 18s rRNA F-5'-CGGAGGTTCGAAGACGATCAG-3'/R-5'-GGGCAGGGACTTAATCAACGC-3'; EF1α F-5'-GAGTCAACAAGATGGACTCCAC-3'/R-5'-GGGTGGTTCAGGATGATGACC-3'; RPLP1α F-5'-GAAGCTAAGGCCCACAATCCG-3'/R-5'-AGAGACCGAAGCCCATGTCG-3'; GAPDH F5'-AAGTATGACTCCACCCACGGC-3'/R-5'CCGAACTCATTATCATACCAGG-3'.

Specific sequences of these common reference genes were retrieved by PCR of barramundi hepatic total RNA using OneStep RT-PCR kit (Qiagen) to perform reverse transcription and PCR. The two reactions were carried out sequentially in the same tube and the Eppendorf Mastercycler® was used for all PCR amplification. All procedures were conducted, according to the manufacturer's protocol. Briefly, each 50 µL reaction components for one-step RT-PCR were as follows: OneStep RT-PCR Buffer (1X); 2 µL of the dNTP Mix; Q-solution (1X); RNase-free water; 2 µL of primers per amplification; 2 µL of RT-PCR Enzyme Mix; 5-10 units/reaction of RNaseOUT™ (Invitrogen) and template RNA (1 µg/reaction). RT-PCR amplification was initiated with a reverse-transcription reaction at 50°C for 30 min and an initial hot start enzyme activation step at 95°C for 15 min followed by 25-40 cycles of denaturation at 94°C for 0.5-1 min, annealing at 55-65°C for 0.5-1 min and extension at 72°C for 1 min and followed by a final extension at 72°C for 10 min. MinElute® PCR Purification Kit (Qiagen) was used to clean up and purify the products of the PCR reactions, according to the manufacturer's instructions and using an Eppendorf microcentrifuge 5415 R (Eppendorf South Pacific Pty. Ltd., NSW, Australia). PCR fragments were visualized on a 1.5% agarose gel (Progen Industries Ltd, Queensland, Australia) and then cloned into a pGEM®-T easy vector system (Promega) and sequenced in both direction (forward and reverse) using M13 universal sequencing primers (F-5'-GTCATAGCTGTTTCCTGTGTG-3'/R-5'-GTCGTGACTGGGAAAACCCTGGCG-3'). Nucleotide sequence analyses were performed using BigDye® terminator sequencing (Applied Biosystems automated 3730 sequencer) at Molecular Pathology Sequencing, Institute of Medical and Veterinary Science (IMVS), SA, Australia. The resulting sequences were compared against those available from the public domain using National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool for nucleotides (BLASTN) and barramundi sequences (β-Actin, 18s rRNA, EF1α and RPLP1α) obtained from liver RNA were deposited in GenBank®. Their cellular functions, qRT-PCR primer pairs, accession numbers and amplicon sizes are listed in Table 4.6.

To eliminate any residual traces of DNA contamination, a total of 1 µg of total RNA for each sample was treated with RQ1 RNase-Free DNase (Promega) and an aliquot of each RNA sample was diluted to a final standardized concentration of 5 ng/µL prior to qRT-PCR analyses. The 5 housekeeping gene candidates was analysed with the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/#housekeepers) which is a Microsoft Excel-based application that evaluates the expression stability of internal control genes based on the principle that the expression ratio of the two stable housekeeping genes should be the same in all samples (265), therefore, the software was used to identify the most stably expressed control genes in a given set of barramundi liver tissues from 6 dietary treatments.

4.4.8 qRT-PCR analysis

Quantification of relative gene expression levels were determined by one-step qRT-PCR using SYBR green fluorescence. Each qRT-PCR reaction consisted of 10 ng of fish total hepatic RNA, 5 µL of 2x QuantiFast® SYBR Green RT-PCR Master Mix, 1 µL of 5 µM (0.5 µM in final concentration) primers, 0.1 µL QuantiFast® RT Mix and supplemented with RNase-free water to final volume of 10 µL. Primer sets used for assessing FADS2 and ELOVL mRNA abundance are shown in Table 4.7. The qRT-PCR conditions, primer validation, quality control and detection procedures were described previously in chapter 2.

4.5 Statistical analysis

All fatty acid composition data and mean normalised expression data between the different dietary groups were tested using one-way ANOVA followed by the Tukey-Kramer multiple comparisons test if *P* value was less than 0.05. Data were sampled from populations which had identical SDs and followed Gaussian distributions. Kruskal-Wallis post test with Dunn's multiple comparison test was applied for non-parametric analyses of groups with N<3 or data which did not pass the normality test. An unpaired t-test was used to examine differences between growth measurements and lipid compositions between initial and after washout period fish. A probability level of 0.05 ($P<0.05$) was used in all tests. All analyses were performed using GraphPad InSat 3.10 software. Detailed selection criteria for statistical methods were previously described in chapter 2.

4.6 Results

4.6.1 Growth performance and feed efficiency

The body weight, length and width of fish upon arrival and after washout feeding are shown in Table 4.8. Over the course of the 3-week washout diet feeding, the fish exhibited an increase in body weight of 1.5 g, fork length of 0.7 cm and width of 3.6 mm.

The growth performance and feed utilization efficienccies were compared for fish fed the 5 experimental diets and the commercial reference diet. During the 4-week experimental period, the overall survival rate ranged between 73.3% (Diet 2) and 97.8% (commercial diet) but survival rate did not differ between the different experimental dietary groups (Diets 1-5), (Table 4.9). The final body weight was also not different between the experimental diets (Diets 1-5) but the commercial diet fed fish were significantly heavier than all fish fed the vegetable oil-based diets, and exhibited a 2.1 fold higher body weight compared to the fish fed vegetable oil-based diets. A similar pattern was observed for other growth parameters including length, width, weight gain and specific growth rate, which were 1.3, 1.3, 4 and 2.8 fold higher, respectively, in fish fed the commercial barramundi diet. There was also a significant difference between the 5 vegetable oil-based experimental diets and fish fed the commercial diet in feed intake with a feed intake of 3.1 fold greater in fish provided with the commercial feed. Feed conversion ratio tended to be lower for the fish on the commercial diet compared to fish fed vegetable oil-based diets but this difference was not statistically significant (P=0.15). Hepatosomatic index, the ratio of liver weight to body weight, provides an indication of the status of energy reserve in fish and this parameter was not different between the different dietary groups (P=0.35) (Table 4.9).

Table 4. 6 qRT-PCR pimers for housekeeping gene selection

Genes	Cellular Function	F/R	Sequence 5'-3'	Accession No.	Amplicon (bp)
β-Actin	Cytoskeleton	F	TGCGTGACATCAAGGAGAAG	GU188683	175
		R	AGGAAGGAAGGCTGGAAGAG		
18S ribosomal RNA (18s rRNA)	Ribosome subunit	F	GTGCATGGCCGTTCTTAGTT	GU188686	150
		R	CTCAATCTCGTGTGGCTGAA		
Elongation factor 1α (EF1α)	Protein biosynthesis	F	CTGGTTCAAGGGATGGAAAA	GU188685	154
		R	TCCAATACCGCCAATTTTGT		
ribosomal protein LP1 (RPLP1α)	Protein biosynthesis	F	AAGGCTGCTGGAGTCACTGT	GU188684	224
		R	ATGTCGTCATCGGACTCCTC		
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Glycolysis enzyme	F	CGACAACTTTGGCATCATTG	GQ507430	238
		R	GTCAACCACTGACACGTTGG		

Table 4. 7 Pimers for qRT-PCR analysis

Genes	F/R	Sequence 5'-3'	Accession. No.	Amplicon (bp)	E[1]
β-Actin	F	TGCGTGACATCAAGGAGAAG	GU188683	175	1.14
	R	AGGAAGGAAGGCTGGAAGAG			
FADS2	F	CTGGTCATCGATCGAAAGGT	GU047383	249	1.18
	R	CTGCGCACATAAAGAGTGGA			
ELOVL	F	GTGCGTCCCTAAACAGCTTC	GU047382	154	1.19
	R	GCACACATTGTCTGGGTCAC			

[1]Amplification efficiency.

Table 4. 8 Mean growth measurements of barramundi before (initial) and after washout period

	Before washout	**After washout**
BW (g)[1]	5.2±0.3**	6.7±0.3
Length (cm)[1]	7.6±0.2	8.3±0.1
Width (mm)[1]	17.5±0.8	21.1±0.4

[1]Values denoted with asterisk superscripts were significantly different from each other (**P<0.01) as determined by t-test (two-tailed). Data are means ± SEM of n=8.

Table 4. 9 Growth and efficiency measurements of barramundi fed the vegetable oil-based diets and the commercial diet

Growth parameters[1]	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Commercial diet**
FBW (g)[2]	10.5±0.4[a]	10.6±0.7[a]	10.2±0.4[a]	11.3±0.5[a]	10.0±0.5[a]	21.9±0.7[b]
Length (cm)[2]	9.5±0.1[a]	9.5 ± 0.2[a]	9.4±0.2[a]	9.8±0.2[a]	9.5±0.2[a]	12.4±0.2[b]
Width (mm)[2]	23.8±0.3[a]	23.7±0.5[a]	23.3±0.4[a]	24.9±0.4[a]	23.7±0.5[a]	30.4±0.3[b]
Weight gain (%)[2]	56.7±6.2[a]	58.3±9.9[a]	52.4±6.5[a]	68.4±8.2[a]	49.8±7.8[a]	227.5±9.9[b]
SGR (%)[2]	0.7±0.1[a]	0.7±0.1[a]	0.6±0.1[a]	0.8±0.1[a]	0.6±0.1[a]	1.8±0.1[b]
FCR[3]	0.9±0.0	1.0±0.0	1.0±0.2	0.8±0.1	1.0±0.1	0.7±0.0
Feed intake (g/d)[3]	0.1±0.0[a]	0.1±0.0[a]	0.1±0.0[a]	0.1±0.0[a]	0.1±0.01[a]	0.4±0.0[b]
HSI (%)[4]	1.5±0.1	1.5±0.1	1.5±0.1	1.3±0.1	1.5±0.1	1.4±0.1
Survival (%)[3]	86.7±7.7	73.3±3.8	88.9±5.9	77.8±8.0	86.6±6.7	97.8±2.2

[1]Growth measurements were calculated using the equations as described in section 4.4.4.
[2]Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Kruskal-Wallis test. Data are means ± SEM of n=24 per group.
[3]FCR and survival (%) was calculated on a tank basis (15 fish per tank and 3 tanks per group). Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Kruskal-Wallis test. Data are means ± SEM of n=3 per group. No significant differences were found between groups.
[4]Values for HSI% were determined by one-way ANOVA with Tukey-Kramer Multiple Comparison test. Values with different superscripts are significantly different from each other (P<0.05). Data are means ± SEM of n=21-24. No significant differences were found between groups.

4.6.2 Tissue fat contents

Liver fat contents (%, w/w) were not significantly altered by the period of feeding of the washout diet however fillet fat content was lower following the 3-week washout diet (Table 4.10). At the end of the 4-week feeding trial, liver fat content was higher in fish fed on the vegetable oil-based diets (Diets 1-5) than in fish fed on the commercial diet, but did not differ between the 5 vegetable oil-based diets (Table 4.11). When the data from all feeding groups were combined, there was a significant inverse relationship between liver fat content and total PUFA content of the diet (Figure 4.1 B). There was no relationship, however, between total dietary PUFA content and fat content of the fillet (Figure 4.1 A).

Table 4. 10 Tissue fat contents of barramundi before (initial) and after washout periods

	Before washout	**After washout**
Fillet fat (%)[1]	1.1±0.1[*]	1.0±0.0
Liver fat (%)[1]	7.5±1.1	10.7±1.5

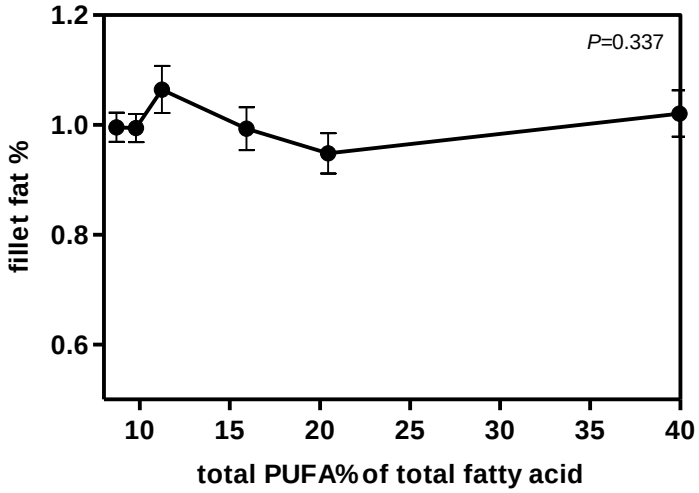
[1]Values denoted with asterisk superscripts were significantly different from each other ([*]$P<0.05$) as determined by two-tailed, unpaired t-test. Data are means ± SEM of n=8.

Table 4. 11 Tissue fats of barramundi fed the vegetable oil-based and the commercial diet

Fat (%)	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**	**Diet 5**	**Commercial diet**
Fillet[1]	1.0±0.0	1.0±0.0	1.1±0.0	1.0±0.0	1.0±0.0	1.0±0.0
Liver[2]	18.0±1.4[b]	19.2±1.0[b]	18.0±1.2[b]	17.0±1.1[b]	15.0±1.0[b]	10.0±0.7[a]

[1]Values with different superscripts are significantly different from each other ($P<0.05$) as determined by one-way ANOVA with Kruskal-Wallis test. Data are means ± SEM of n=24 per group. No differences were found between groups.
[2]Values with different superscripts are significantly different from each other ($P<0.05$) as determined by one-way ANOVA with Tukey-Kramer Multiple Comparison test. Data are means ± SEM of n=24 for all dietary groups except n=21 for dietary group 5 (Diet 5).

A



B

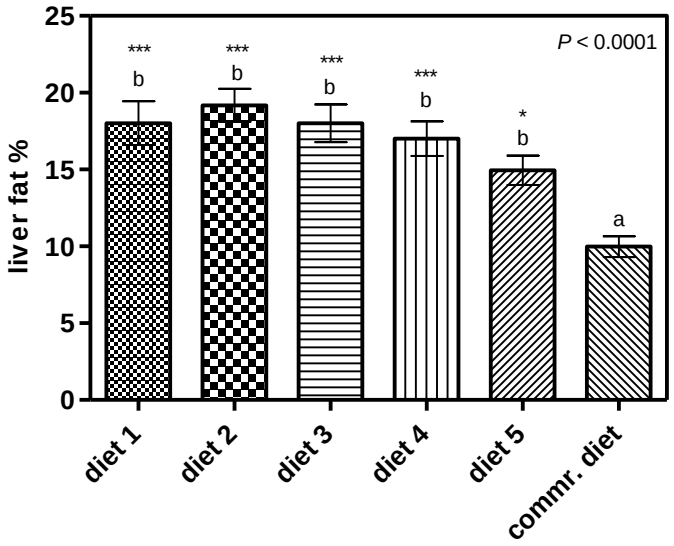


Figure 4. 1 Fat contents of fillet (A) and liver (B). Statistical analysis for fillet fat contents was determined by one-way ANOVA with Kruskal-Wallis test. Data are means ± SEM of n=24 per group. Statistical analysis for liver fat contents was determined by one-way ANOVA with Tukey-Kramer Multiple Comparison test. Data are means ± SEM of n=24 for all dietary groups except n=21 for dietary group 5 (Diet 5). Values denoted with asterisk superscripts were significantly different from each other ($^{*}P<0.05$ and $^{***}P<0.001$).

4.6.3 Effect of 3-week washout diet on tissue fatty acid compositions

In the fillet and liver tissues of barramundi fingerlings previously fed fish oil- and fish meal-based commercial diet, the fatty acid composition of the phospholipid fractions changed significantly after the 3-week washout period (Table 4.12 and 4.13). Compared to the pre-washout fatty acid composition, the level of n-3 LCPUFA originating from dietary fish oil and fishmeal, particularly EPA, DPA and DHA decreased significantly, while total monoenes (16:1 n-7, 18:1 n-9 and 18:1 n-7), which were present at high levels in the washout diet (macadamia oil) increased by 1.7 fold and 2.8 fold in fillet (Table 4.12) and liver (Table 4.13), respectively. The level of total saturates decreased in both fillet and liver phospholipids during the washout period. Levels of LA and total n-6 decreased in fillet phospholipids but LA level increased and total n-6 content was unchanged in liver phospholipids in fish after 3 weeks of the washout diet. Most importantly, levels of n-3 series fatty acids, ALA, EPA, DPA and DHA in both fillet (Table 4.12) and liver (Table 4.13) phospholipid fractions decreased significantly in fish fed the washout diet for 3 weeks. Among these, ALA in fillet phospholipids decreased 2.8 fold and that in liver phospholipids was below the detection level after the washout diet period. Moreover, levels of EPA, DPA and DHA decreased by 1.5-1.2 fold in fillet phospholipids (Table 4.12) whereas in the liver phospholipid fraction the n-3 LCPUFA content decreased by 2.4-1.6 fold (Table 4.13). Figure 4.2 A shows that, with the exception of total monoenes, the proportion of all of fatty acid series including total saturates, total n-6, total n-3 and total PUFA in fillet phospholipid fraction decreased significantly in fish fed the washout diet. Similar effects were also observed in liver phospholipids, with the exception of total n-6 (Figure 4.2 B).

The fillet triglyceride had a lower percentage of 16:0, LA, ALA and all n-3 LCPUFA following the 3-week washout period, and the total saturates, total n-6, total n-3 and total PUFA content of fillet triglyceride decreased by 1, 2.4, 2.7 and 2.5 fold, respectively, whereas total monoenes increased by 1.8 fold (Table 4.14 and Figure 4.3 A). Similar changes were observed in the liver triglyceride fraction (Table 4.15 and Figure 4.3 B).

Saturates and n-3 fatty acids were the two most abundant fatty acid series in both the fillet and liver phospholipid fractions in fish prior to feeding the washout diet, whereas saturates and monoenes are the two major fatty acid groups in fish after the 3 weeks on the washout diet (Table 4.12, 4.13 and Figure 4.2). In contrast, saturates and monoenes were the most abundant fatty acids in the triglyceride fractions of fillet and liver both before and after the 3 weeks on

the washout diet. The monoene content, however, was increased by 1.2 fold and 1.9 fold in fillet and liver triglyceride, respectively, in fish fed the washout diet (Table 4.14, 4.15 and Figure 4.3).

In summary, after being fed for 3 weeks on a washout diet which contained very low levels of PUFA, high monoenes and no n-3 LCPUFA, there was a significant decrease in the levels of all of major n-3 LCPUFA including EPA, DPA and DHA and increase in monoenes content in both liver and fillet of barramundi fingerlings. This thus resulted in a decrease in total n-3 fatty acid content and increase in the proportion of monoenes including 16:1 n-7, 18:1 n-9 and 18:1 n-7, compared to fish fed on the standard commercial barramundi diet, which are based largely on fish oils.

Table 4. 12 Fatty acid compositions of barramundi fillet phospholipids before and after the 3 weeks on the washout diet

Fillet phospholipid	Before washout	After washout
Fatty acids (%)[1]		
16:0	18.6±0.1	16.6±0.2***
18:0	11.0±0.1	10.4±0.2**
Total saturates	36.2±0.2	31.6±0.4***
16:1 n-7	0.9±0.1	2.8±0.4**
18:1 n-9	13.5±0.2	23.2±0.9***
18:1 n-7	2.9±0.0	3.4±0.1***
Total monoenes	18.9±0.2	31.3±1.4***
18:2 n-6 (LA)	8.3±0.1	7.3±0.2***
18:3 n-6	0.4±0.0	0.4±0.1
20:2 n-6	0.3±0.0	N.D.
20:3 n-6	0.3±0.0	0.4±0.0*
20:4 n-6 (AA)	2.8±0.1	2.6±0.1
22:2 n-6	< 0.05	N.D.
22:4 n-6	0.2±0.0	0.1±0.0*
Total n-6	12.3±0.1	10.8±0.2***
18:3 n-3 (ALA)	0.4±0.0	0.1±0.0***
18:4 n-3	0.1±0.0	N.D.
20:5 n-3 (EPA)	4.5±0.0	3.1±0.1***
22:5 n-3 (DPA)	2.2±0.1	1.7±0.1***
22:6 n-3 (DHA)	24.7±0.3	19.8±0.8***
Total n-3	31.9±0.2	24.7±1.0***
Total PUFA	44.2±0.3	35.5±1.0***

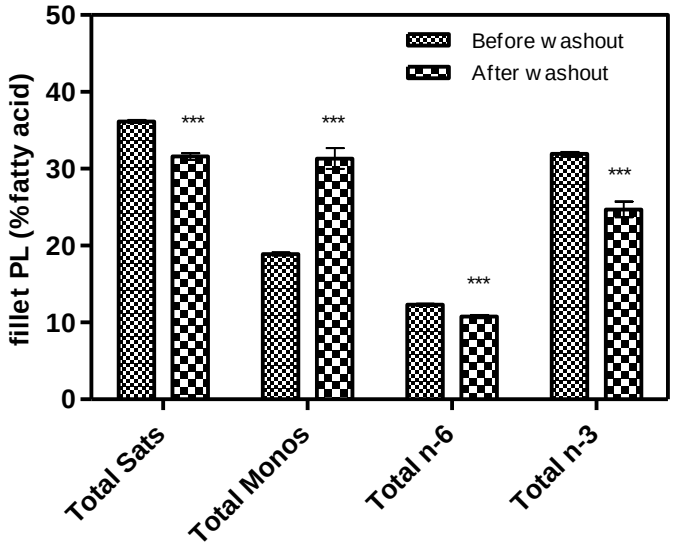
[1]Fatty acid (%) is present as % of total fatty acids. Values denoted with asterisk superscripts were significantly different from each other (*P<0.05, **P<0.01 and ***P<0.001) as determined by a two-tailed, unpaired t-test. Data are means ± SEM of n=9-10.

Table 4. 13 Fatty acid compositions of barramundi liver phospholipids before and after the 3 weeks on the washout diet

Liver phospholipid	Before washout	After washout
Fatty acids (%)[1]		
16:0	18.5±0.3	15.0±0.7***
18:0	15.8±0.5	14.7±0.5
Total saturates	37.9±0.4	32.0±0.5***
16:1 n-7	< 0.05	1.3±0.4***
18:1 n-9	7.0±0.1	22.6±2.0***
18:1 n-7	1.9±0.0	3.1±0.1***
Total monoenes	10.3±0.1	28.7±2.4***
18:2 n-6 (LA)	7.6±0.3	8.8±0.3*
18:3 n-6	0.5±0.1	0.8±0.1*
20:2 n-6	0.2±0.0	N.D.
20:3 n-6	0.4±0.1	1.6±0.3*
20:4 n-6 (AA)	5.4±0.1	4.2±0.2***
22:2 n-6	< 0.05	N.D.
22:4 n-6	0.1±0.0	0.3±0.1*
Total n-6	14.3±0.3	15.6±0.6
18:3 n-3 (ALA)	0.3±0.0	N.D.
18:4 n-3	N.D.	0.1±0.1
20:5 n-3 (EPA)	5.8±0.1	2.4±0.4***
22:5 n-3 (DPA)	2.1±0.1	1.4±0.1***
22:6 n-3 (DHA)	28.5±0.4	18.0±2.2***
Total n-3	36.8±0.3	22.3±2.8***
Total PUFA	51.2±0.3	37.9±2.3***

[1]Fatty acid (%) is present as % of total fatty acids. Values denoted with asterisk superscripts were significantly different from each other (*P<0.05 and ***P<0.001) as determined by a two-tailed, unpaired t-test. Data are means ± SEM of n=9-10.

A



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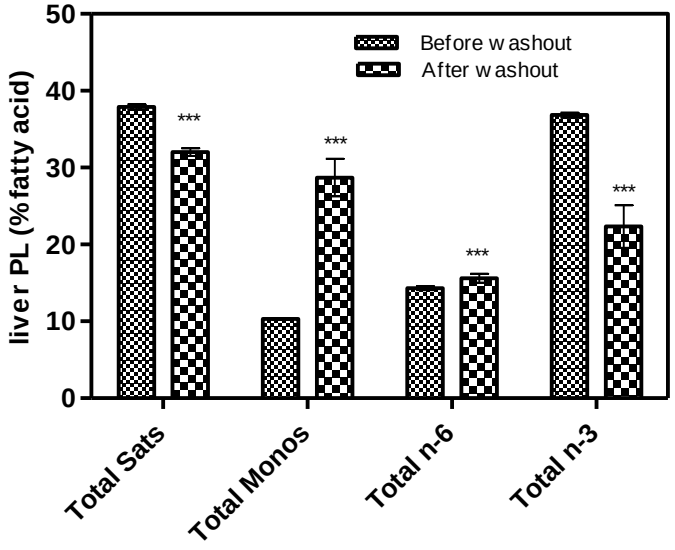

Figure 4. 2 Fatty acid compositions of fillet (A) and liver (B) phospholipids (PL). Statistical analysis for fatty acids was determined by a two-tailed, unpaired t-test. Data are means ± SEM of n=9-10 per group. Values with different asterisk superscripts are significantly different from each other ($^{***}P<0.001$).

Table 4. 14 Fatty acid compositions of barramundi fillet triglycerides before and after the 3 weeks on the washout diet

Fillet triglyceride	Before washout	After washout
Fatty acids (%)[1]		
16:0	22.4±0.5	20.7±0.8*
18:0	7.1±0.4	7.5±0.5
Total saturates	34.0±0.7	29.7±1.0*
16:1 n-7	3.8±0.1	8.9±0.5***
18:1 n-9	21.3±0.2	42.7±1.3***
18:1 n-7	2.9±0.1	2.5±0.8
Total monoenes	30.4±0.2	54.1±2.0***
18:2 n-6 (LA)	16.3±0.4	7.6±1.2***
18:3 n-6	0.8±0.0	N.D.
20:2 n-6	N.D.	N.D.
20:3 n-6	0.2±0.1	N.D.
20:4 n-6 (AA)	0.6±0.1	N.D.
22:2 n-6	N.D.	N.D.
22:4 n-6	0.1±0.0	N.D.
Total n-6	18.0±0.4	7.6±1.2***
18:3 n-3 (ALA)	1.3±0.0	N.D.
18:4 n-3	0.3±0.1	0.4±0.2
20:5 n-3 (EPA)	3.8±0.1	1.1±0.3***
22:5 n-3 (DPA)	1.6±0.1	0.6±0.2***
22:6 n-3 (DHA)	8.9±0.3	3.9±0.7***
Total n-3	16.1±0.5	6.0±1.1***
Total PUFA	34.1±0.7	13.6±1.8***

[1]Fatty acid (%) is present as % of total fatty acids. Values denoted with asterisk superscripts were significantly different from each other (*P<0.05 and ***P<0.001) as determined by a two-tailed, unpaired t-test. Data are means ± SEM of n=9-10.

Table 4. 15 Fatty acid compositions of barramundi liver triglycerides before and after the 3 weeks on the washout diet

Liver triglyceride	Before washout	After washout
Fatty acids (%)[1]		
16:0	21.2±0.1	18.9±0.8*
18:0	5.8±0.3	5.2±0.3
Total saturates	32.3±0.3	27.5±1.1***
16:1 n-7	3.6±0.1	9.5±0.4***
18:1 n-9	22.0±0.3	43.6±1.4***
18:1 n-7	2.7±0.0	3.9±0.1***
Total monoenes	31.3±0.2	60.1±2.0***
18:2 n-6 (LA)	17.0±0.5	6.2±1.2***
18:3 n-6	1.4±0.1	1.4±0.1
20:2 n-6	0.3±0.0	N.D.
20:3 n-6	0.3±0.0	< 0.05***
20:4 n-6 (AA)	0.7±0.0	< 0.05***
22:2 n-6	0.1±0.0	N.D.
22:4 n-6	0.2±0.0	< 0.05***
Total n-6	20.0±0.6	7.7±1.2***
18:3 n-3 (ALA)	1.3±0.0	0.1±0.1***
18:4 n-3	0.7±0.0	< 0.05***
20:5 n-3 (EPA)	3.6±0.2	0.8±0.2***
22:5 n-3 (DPA)	1.9±0.1	0.5±0.1***
22:6 n-3 (DHA)	7.9±0.4	2.1±0.6***
Total n-3	15.7±0.5	3.8±1.0***
Total PUFA	35.7±0.3	11.4±2.2***

[1]Fatty acid (%) is present as % of total fatty acids. Values denoted with asterisk superscripts were significantly different from each other (*P<0.05 and ***P<0.001) as determined by a two-tailed, unpaired t-test. Data are means ± SEM of n=9-10.

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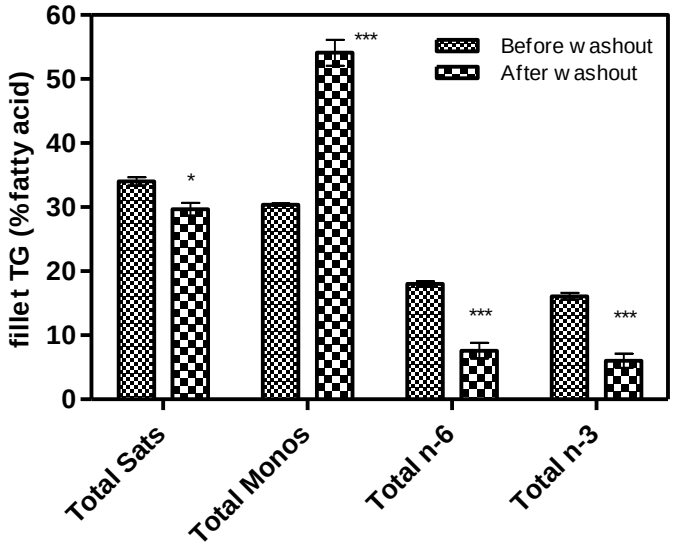

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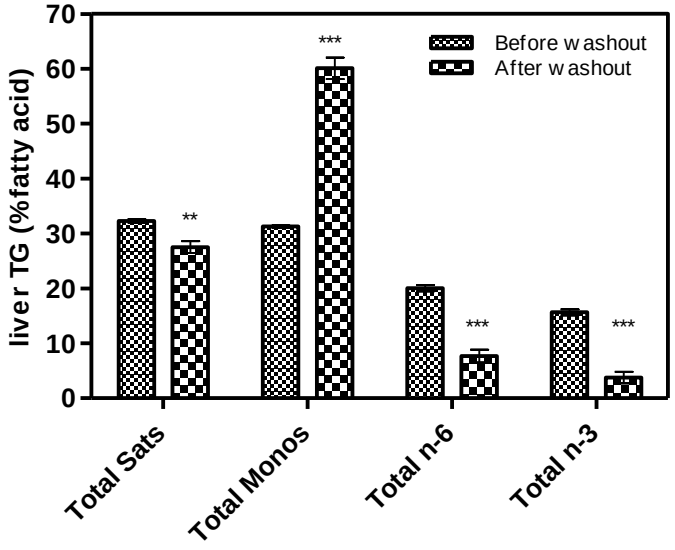


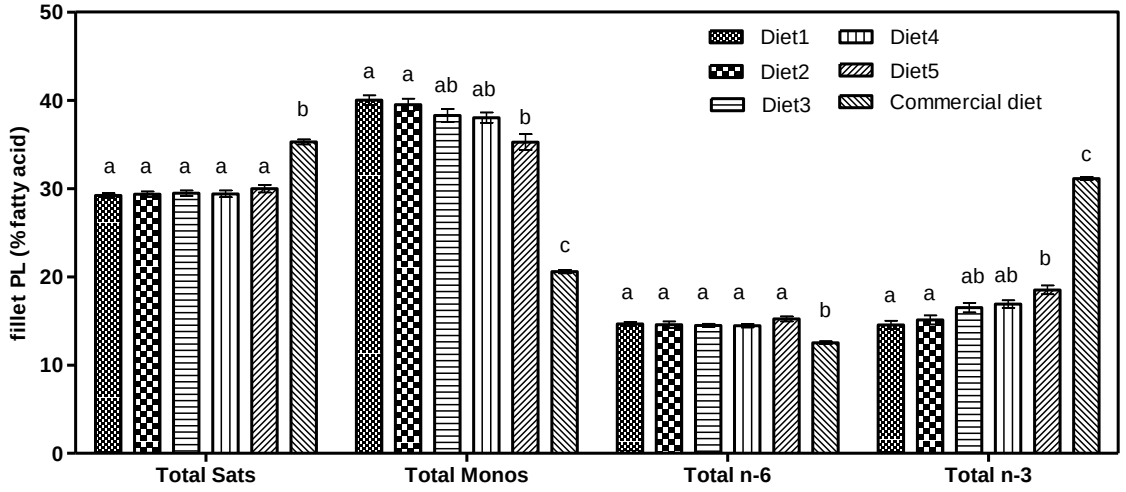
Figure 4. 3 Fatty acid compositions of fillet (A) and liver (B) triglycerides (TG). Statistical analysis for fatty acids was determined by a two-tailed, unpaired t-test. Data are means ± SEM of n=9-10 per group. Values with different superscripts are significantly different from each other ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$).

4.6.4 Effect of dietary treatments (vegetable oil-based and commercial feeds) on tissue fatty acid compositions

4.6.4.1 Fillet phospholipid fraction

There were no significant differences in total saturates among fish fed experimental diets 1 to diet 5, however the total saturates were about 1.2 fold higher in fish fed the commercial diet compared to those fed on any of the vegetable oil-based diets. The total n-3 fatty acid content in the fillet phospholipids increased with increasing dietary ALA concentration among experimental diets 1 to 5 however the average level of total n-3 was still 1.9 fold lower in the diet containing the highest ALA level than that in fish fed the commercial diet (Table 4.16 and Figure 4.4 A). No differences were observed in fillet phospholipid content of total n-6 fatty acids with increasing ALA content in fisf fed the 5 experimental diets. LA levels were constant but AA levels increased with increasing dietary ALA in fillet phospholipids of the fish fed the 5 experimental diets (Diets 1-5) (Table 4.16). Levels of total n-6 in fillet phospholipids were lower in fish fed the commercial diet compared with fish fed any of the 5 experimental diets (Figure 4.4 A). Fish fed the commercial reference diet had, on average, 2.4, 1.7 and 2 fold higher levels of EPA, DPA and DHA, respectively, in their fillet phospholipids than fish fed the vegetable oil-based diets (Diets 1-5) (Table 4.16 and Figure 4.4 B). No significant differences were observed in the levels of these n-3 LCPUFA in the fillet phospholipids among fish fed the 5 experimental vegetable oil-based diets (Table 4.16), however these fish fed diets 3, 4 and 5 had significantly higher levels of ALA in their fillet phospholipids compared to fish fed the commercial diet and the ALA content increased with increasing ALA content in the diet (Figure 4.4 B). Thus, fish fed diet 5 (dietary ALA 3.2%en) had the highest level of ALA in their fillet phospholipid fraction and this level was 12.2 fold higher than that in fish fed diet 1 (dietary ALA 0.1%en) (Table 4.16).

A



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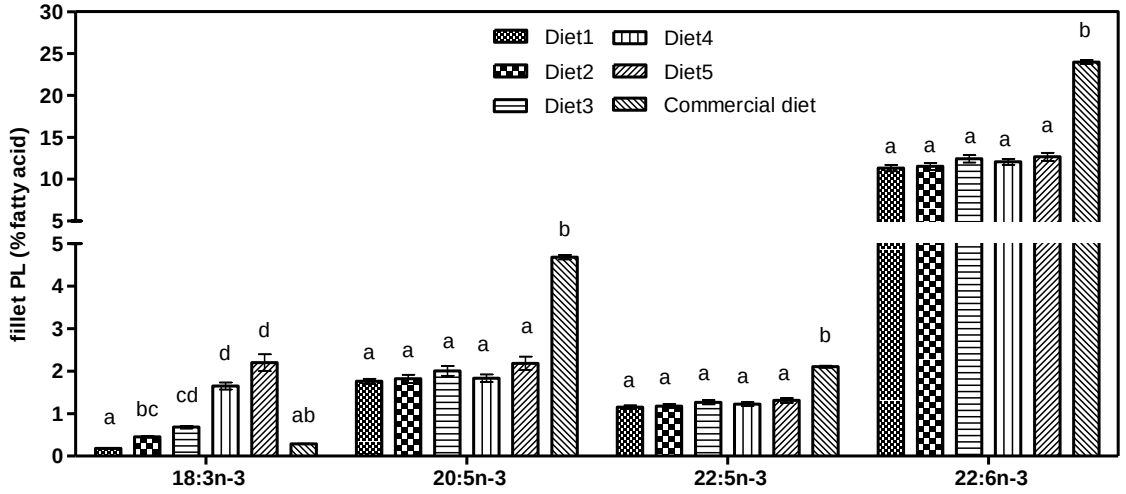


Figure 4. 4 Major fatty acid series (A) and n-3 fatty acids (B) of fillet phospholipids (PL). Data are means ± SEM of n=24 for all dietary groups. Values with different superscripts are significantly different from each other (P<0.05).

Table 4. 16 Fatty acid compositions of fish fillet phospholipids

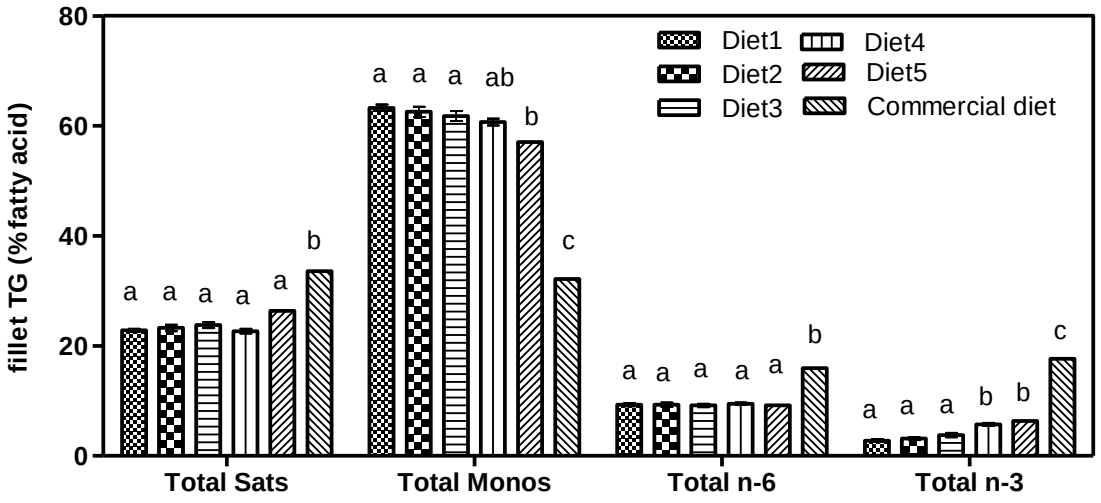
Fillet phospholipid	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Commercial diet
LA:ALA Ratio	21.9	4.7	2.9	1.2	0.7	8.8
LA%en	2.5	2.3	2.4	2.5	2.3	3.3
ALA%en	0.1	0.5	0.8	2.2	3.2	0.4
Fatty acids (%)[1]						
16:0	17.2±0.2^{a}	17.2±0.2^{a}	16.7±0.2^{a}	16.6±0.2^{a}	16.9±0.3^{a}	20.5±0.2^{b}
18:0	8.5±0.1^{a}	8.6±0.1^{a}	9.0±0.1^{b}	9.1±0.1^{b}	9.3±0.1^{b}	10.7±0.1^{c}
Total saturates	29.2±0.3^{a}	29.4±0.3^{a}	29.5±0.3^{a}	29.4±0.4^{a}	30.0±0.4^{a}	35.3±0.3^{b}
16:1 n-7	3.4±0.1^{a}	3.4±0.1^{a}	3.2±0.1^{a}	3.1±0.1^{ab}	2.7±0.1^{b}	1.3±0.1^{c}
18:1 n-9	30.9±0.4^{a}	30.2±0.5^{a}	29.3±0.5^{ab}	29.0±0.4^{ab}	27.0±0.6^{b}	14.8±0.2^{c}
18:1 n-7	3.3±0.1^{a}	3.3±0.1^{a}	3.3±0.1^{a}	3.3±0.1^{a}	3.0±0.1^{a}	2.5±0.1^{b}
Total monoenes	40.0±0.6^{a}	39.5±0.7^{a}	38.3±0.7^{ab}	38.0±0.6^{ab}	35.3±0.9^{b}	20.6±0.2^{c}
18:2 n-6 (LA)	10.1±0.2^{a}	10.1±0.3^{a}	9.9±0.2^{a}	10.0±0.2^{a}	10.4±0.2^{a}	7. 3±0.2^{b}
18:3 n-6	0.8±0.0^{a}	0.7±0.0^{ab}	0.7±0.0^{b}	0.7±0.0^{ab}	0.6±0.0^{b}	0.2±0.0^{c}
20:2 n-6	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.2±0.0^{b}
20:3 n-6	0.7±0.0^{a}	0.6±0.0^{ab}	0.6±0.0^{bc}	0.6±0.0^{bc}	0.5±0.0^{c}	0.4±0.0^{d}
20:4 n-6 (AA)	2.0±0.1^{a}	2.09±0.1^{a}	2.2±0.1^{ab}	2.1±0.1^{ab}	2.5±0.1^{b}	2.8±0.0^{c}
22:4 n-6	0.2±0.0^{a}	0.2±0.0^{a}	0.2±0.0^{a}	0.2±0.0^{a}	0.2±0.0^{a}	0.2±0.0^{b}
22:5 n-6	0.9±0.0^{a}	0.9±0.0^{a}	0.9±0.0^{a}	0.9±0.0^{a}	1.0±0.1^{a}	1.5±0.0^{b}
Total n-6	14.7±0.2^{a}	14.6±0.4^{a}	14.5±0.2^{a}	14.5±0.2^{a}	15.2±0.3^{a}	12.5±0.2^{b}
18:3 n-3 (ALA)	0.2±0.0^{a}	0.5±0.0^{bc}	0.7±0.0^{cd}	1.7±0.1^{d}	2.2±0.2^{d}	0.3±0.0^{ab}
18:4 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:3 n-3	N.D.	N.D.	N.D.	N.D.	< 0.05	< 0.05
20:5 n-3 (EPA)	1.8±0.1^{a}	1.8±0.1^{a}	2±0.1^{a}	1.8±0.1^{a}	2.2±0.2^{a}	4.7±0.1^{b}
22:5 n-3 (DPA)	1.2±0.0^{a}	1.2±0.1^{a}	1.3±0.1^{a}	1.2±0.1^{a}	1.3±0.1^{a}	2.1±0.0^{b}
22:6 n-3 (DHA)	11.3±0.4^{a}	11.5±0.4^{a}	12.4±0.4^{a}	12.1±0.4^{a}	12.7±0.5^{a}	24.0±0.2^{b}
Total n-3	14.6±0.5^{a}	15.1±0.5^{a}	16.5±0.6^{ab}	16.9±0.4^{ab}	18.5±0.5^{b}	31.1±0.2^{c}
Total PUFA	29.2±0.5^{a}	29.7±0.6^{a}	31.0±0.5^{ab}	31.4±0.4^{ab}	33.7±0.7^{b}	43.7±0.2^{c}

[1]Fatty acid (%) is present as % of total fatty acids. Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer or Kruskal-Wallis test. Data are means ± SEM of n=24 per group.

4.6.4.2 Fillet triglyceride fraction

The overall fatty acid composition in fillet triglyceride of fish fed the vegetable oil-based diets 1 to 5 and the commercial diet are shown in Figure 4.5 and Table 4.17. No significant differences were observed in the content of total saturates and total n-6 between the 5 vegetable oil-based diets. Total saturates and total n-6 in the fillet triglyceride fractions of fish fed the diets 1 to 5 were approximately 23.8% and 9.3%, respectively (Table 4.17). The pattern of fatty acid distribution was similar to that in fillet phospholipids (Figure 4.4). Total monoenes decreased and total n-3 increased significantly in the fillet triglyceride fractions with increasing dietary ALA content in the experimental vegetable oil-based diets 1 to 5 (Figure 4.5 A). Fish fed the commercial diet had 1.4, 1.7 and 4.1 fold higher levels of total saturates, total n-6 and total n-3 but 1.9 fold lower levels of monoenes in the fillet triglyceride fractions compared to fish fed the vegetable oil-based diets (Table 4.17). For the individual n-3 fatty acids, no significant differences were observed in fillet triglyceride EPA, DPA and DHA levels in fish fed the vegetable oil-based feeds (Diets 1-5), however, the levels were 10.2 fold, 6.9 fold and 6.5 fold higher for EPA, DPA and DHA, respectively, in fish fed the commercial diet compared with those fed the experimental diets 1 to 5 (Figure 4.5 B and Table 4.17). ALA levels in fillet triglycerides increased significantly with increasing dietary ALA content (Figure 4.5 B). Fillet triglyceride ALA levels in the fish fed on the commercial diet (ALA%en 0.4) were lower than those in fish fed on the high ALA vegetable oil-based diets 4 to 5 (ALA%en from 2.2 to 3.2) but similar to those in fish fed on diet 3 (ALA%en 0.8).

A



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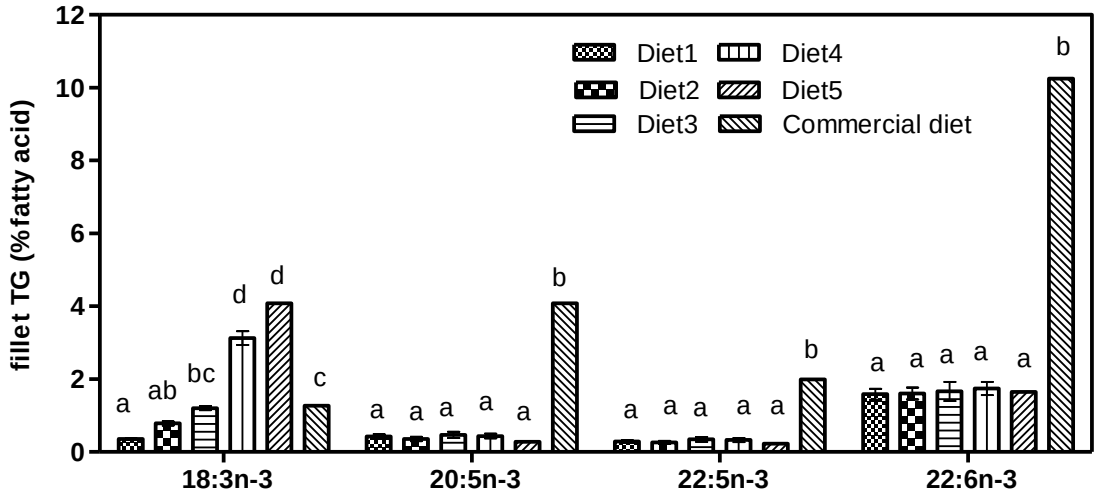


Figure 4. 5 Major fatty acid series (A) and n-3 fatty acids (B) of fillet triglycerides (TG). Data are means ± SEM of n=24 for all dietary groups. Values with different superscripts are significantly different from each other (P<0.05).

Table 4. 17 Fatty acid compositions of fish fillet triglycerides

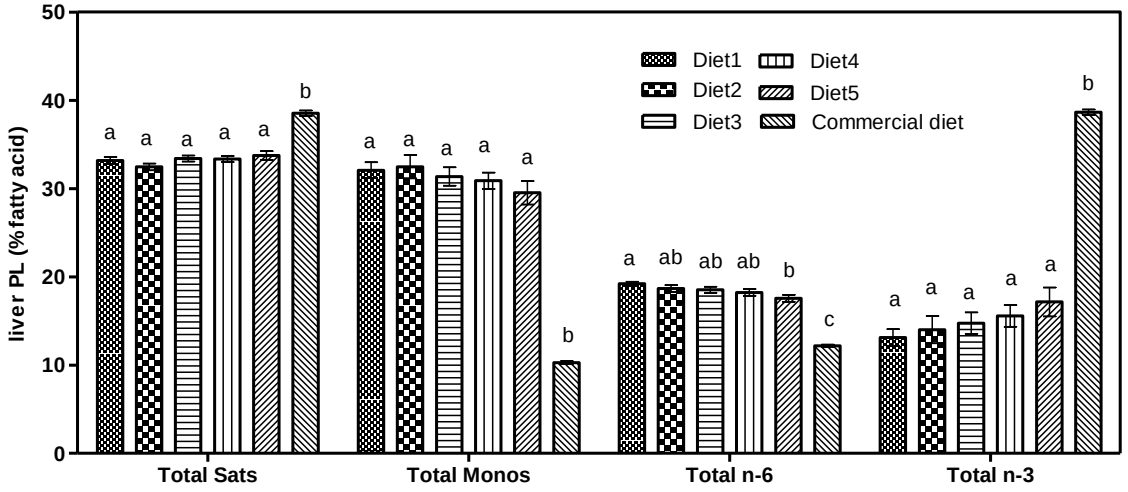
Fillet triglyceride	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Commercial diet
LA:ALA Ratio	21.9	4.7	2.9	1.2	0.7	8.8
LA%en	2.5	2.3	2.4	2.5	2.3	3.3
ALA%en	0.1	0.5	0.8	2.2	3.2	0.4
Fatty acids (%)[1]						
16:0	14.6±0.2^{a}	14.9±0.4^{a}	14.8±0.3^{a}	14.5±0.3^{a}	17.0±1.1^{a}	22.0±0.2^{b}
18:0	4.8±0.1^{ab}	5.1±0.2^{ab}	5.4±0.3^{abc}	4.9±0.2^{ab}	6.3±0.5^{bc}	5.7±0.1^{c}
Total saturates	22.9±0.2^{a}	23.3±0.6^{a}	23.8±0.5^{a}	22.7±0.4^{a}	26.4±1.5^{a}	33.6±0.3^{b}
16:1 n-7	11.3±0.2^{a}	11.3±0.4^{a}	10.7±0.3^{ab}	10.8±0.3^{ab}	9.2±0.5^{b}	5.1±0.1^{c}
18:1 n-9	46.0±0.5^{a}	45.2±0.6^{a}	45.1±0.7^{a}	43.9±0.4^{ab}	42.1±0.6^{b}	22.0±0.3^{c}
18:1 n-7	3.4±0.0^{a}	3.6±0.0^{b}	3.5±0.0^{ab}	3.3±0.0^{a}	3.3±0.1^{a}	2.8±0.0^{c}
Total monoenes	63.3±0.6^{a}	62.6±0.9^{a}	61.8±0.9^{a}	60.7±0.6^{ab}	57.1±1.0^{b}	32.1±0.3^{c}
18:2 n-6 (LA)	8.0±0.2^{a}	8.0±0.4^{a}	8.1±0.3^{a}	8.5±0.2^{a}	8.5±0.3^{a}	13.9±0.2^{b}
18:3 n-6	0.8±0.0^{a}	0.7±0.0^{ab}	0.6±0.0^{bc}	0.7±0.0^{abc}	0.5±0.1^{cd}	0.4±0.0^{d}
20:2 n-6	< 0.05^{a}	< 0.05^{a}	< 0.05^{a}	< 0.05^{a}	< 0.05^{a}	0.1±0.0^{b}
20:3 n-6	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{b}	0.2±0.0^{c}
20:4 n-6 (AA)	0.3±0.1^{a}	0.4±0.1^{a}	0.3±0.1^{a}	0.2±0.0^{a}	0.1±0.0^{a}	0.7±0.0^{b}
22:4 n-6	N.D.	N.D.	N.D.	N.D.	N.D.	0.1±0.0
22:5 n-6	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	< 0.05^{a}	< 0.05^{a}	0.69±0.0^{b}
Total n-6	9.3±0.3^{a}	9.3±0.4^{a}	9.2±0.3^{a}	9.5±0.2^{a}	9.2±0.3^{a}	16.0±0.2^{b}
18:3 n-3 (ALA)	0.4±0.0^{a}	0.8±0.1^{ab}	1.2±0.1^{bc}	3.1±0.2^{d}	4.1±0.5^{d}	1.3±0.0^{c}
18:4 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	0.3±0.1
20:3 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	< 0.05
20:5 n-3 (EPA)	0.4±0.1^{a}	0.4±0.1^{a}	0.5±0.1^{a}	0.4±0.1^{a}	0.3±0.1^{a}	4.18±0.1^{b}
22:5 n-3 (DPA)	0.3±0.0^{a}	0.3±0.0^{a}	0.3±0.1^{a}	0.3±0.1^{a}	0.2±0.1^{a}	2.0±0.0^{b}
22:6 n-3 (DHA)	1.3±0.2^{a}	1.6±0.2^{a}	1.7±0.3^{a}	1.7±0.2^{a}	1.7±0.3^{a}	10.3±0.2^{b}
Total n-3	2.8±0.3^{a}	3.1±0.3^{a}	3.8±0.4^{a}	5.7±0.3^{b}	6.3±0.5^{b}	17.7±0.3^{c}
Total PUFA	12.1±0.5^{a}	12.4±0.6^{a}	12.9±0.7^{a}	15.2±0.4^{b}	15.5±0.7^{b}	33.6±0.3^{c}

[1]Fatty acid (%) is present as % of total fatty acids. Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer or Kruskal-Wallis test. Data are means ± SEM of n=24 per group.

4.6.4.3 Liver phospholipid fraction

Effects of the vegetable oil-based diets and the commercial diet on fatty acid composition of liver phospholipids are shown in Figure 4.6. The saturated fatty acids 16:0 and 18:0 contributed equally to the total saturate content, which was approximately 30% of total fatty acids for the liver phospholipids in fish from all dietary treatments. There were no significant differences in total saturate content in liver phospholipid in fish fed the vegetable oil-based diets 1 to 5. Similarly, there was no difference in the total monoenes and total n-3 fatty acid content of liver phospholipids between the fish fed the 5 vegetable oil-based diets. The total n-6 level decreased with increasing dietary ALA content in fish fed the diets 1 to 5 (Figure 4.6 A and Table 4.18). Fish fed the commercial diet had lower total monoenes and total n-6 content, but a higher proportions of saturated and n-3 fatty acids (Figure 4.6 A and Table 4.18), particularly n-3 LCPUFA EPA, DPA and DHA (Figure 4.6 B and Table 4.18), compared to fish fed the vegetable oil-based diets. No significant differences were observed in liver phospholipid content of LA, 18:3 n-6 or any other n-6 LCPUFA between fish fed diets 1 to 5 (Table 4.18). The ALA content of liver phospholipids increased progressively with increasing dietary ALA content in fish fed the diets 1 to 5. Moreover, the liver phospholipid content of the ALA desaturation product 18:4 n-3 in fish fed the diets 4 and 5 appeared to increase with increasing dietary ALA levels (Table 4.18). When compared with fish fed the commercial diet, the EPA, DPA and DHA levels were 4.8, 2 and 2.7 fold lower in fish fed on the vegetable oil-based diets (Table 4.18).

A



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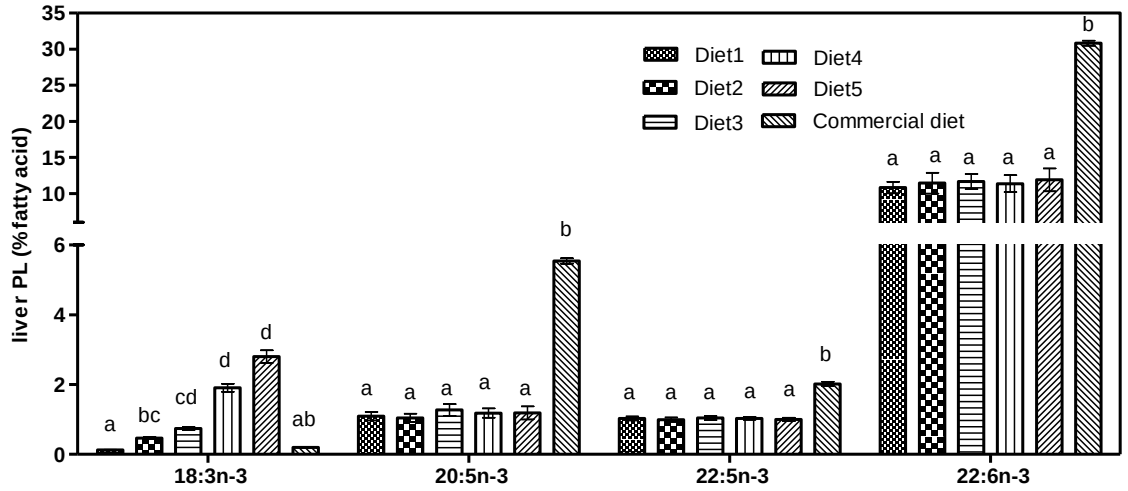


Figure 4. 6 Major fatty acid series (A) and n-3 fatty acids (B) of liver phospholipids. Data are means ± SEM of n=24 for all dietary groups except n=21 for dietary group 5 (Diet 5). Values with different superscripts are significantly different from each other (P<0.05).

Table 4. 18 Fatty acid compositions of fish liver phospholipids

Liver phospholipid	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Commercial diet
LA:ALA Ratio	21.9	4.7	2.9	1.2	0.7	8.8
LA%en	2.5	2.3	2.4	2.5	2.3	3.3
ALA%en	0.1	0.5	0.8	2.2	3.2	0.4
Fatty acids (%)[1]						
16:0	14.4±0.3^{a}	14.3±0.4^{a}	14.5±0.4^{a}	14.4±0.4^{a}	15.2±0.4^{a}	19.7±0.2^{b}
18:0	14.6±0.2	14.3±0.3	14.9±0.3	14.9±0.3	14.9±0.4	15.0±0.3
Total saturates	33.2±0.4^{a}	32.5±0.4^{a}	33.4±0.4^{a}	33.4±0.3^{a}	33.8±0.8^{a}	38.5±0.3^{b}
16:1 n-7	2.4±0.1^{a}	2.6±0.1^{a}	2.3±0.1^{a}	2.3±0.1^{a}	2.3±0.1^{a}	0.72±0.0^{b}
18:1 n-9	24.9±0.8^{a}	24.8±1.1^{a}	24.1±0.9^{a}	23.2±0.8^{a}	21.8±1.1^{a}	6.3±0.1^{b}
18:1 n-7	2.6±0.0^{a}	2.7±0.1^{a}	2.6±0.1^{a}	2.5±0.0^{a}	2.4±0.1^{a}	1.6±0.0^{b}
Total monoenes	32.1±0.9^{a}	32.5±1.3^{a}	31.4±1.1^{a}	30.9±0.9^{a}	29.5±1.3^{a}	10.3±0.2^{b}
18:2 n-6 (LA)	11.2±0.2^{a}	11.1±0.3^{a}	11.2±0.3^{a}	11.20.3^{a}	10.5±0.3^{a}	4.6±0.1^{b}
18:3 n-6	1.6±0.1^{a}	1.5±0.1^{a}	1.3±0.1^{a}	1.4±0.1^{a}	1.4±0.1^{a}	0.4±0.0^{b}
20:2 n-6	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.2±0.0^{b}
20:3 n-6	2.2±0.2^{a}	2.0±0.2^{a}	1.8±0.2^{a}	1.5±0.1^{a}	1.5±0.2^{a}	0.6±0.1^{b}
20:4 n-6 (AA)	3.0±0.1^{a}	2.9±0.1^{a}	3.0±0.1^{a}	2.9±0.1^{a}	2.9±0.1^{a}	4.8±0.1^{b}
22:4 n-6	0.3±0.0^{a}	0.2±0.0^{a}	0.3±0.0^{a}	0.3±0.0^{a}	0.3±0.0^{a}	0.1±0.0^{b}
22:5 n-6	0.9±0.0^{a}	0.8±0.1^{a}	0.9±0.1^{a}	0.9±0.1^{a}	0.9±0.1^{a}	1.4±0.0^{b}
Total n-6	19.2±0.2^{a}	18.7±0.4^{ab}	18.5±0.3^{ab}	18.2±0.4^{ab}	17.6±0.4^{b}	12.2±0.2^{c}
18:3 n-3 (ALA)	0.1±0.0^{a}	0.5±0.0^{bc}	0.7±0.0^{cd}	1.9±0.1^{d}	2.8±0.2^{d}	0.2±0.0^{ab}
18:4 n-3	< 0.05^{a}	< 0.05^{a}	< 0.05^{a}	0.1±0.1^{ab}	0.3±0.1^{b}	0.1±0.0^{a}
20:3 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:5 n-3 (EPA)	1.1±0.1^{a}	1.0±0.1^{a}	1.3±0.2^{a}	1.2±0.1^{a}	1.2±0.2^{a}	5.5±0.1^{b}
22:5 n-3 (DPA)	1.0±0.1^{a}	1.0±0.1^{a}	1.0±0.1^{a}	1.0±0.0^{a}	1.0±0.1^{a}	2.0±0.1^{b}
22:6 n-3 (DHA)	10.8±0.8^{a}	11.5±1.5^{a}	11.7±1.0^{a}	11.4±1.2^{a}	11.9±1.6^{a}	30.8±0.4^{b}
Total n-3	13.1±1.0^{a}	14.0±1.6^{a}	14.7±1.2^{a}	15.6±1.2^{a}	17.2±1.6^{a}	38.6±0.3^{b}
Total PUFA	32.3±0.8^{a}	32.7±1.3^{a}	33.3±1.0^{a}	33.8±0.9^{a}	34.7±1.3^{a}	50.8±0.3^{b}

[1]Fatty acid (%) is present as % of total fatty acids. Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer or Kruskal-Wallis test. Data are means ± SEM of n=24 per group.

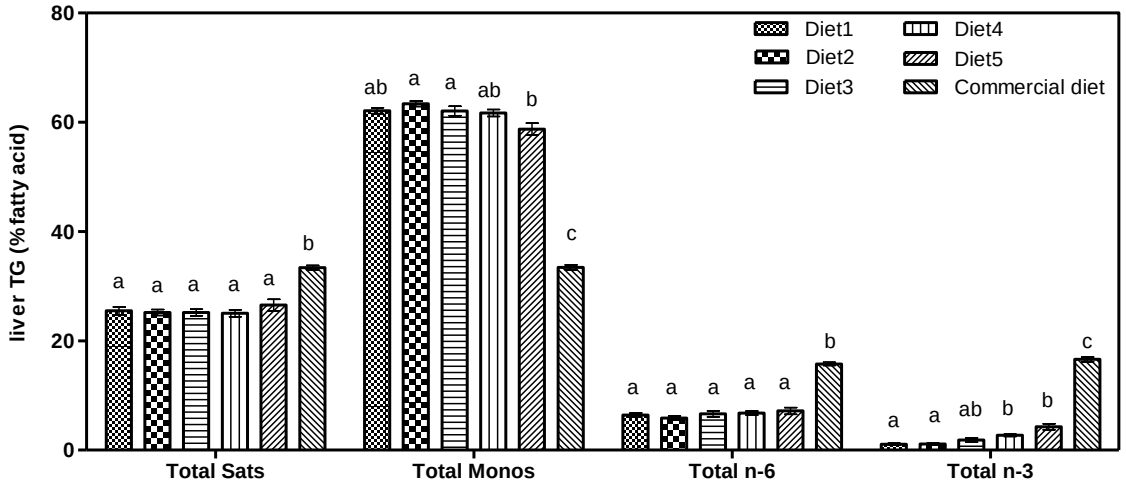
4.6.4.4 Liver triglyceride fraction

Total fatty acid composition in liver triglycerides of fish fed the vegetable oil-based diets (Diets 1-5) and the commercial diet are shown in Figure 4.7 and Table 4.19. No significant differences were observed in the level of total saturates and total n-6 fatty acids between the 5 vegetable oil-based diets. Total saturates and total n-6 in liver triglycerides of fish fed the diets 1 to 5 were approximately 25.5% and 6.6% respectively (Table 4.19). The fatty acid composition followed a similar trend to that in the fillet triglyceride fractions (Figure 4.5). Total monoenes appeared to decrease and total n-3 appeared to increase significantly in liver triglycerides with increasing dietary ALA levels (Figure 4.7 A). Fish fed the commercial diet had a 1.3, 2.4 and 7.5 fold higher content of total saturates, total n-6 and total n-3 and 1.8 fold lower total monoene content in liver triglycerides than the vegetable oil-based diets. For the individual n-3 fatty acids, no significant differences were observed in EPA, DPA and DHA levels in liver triglycerides of fish fed the vegetable oil-based feeds (Diets 1-5), however, EPA, DPA and DHA levels were 22.2 fold, 15.5 fold and 15.3 fold lower respectively, compared to levels in fish fed the commercial diet (Figure 4.7 B and Table 4.19). ALA levels in liver triglycerides (Figure 4.7 B and Table 4.19) increased significantly with increasing dietary ALA content.

In summary, fillet and liver phospholipid fractions both shared similar fatty acid profiles and responses to the different experimental diets. A similar phenomenon was also observed in fillet and liver triglyceride fractions. There were no significant difference in the levels of total saturates or any of n-3 LCPUFA EPA, DPA and DHA in tissues between the different vegetable oil-based diets (Diets 1-5) (Figure 4.4 and 4.6). There were also no significant differences in total n-6 fatty acid content in fillet phospholipid and triglyceride as well as liver triglyceride fractions among the 5 vegetable oil-based diets, however fish fed on the diet 5 had significantly lower total n-6 content in liver phospholipid compared to the fish fed on diet 1 (Table 4.18). In fish fed the vegetable oil-based diets, the ALA content of the fillet and liver increased whilst the monoene content decreased with increasing dietary ALA content (Figure 4.4-4.7 and Table 4.16-4.19). Compared to fish fed the commercial fish diet, however, the vegetable oil-based diets were all associated with lower EPA, DPA and DHA contents in both the fillet and the liver, particularly the triglyceride fractions (Figure 4.4-4.7 and Table 4.16-4.19). These results suggest that ALA is the only n-3 fatty acid that accumulated in fish tissues to any significant extent as dietary ALA content increased, and that there was no

corresponding increase in the n-3 LCPUFA content of these tissues in response to increased ALA in the diets (Figure 4.8 and 4.9).

A



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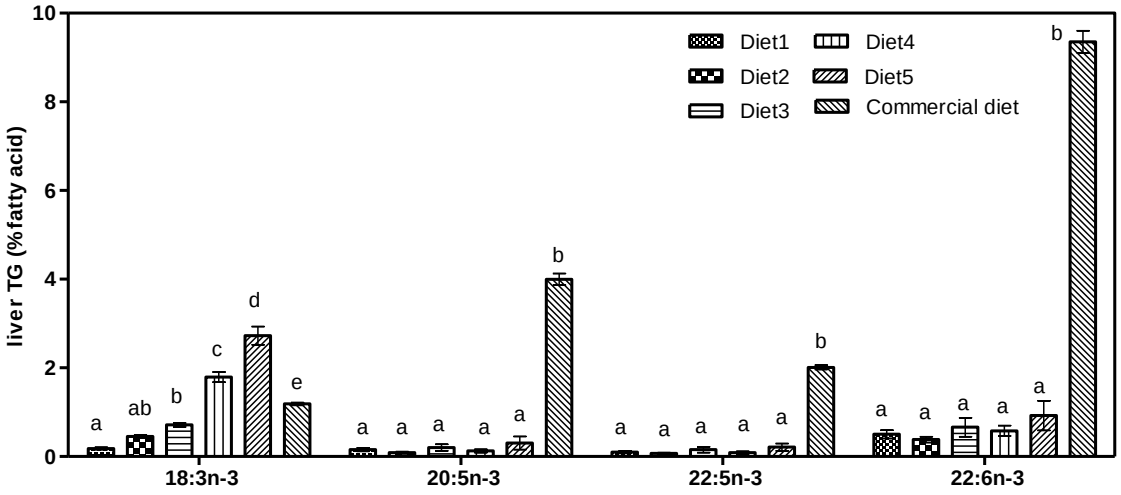

Figure 4. 7 Major fatty acid series (A) and n-3 fatty acids (B) of liver triglycerides (TG). Data are means ± SEM of n=24 for all dietary groups except n=21 for dietary group 5 (Diet 5). Values with different superscripts are significantly different from each other (P<0.05).

Table 4. 19 Fatty acid compositions of fish liver triglycerides

Liver triglyceride	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Commercial diet
LA:ALA Ratio	21.9	4.7	2.9	1.2	0.7	8.8
LA%en	2.5	2.3	2.4	2.5	2.3	3.3
ALA%en	0.1	0.5	0.8	2.2	3.2	0.4
Fatty acids (%)[1]						
16:0	17.3±0.6^{a}	17.3±0.8^{a}	16.7±0.5^{a}	16.8±0.5^{a}	17.9±0.9^{a}	22.3±0.3^{b}
18:0	4.5±0.1^{a}	4.4±0.2^{a}	4.7±0.1^{a}	4.7±0.1^{a}	5.1±0.2^{a}	5.9±0.1^{b}
Total saturates	25.5±0.7^{a}	25.2±0.5^{a}	25.2±0.6^{a}	25.1±0.6^{a}	26.6±1.1^{a}	33.4±0.4^{b}
16:1 n-7	9.3±0.3^{a}	9.4±0.4^{a}	9.2±0.2^{ab}	8.8±0.3^{ab}	8.3±0.3^{b}	4.7±0.1^{c}
18:1 n-9	46.0±0.4^{a}	47.3±1.0^{a}	45.8±0.6^{ab}	45.6±0.4^{abc}	43.3±0.8^{c}	23.1±0.4^{d}
18:1 n-7	3.6±0.0^{ab}	3.5±0.2^{a}	3.6±0.1^{ab}	3.5±0.0^{bc}	3.4±0.1^{c}	2.9±0.0^{d}
Total monoenes	63.1±0.6^{ab}	63.4±0.5^{a}	62.1±0.9^{a}	61.7±0.6^{ab}	58.7±1.1^{b}	33.4±0.5^{c}
18:2 n-6 (LA)	4.6±0.4^{a}	4.3±0.3^{a}	5.0±0.5^{a}	5.2±0.4^{a}	5.6±0.6^{a}	13.4±0.3^{b}
18:3 n-6	1.6±0.1^{a}	1.5±0.1^{a}	1.5±0.1^{a}	1.5±0.1^{a}	1.4±0.1^{a}	0.7±0.0^{b}
20:2 n-6	< 0.05^{a}	N.D.	< 0.05^{a}	< 0.05^{a}	N.D.	0.1±0.0^{b}
20:3 n-6	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{a}	0.2±0.0^{b}
20:4 n-6 (AA)	< 0.05^{a}	< 0.05^{a}	0.1±0.0^{a}	< 0.05^{a}	0.1±0.0^{a}	0.7±0.0^{b}
22:4 n-6	N.D.	N.D.	N.D.	N.D.	N.D.	0.1±0.0
22:5 n-6	< 0.05^{a}	< 0.05^{a}	< 0.05^{a}	< 0.05^{a}	0.1±0.0^{a}	0.6±0.0^{b}
Total n-6	6.4±0.4^{a}	5.9±0.4^{a}	6.6±0.6^{a}	6.8±0.4^{a}	7.2±0.6^{a}	15.8±0.3^{b}
18:3 n-3 (ALA)	0.2±0.0^{a}	0.5±0.0^{ab}	0.7±0.0^{b}	1.8±0.1^{c}	2.7±0.2^{d}	1.2±0.0^{e}
18:4 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	0.5±0.1
20:3 n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:5 n-3 (EPA)	0.2±0.0^{a}	0.1±0.0^{a}	0.2±0.1^{a}	0.1±0.0^{a}	0.3±0.2^{a}	4.0±0.1^{b}
22:5 n-3 (DPA)	0.1±0.0^{a}	0.1±0.0^{a}	0.2±0.1^{a}	0.1±0.0^{a}	0.2±0.1^{a}	2.0±0.1^{b}
22:6 n-3 (DHA)	0.5±0.1^{a}	0.4±0.1^{a}	0.7±0.2^{a}	0.6±0.1^{a}	0.9±0.3^{a}	9.4±0.3^{b}
Total n-3	1.1±0.2^{a}	1.1±0.1^{a}	1.9±0.4^{ab}	2.7±0.2^{b}	4.3±0.6^{b}	16.6±0.4^{c}
Total PUFA	7.5±0.6^{a}	7.0±0.5^{a}	8.5±0.9^{ab}	9.5±0.6^{ab}	11.4±1.2^{b}	32.4±0.5^{c}

[1]Fatty acid (%) is present as % of total fatty acids. Values with different superscripts are significantly different from each other (P<0.05) as determined by one-way ANOVA with Tukey-Kramer or Kruskal-Wallis test. Data are means ± SEM of n=24 per group.

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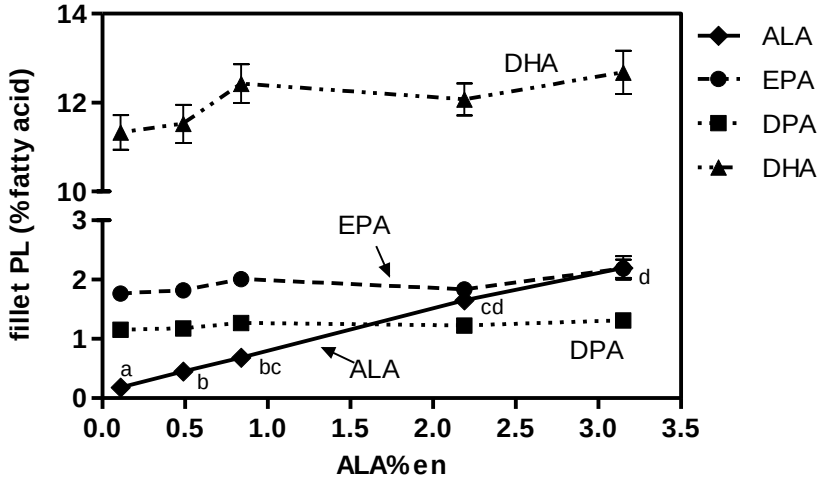

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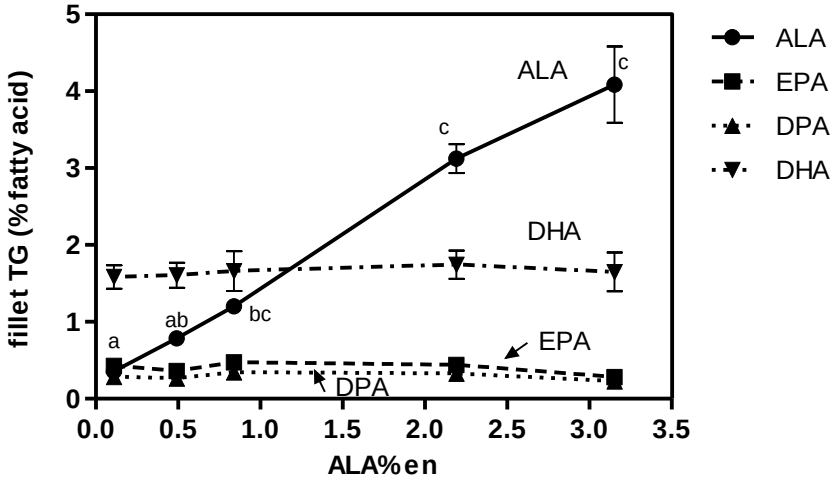
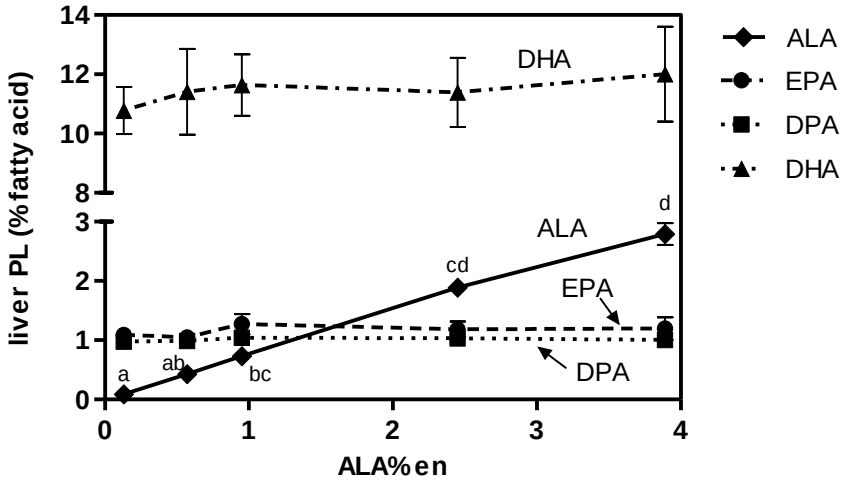


Figure 4. 8 N-3 fatty acids of fillet phospholipids (PL) (A) and triglycerides (TG) (B) in fish fed the diets 1 to 5 with increasing dietary ALA. Data are means ± SEM of n=24 for all dietary groups. Statistical analysis for fatty acid compositions was determined by one-way ANOVA with Tukey-Kramer Multiple or Kruskal-Wallis test. Values with different superscripts are significantly different from each other (P<0.05).

A



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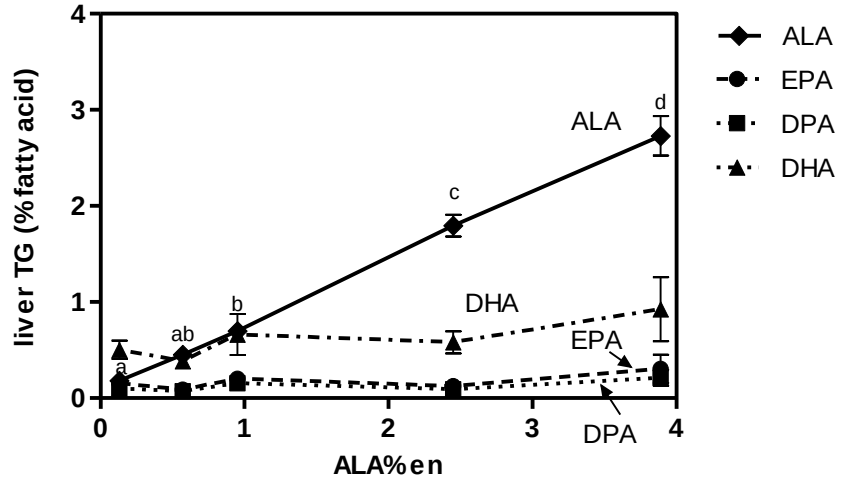


Figure 4. 9 N-3 fatty acids of liver phospholipids (PL) (A) and triglycerides (TG) (B) in fish fed the diets 1 to 5 with increasing dietary ALA. Statistical analysis for fatty acid compositions was determined by one-way ANOVA with Tukey-Kramer Multiple or Kruskal-Wallis test. Values with different superscripts are significantly different from each other (P<0.05). Data are means ± SEM of n=21-24 for all dietary groups.

4.6.5 Effect of dietary treatments (vegetable oil-based and commercial feeds) on tissue fatty acid profiles in individual fish

Scatter plots of fatty acid compositions of the individual fish fed with the 5 vegetable oil-based diets and the commercial diets are presented in Figure 4.10 and 4.11. The results show remarkable diversities in fatty acids in tissues of individual fish, particularly in the liver, after the period of feeding on the vegetable oil-based diets (Figure 4.10 and 4.11 A-E). In contrast, the diversity of fatty acid composition between individual fish was negligible in fish fed the commercial diet (Figure 4.10 and 4.11 F). In the fish fed on the commercial diet, the major n-3 LCPUFA was DHA which ranged from 26.7 to 34% of total fatty acid in livers (Figure 4.10 F) and 21.5 to 26.1% of total fatty acids in fillets (Figure 4.11 F). On the other hand, though DHA was still the main n-3 LCPUFA in the both fillet and liver, the level varied massively from 5.9 to 21.2% in liver phospholipids of the fish fed on the diet 1 (Figure 4.10 A) and 4.2 to 34.3% in fish fed on the diet 5 (Figure 4.10 E). Similar ranges were also observed in liver phospholipid DHA content among fish fed the diets 2 to 4 (Figure 4.10 B-D). When the differences between individual fish in liver phospholipid fatty acid contents were expressed as coefficient of variation (CV%) values, the average CV% was as high as 61.1±3.3% for EPA, 25.1±1.8% for DPA and 50.6±5.0% for DHA in the fish fed any of the vegetable oil-based diets compared with a CV% of only 6.9%, 15% and 5.6% for EPA, DPA and DHA, respectively, in the fish fed the commercial diet (Figure 4.10). Similar results were also observed in fillet phospholipid in which the average CV% were smaller than those in liver phospholipid, with 25.6±2.9% for EPA, 19±0.8% for DPA and 17.1±0.7% for DHA in fillet phospholipids of fish fed the vegetable oil-based diets (Figure 4.11). In contrast, the CV% values in fillet phospholipid of fish fed on the commercial diet were much lower than those in the same tissue of fish fed on the vegetable oil-based feeds, with CV% of 5.5% for EPA, 5.4% for DPA and 4.9% for DHA (Figure 4.11). Furthermore, similar diversities in fatty acid composition were also found in fillet phospholipids of fish fed on the 5 vegetable oil-based diets, however to a lesser extent than in the liver. For example, the level of DHA ranged from 8.8 to 16% of total fatty acids in fillet phospholipid of the fish fed on the diet 1 (Figure 4.11 A) and 8.9 to 17.6% of total fatty acids in fish fed the diet 5 (Figure 4.11 E).

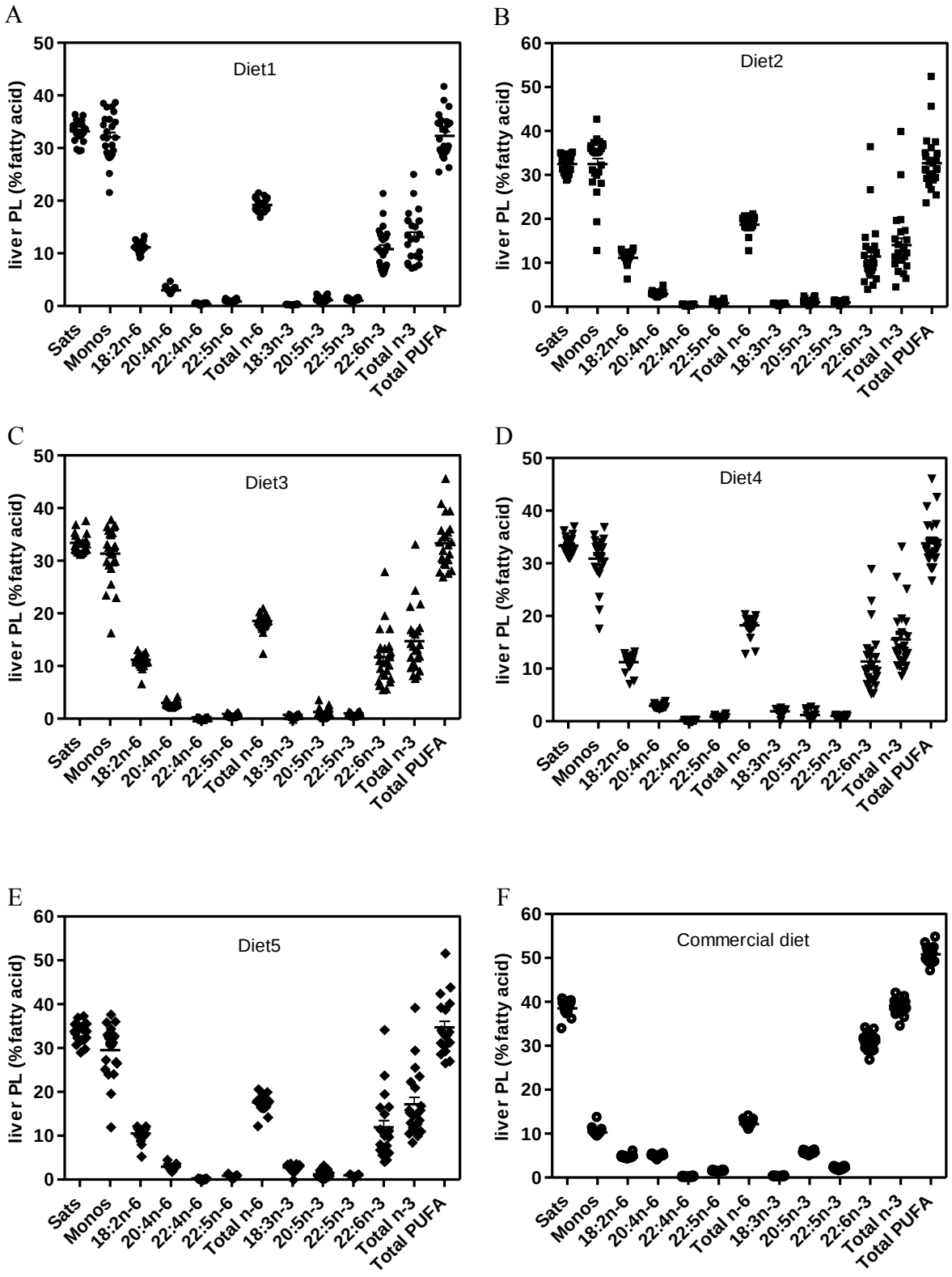


Figure 4. 10 Individual variation in the fatty acid composition of liver phospholipids (PL) in fish fed the 5 vegetable oil-based diets and the commercial diet. Scatter plots were plotted using fatty acid data from 20-24 fish per dietary treatments (A-F).

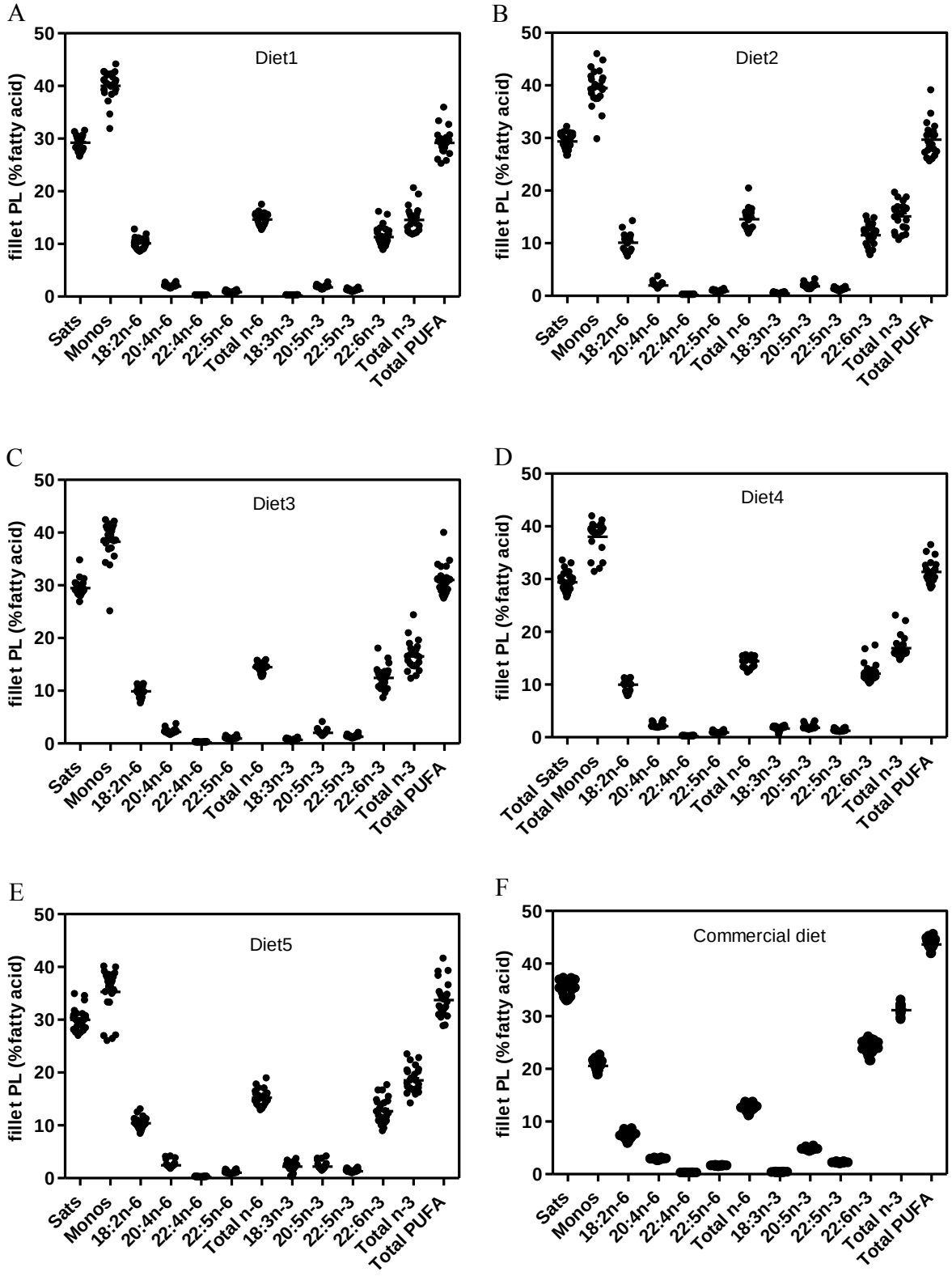


Figure 4. 11 Individual variation in the fatty acid composition of fillet phospholipids (PL) in fish fed the 5 vegetable oil-based diets and the commercial diet. Scatter plots were plotted using fatty acid data from 20-24 fish per dietary treatments (A-F).

4.6.6 Gene stability measure and ranking of selected housekeeping genes

In this study, the geNorm expression stability analysis indicates that β-Actin and 18s rRNA had the highest stability value (Figure 4.12). However, due to the low threshold cycle (Ct) value (high mRNA abundance) of 18s rRNA (Ct<6) at the RNA concentration that I tested for all housekeeping and target genes, β-Actin, which had a Ct value of 16, was chosen as an internal control for assessing gene expression levels of barramundi FADS2 and ELOVL.

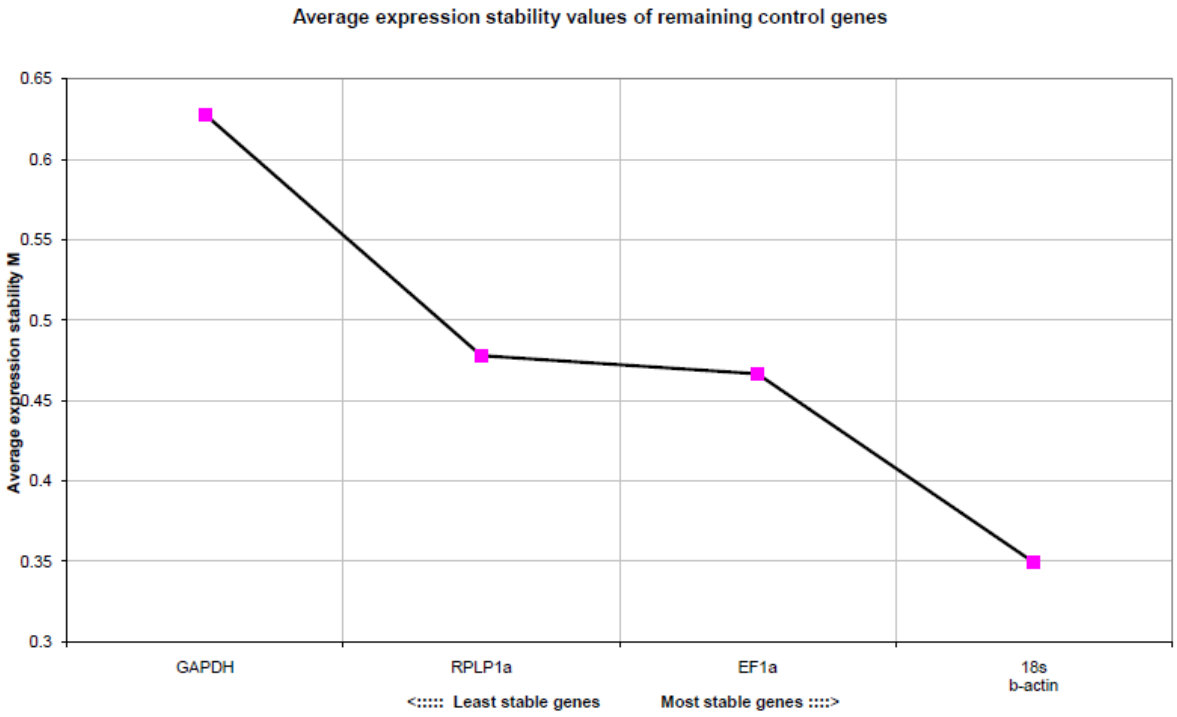
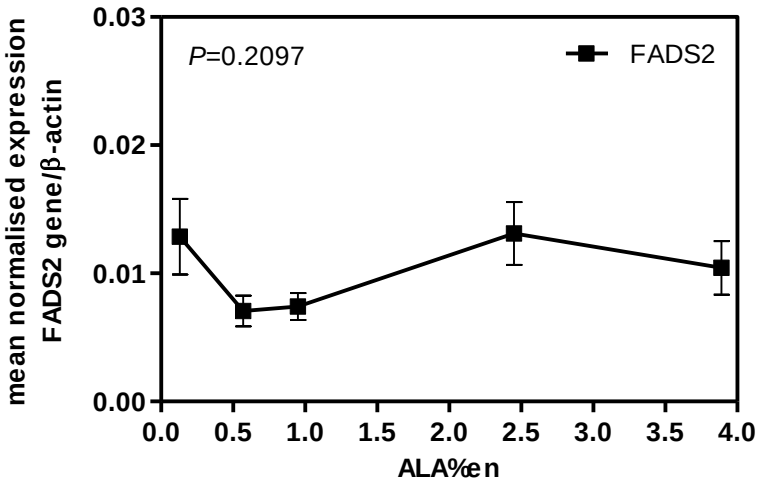


Figure 4. 12 Average expression stability values (M) of five housekeeping gene candidates during stepwise exclusion of the least stable control gene were calculated by the geNorm program. Low M values correspond to high expression stability.

4.6.7 Effect of dietary treatments (vegetable oil-based and commercial feeds) on mRNA expression levels of FADS2 and ELOVL

The effect of dietary ALA content on the mRNA expression of two of the most important enzymes in the LCPUFA synthetic pathway, Δ6 desaturase (FADS2) and elongase (ELOVL) is shown in Figure 4.13. FADS2 (Figure 4.13 A) and ELOVL (Figure 4.13 B) mRNA expression was not different between the vegetable oil-based diets across the range of dietary ALA content of 0.1%en to 3.2%en. Despite the fact that there were no significant differences in gene expression levels of hepatic FADS2 and ELOVL among the fish fed on the vegetable oil-based diets, the expression of both FADS2 (Figure 4.14 A) and ELOVL (Figure 4.14 B) mRNA was higher in fish fed on any of the vegetable oil-based diets compared to those fed on the commercial diet. On average, the relative expression of the FADS2 gene was 9.96 fold (Figure 4.14 A) and ELOVL gene was 3.12 fold (Figure 4.14 B) higher in fish fed on the vegetable oil-based diets than in the fish fed on the commercial diet, irrespective of the dietary ALA content.

A



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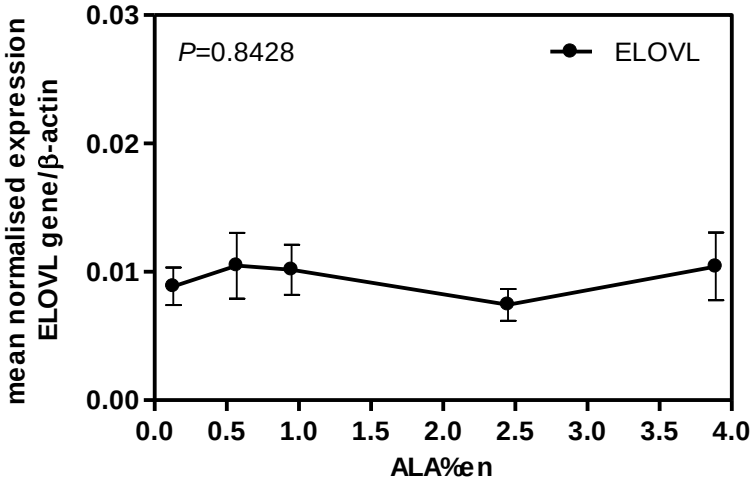
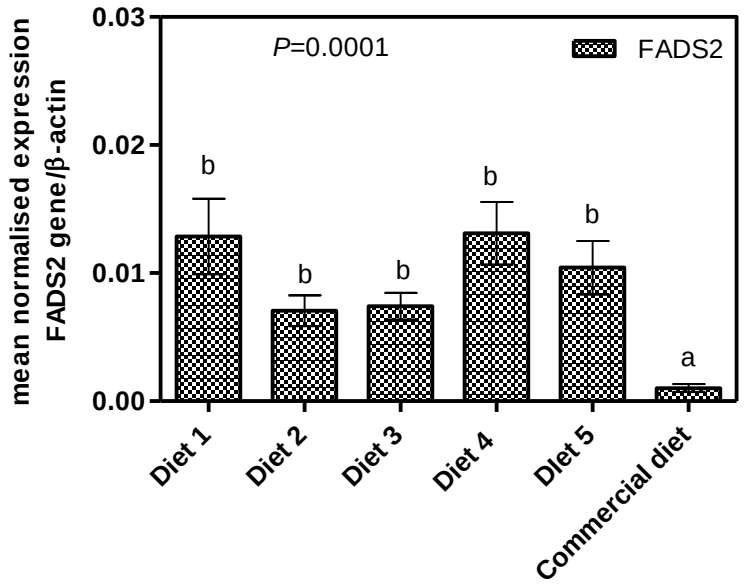


Figure 4. 13 The relative gene expression of FADS2 (A) and ELOVL (B) in liver of fish fed the 5 vegetable oil-based diets (Diets 1-5) with increasing dietary ALA. Relative transcript (mRNA) levels were determined by qRT-PCR and normalised by housekeeping gene β-Actin. Abundance of mRNA is expressed as the mean normalised expression value. Statistical analysis was determined by one-way ANOVA with Tukey-Kramer Multiple or Kruskal-Wallis test. No significant differences were found between groups. Data are mean ± SEM (n=21-24).

A



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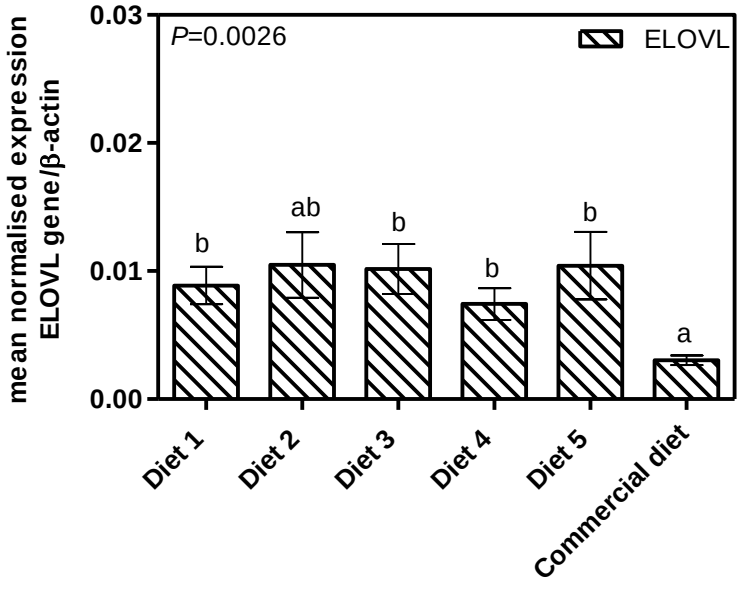


Figure 4. 14 The relative gene expression of FADS2 (A) and ELOVL (B) in liver of fish fed the 5 vegetable oil-based diets (Diets 1-5) with increasing dietary ALA and the commercial reference diet. Relative transcript (mRNA) levels were determined by qRT-PCR and normalised by housekeeping gene β-Actin. Abundance of mRNA is expressed as the mean normalised expression value. Statistical analysis was determined by one-way ANOVA with Tukey-Kramer Multiple or Kruskal-Wallis test. Values with different superscripts are significantly different from each other (P<0.05). Data are mean ± SEM (n=21-24).

4.6.8 Relationship between liver phospholipid DHA content and fish body weight of fish fed on different dietary treatments (vegetable oil-based and commercial feeds)

The relationships between DHA level of barramundi liver phospholipid and the body weight of the fish are presented in Figure 4.15. Linear regression analysis showed that there was no significant correlation between liver DHA content (% fatty acid) and body weight in fish fed either the vegetable oil-based diets (Figure 4.15 A-E) or the fish oil-based diet (Figure 4.15 F).

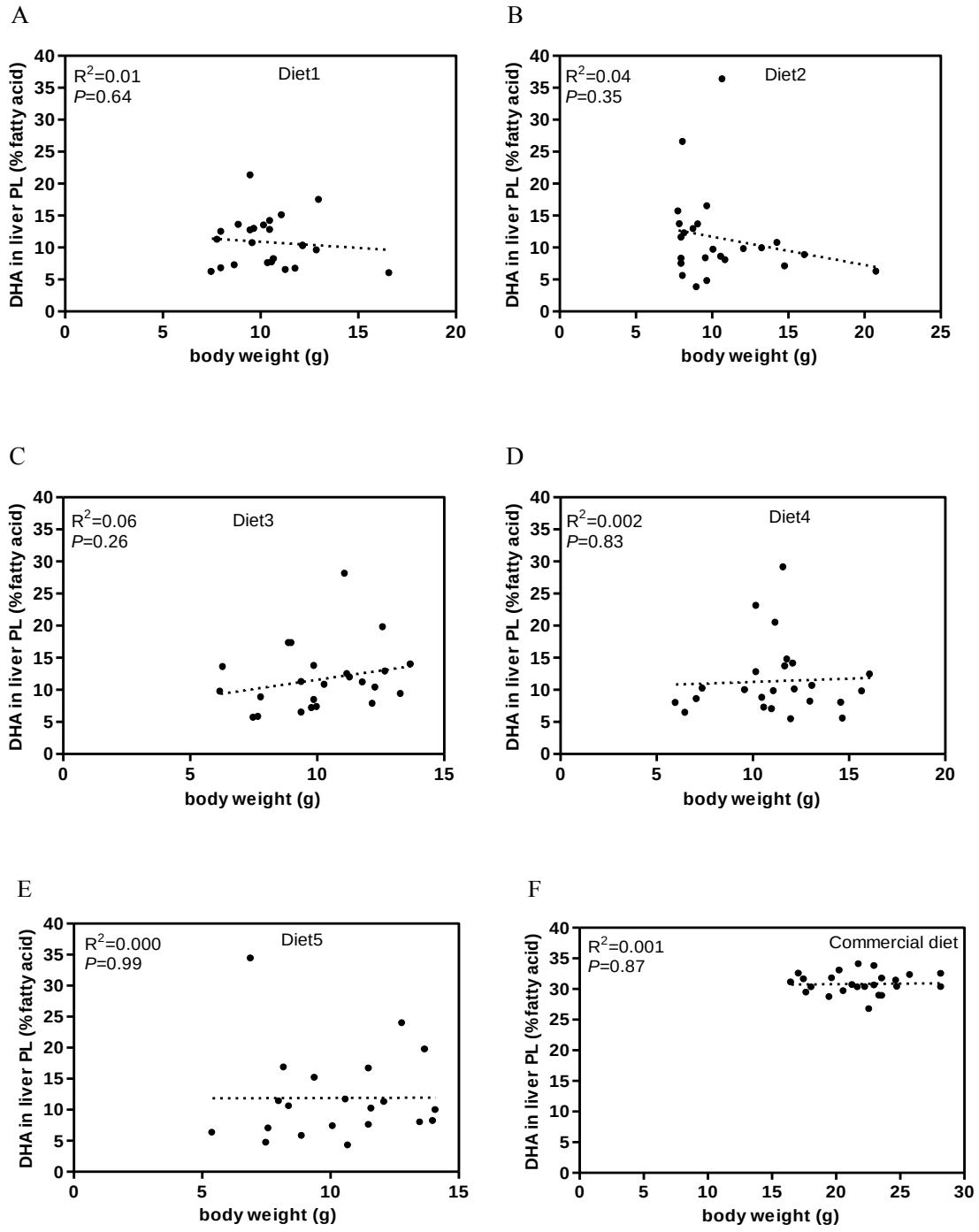


Figure 4. 15 Correlations between liver phospholipid (PL) DHA levels and fish body weight in fish fed the 5 vegetable oil-based diets (Diets 1-5) and the commercial reference diet. Scatter plots were plotted using fatty acid data from 20-24 fish per dietary treatments (A-F).

4.6.9 Relationship between liver phospholipid DHA content and mRNA expression levels of FADS2 and ELOVL of fish fed on different dietary treatments (vegetable oil-based diets and commercial feed)

The relationships between DHA level of barramundi liver phospholipid and mRNA levels of FADS2 and ELOVL of the fish are presented in Figures 4.16 and 4.17, respectively. Linear regression analysis showed that there was no significant correlation between liver DHA content (% fatty acid) and hepatic gene expression of either gene in fish fed the vegetable oil-based diets (Figures 4.16 and 4.17 A-E) or the fish oil-based commercial diet (Figures 4.16 and 4.17 F). However, liver DHA levels in fish fed vegetable oil-based diets tended (P=0.07) to be inversely related to FADS2 and ELOVL expression, such that the DHA% of total fatty acids decreased with increasing relative FADS2 and ELOVL expression; but this relationship was not statistically significant.

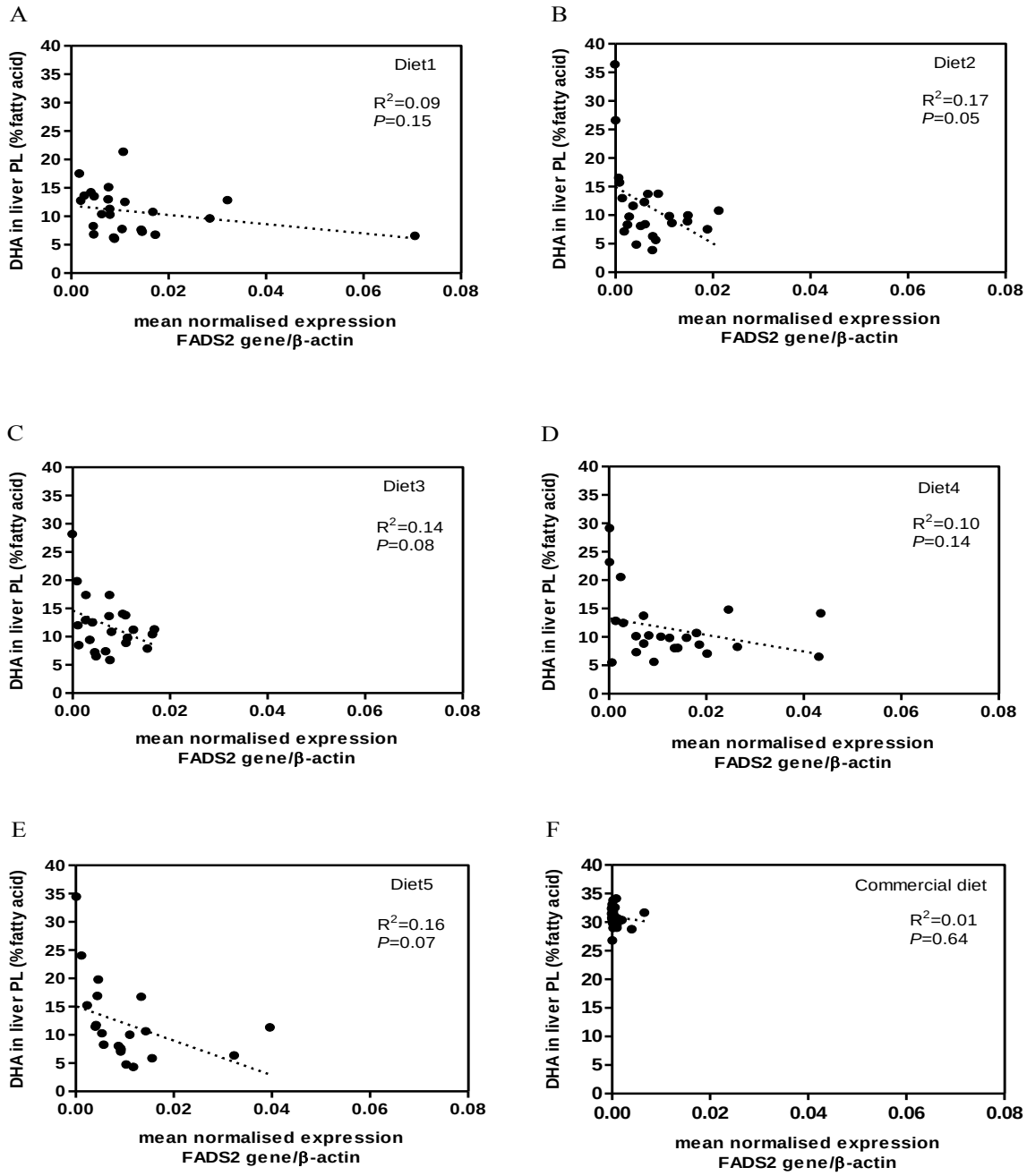


Figure 4. 16 Correlations between liver phospholipid (PL) DHA levels and hepatic gene expression levels of FADS2 in fish fed the 5 vegetable oil-based diets (Diets 1-5) and the commercial reference diet. Scatter plots were plotted using fatty acid data from 20-24 fish per dietary treatments (A-F).

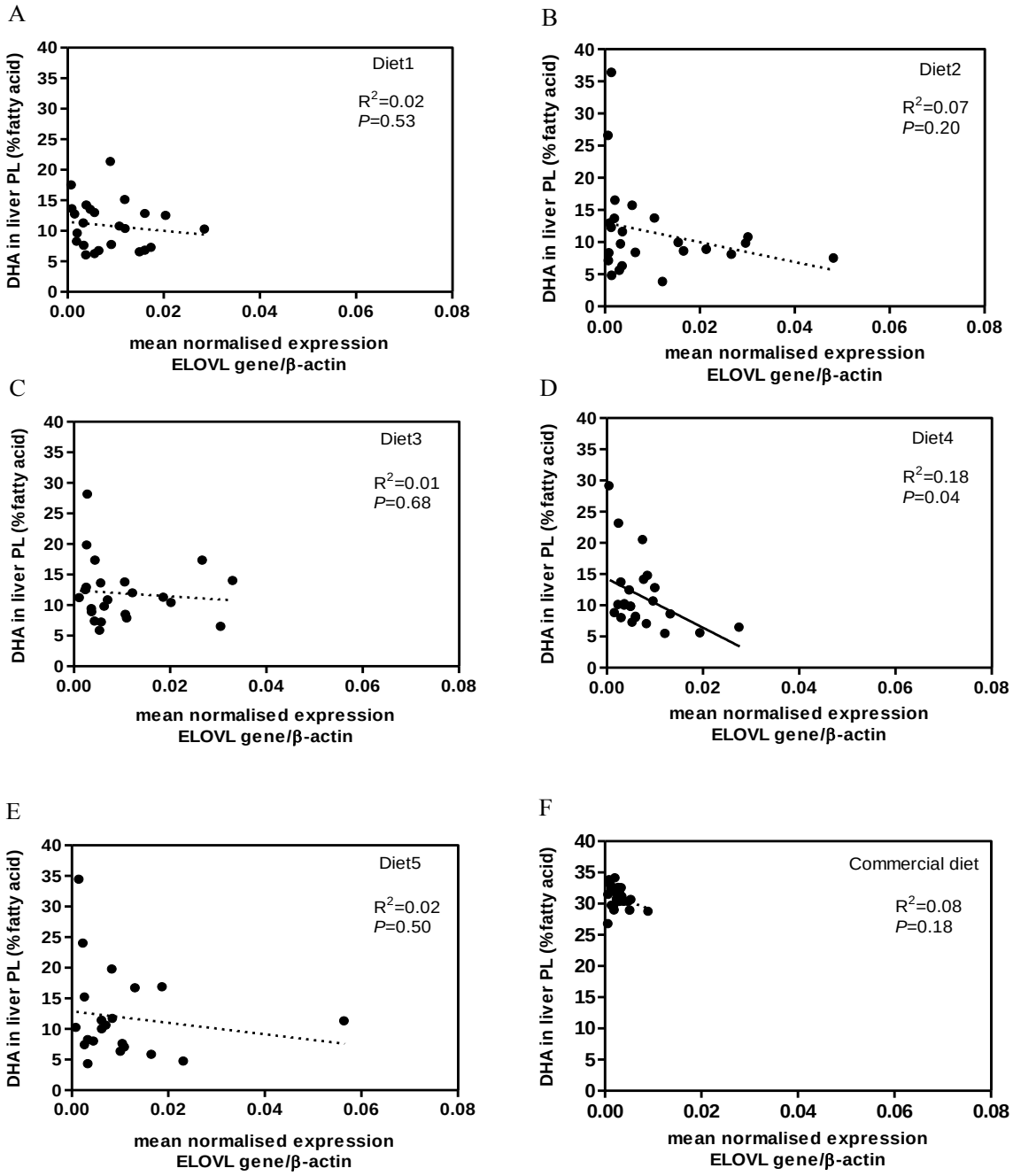


Figure 4. 17 Correlations between liver phospholipid (PL) DHA levels and hepatic gene expression levels of ELOVL in fish the 5 vegetable oil-based diets (Diets 1-5) and the commercial reference diet. Scatter plots were plotted using fatty acid data from 20-24 fish per dietary treatments (A-F).

4.7 Discussion

Tucker *et. al*. (266) found that when barramundi with body weights between 9-62 g were fed with diets of similar fatty acid and dietary composition but containing different levels of fat (9% and 13%, w/w of diets), the growth rate of fish was similar but feed conversion was significantly lower when the fat content of the diet was increased. In another study, which compared diets with three lipid levels (6, 12 and 18%, w/w of diets) at a fixed protein content of 43%, the highest weight gains and best feed conversion were observed in juvenile barramundi fed diets containing 20% carbohydrate with 12% or 18% fat (267). The improvement in growth rate and feed efficiency at higher lipid contents of the diet suggested an energy dependant effect of fat on growth. In the current study, an average of 12.7% of fat from vegetable oils and 49.9% of protein from defatted poultry meals and soybean protein concentrates were included in the 5 experimental diets compared with the commercial diet which contained 10% and 48.7% of marine derived fat and protein, respectively. The results indicated that feeding barramundi fingerlings diets based on vegetable oils as compared to marine oils had negative effects on the fish growth, with the weight gain 2 fold lower and feed conversion rate (FCR) 1.3 fold higher in these fish compared with fish fed commercial diets. In animal husbandry, FCR is a measure of an animal's efficiency in converting feed mass into increased body mass, and is calculated by dividing the mass of food eaten by the body mass gain, over a defined period of time, and as a result a low FCR is indicative of more efficient conversion of feed into growth. In this study, the growth rate, length, width, weight gain and specific growth rate were all significantly lower in fish fed the vegetable oil-based diets than that of fish fed the commercial diet and this decreased growth performance occurred despite the fact that total fat levels in the vegetable oil-based diets were ~3% higher than in the commercial diet (Table 4.4 and 4.9). This implies, therefore, that fat or energy content may not be the major determinant for barramundi growth and performance, and that the type of lipid in the diet, or other nutrients in the diet also play an important role. The commercial diet contained ~2.35% (w/w) of n-3 LCPUFA, predominately EPA and DHA, however none of the major n-3 and n-6 LCPUFA was present at a detectable levels in the vegetable oil-based diets. There have been numerous studies indicating that most aquatic species have some form of demand for n-3 LCPUFA particularly EPA and DHA as essential dietary nutrients. However, the amount of these n-3 fatty acids in the diet and how it is influenced by the presence of other fatty acids in the diets appear to vary among different fish species (268). Results from my study suggest that the n-3 LCPUFA are indeed essential for optimal growth

and development, and that this requirement for n-3 LCPUFA is independent of the total dietary fat content.

Previous studies have suggested that the requirements of fish for n-3 LCPUFA are higher for marine than for freshwater species, and also higher for cool than for warm water species. Diadromous species may be able to obtain their fatty acid requirements from C18 PUFA or have a reduced requirement for n-3 LCPUFA and some freshwater fish appear to have no demand for n-3 LCPUFA at all (269-271). The limited ability to digest and utilise dietary PUFA or the low desaturation/elongation activities of marine/diadromous species is inherently associated with its marine environment and evolution. The differences between marine and freshwater fish can be accounted for by considering their natural diets in the food chain and particularly whether a species is carnivorous, omnivorous or herbivorous. Taking an extreme carnivorous marine species turbot as an example, the fish consumes large amounts of n-3 LCPUFA through eating other fish and consequently there is no requirement for turbot to convert their limited intake of ALA and LA in their diet to EPA and DHA, therefore, the capacity for LCPUFA conversion has been reduced or completely lost during evolution (40). In contrast, freshwater invertebrate organisms or aquatic insect larvae used commonly as fish feed tend to have higher levels of LA, ALA, AA and EPA but considerably less DPA and DHA (41-43), thus, C18 PUFA are at least present in the food chain of freshwater species and correlates with the capability of freshwater fish to convert ALA and LA to the biologically active C20 and C22 LCPUFA. In the case of barramundi, studies from the 1980s by Buranapanidgit *et. al.* (272, 273) as cited by Glencross and Rutherford (268) indicated that dietary LCPUFA levels, primarily as a mix of EPA and DHA, of 1-1.7% (w/w) of the diets were adequate to maintain good growth. Early studies by Dhert *et. al.* suggested that barramundi larvae fed on a n-3 LCPUFA fortified live feed had a superior physiological condition which was reflected by significantly lower mortality figures during a stress test (274). Glencross and Rutherford (268) examined a series of diets with varying DHA levels in juvenile barramundi and observed that 0.2% of n-3 LCPUFA resulted in the best growth, and emphasized that both EPA and DHA were required. A similar study examined the effects of mixing fish oil with soybean oil to alter the n-3 and n-6 ratio and simultaneously reduce the levels of n-3 LCPUFA in barramundi diets and results from this study showed that the increasing levels of soybean oil had little effect on fish growth, however a sign of EFA deficiency such as shock-like response was observed where there were low levels of n-3 LCPUFA (275). Another study in which authors examined total lipid levels reported that fish

fed diets with 5% lipid, regardless of the protein content, showed abnormal reddening of the fins (fin erosion), indicating a deficiency in total lipids, n-3 LCPUFA or both (257). Similar signs of EFA deficiency have also been reported in barramundi fed diets containing 0.035-0.068% (w/w) n-3 LCPUFA but not in those fed diets containing 0.13% (w/w) n-3 LCPUFA (272). In this current study, though the fin erosion was not observed, growth retardation was significant in fish fed the vegetable oil-based diets (n-3 LCPUFA-free) compared to fish fed the commercial diet (n-3 LCPUFA-rich). Moreover, higher mortality (Table 4.9) and increased liver fat content (Table 4.11) were both observed in the vegetable oil-based diet fed fish. These responses have been reported to be typical signs of LCPUFA deficiency in other fish species (269, 276).

The primary aim of this study was to determine whether feeding barramundi with vegetable oil-based diets containing high levels of ALA affects the n-3 content of tissues and gene expression of the key fatty acid enzymes (FADS2 and ELOVL). The analysis of fatty acids in phospholipid and triglyceride fractions of tissues of juvenile barramundi fed different diets in this study confirmed that the fatty acid composition of the fillet and liver are directly influenced by the dietary lipid composition as described previously by others (277, 278). The primary representative fatty acids in the washout diet were monoenes (Table 4.5) and therefore more monoenes were found in tissues of fish fed the washout diets compared with fish fed on commercial diets (before washout diet feeding) (Figure 4.2 and Figure 4.3). The major fatty acids in the 5 vegetable oil-based diets were monoenes and short chain PUFA (LA and ALA) (Table 4.5) and these fatty acids were shown to accumulate in fish fillet and liver (Table 4.16-4.19). However, fatty acid intermediates 18:4 n-3, 18:3 n-6, 20:3 n-6 and 22:4 n-6 were detected at higher levels in liver phospholipid after a 3-week acclimation during which fish were fed on a washout diet. Similar accumulation was also observed in both fillet and liver phospholipid fractions in fish fed the 5 vegetable oil-based diets, the 18:4 n-3, 20:3 n-3 and 20:2 n-6 levels, however, were low or in some cases even below the limit of detection, and levels of all n-3 LCPUFA in fish fed the vegetable oil-based diets were even lower than the levels in fish fed the washout diets, suggesting the limited capacity for fatty acid desaturation and elongation in barramundi.

In other studies, when barramundi were fed on three diets containing different oil sources including fish oil, echium oil (rich in 18:4 n-3) or rapeseed oil (rich in monoenes and LA), a fatty acid mass balance of fish whole body showed that feeding barramundi on 18:4 n-3 rich

feed bypassed the first rate-limiting step in n-3 LCPUFA pathway. The fish, however, showed no accumulation of EPA and DHA; and ALA and 18:4 n-3 were the two major n-3 fatty acid accumulated in the whole body of echium oil fed barramundi (279). A study by Izquierdo *et. al.* (192) showed that it is possible to substitute 60% of the fish oil in diets with other vegetable oil sources in another diadromous species, the European sea bass (*Dicentrarchus labrax*), with a range of vegetable oils, without negative effects on fish performance and flesh quality. The replacement of fish oil, however, resulted in a reduction in n-3 LCPUFA content, particularly EPA, in fish muscle by nearly 50%. A more recent study in the European sea bass, indicated that total substitution of a fish meal and fish oil with a blend of vegetable meal (corn, soybean, wheat and lupin) and flaxseed oil in the diets resulted in nutritional deficiency, growth retardation and significant decreases in all n-3 LCPUFA by about 3 fold compared with the n-3 LCPUFA levels in fish fed fish meal/fish oil-based diets (280). In contrast, the results reported by Alhazzaa *et. al.* (279), who performed whole body fatty acid mass balance (appearance/disappearance) analysis for juvenile barramundi fed different diets, suggested that fish receiving higher levels of monoenes and PUFA (ALA and LA) in diets with lower n-3 LCPUFA levels had a higher Δ6 desaturase apparent activity than fish fed fish oil diets. Interestingly, when fish were fed diets rich in 18:4 n-3 and 18:3 n-6 and low in n-3 LCPUFA, but which still contained high levels of ALA and LA, the apparent Δ6 desaturase activity was very low, whilst Δ5 desaturase and elongase activities were high; however, no further increases in the n-3 LCPUFA content in tissues was observed in fish fed vegetable oil diets (279).

In this study, the comparison between the 5 vegetable oil-based diets with increasing ALA levels revealed that fish fed with the vegetable oil-based diets were completely depleted in n-3 LCPUFA and that this was not corrected by including increased amounts of ALA in the diet. Increasing dietary ALA content also had no effect on hepatic FADS2 and ELOVL mRNA levels (Figure 4.13). However, I reported that hepatic mRNA expression of FADS2 and ELOVL genes was higher in fish fed on the vegetable oil-based diets compared with those consuming the commercial diet (Figure 4.14). These results are consistent with the results of a previous study in European sea bass in which fish were fed on a diet in which 60% of the dietary fish oil was substituted with rapeseed oil or flaxseed oil. This resulted in a 5 fold up-regulation of the FADS2 gene compared with fish who were fed the standard (100% fish oil) diets (281). In another similar study, the mRNA expression of FADS2 in marine gilthead sea bream fed soybean and rapeseed oils were increased by 5.8 and 6.6 fold, respectively,

compared with fish consuming fish oil-based diets (282). However, in the same study, feeding the sea bream with same amount of flaxseed oil resulted in a much more modest increase in mRNA abundance of the FADS2, which was only increased by 2.3 fold compared with the expression level in fish fed fish oil diets. Doubling the amount of flaxseed oil in the diet completely inhibited FADS2 gene expression, however, the enzyme activity remained the same (43). In contrast, feeding freshwater fish, such as zebra fish and Nile tilapia (*Oreochromis niloticus*) with vegetable oil diets which provided 1% (w/w) of both LA and ALA, increased hepatocyte Δ6 desaturase activity in both fish; in zebra fish, the main effect of the vegetable oil diet was to increase Δ6 desaturase activity and to production of the ALA desaturation product, 18:4 n-3. The same diet in tilapia, however, was able to induce activity of all enzymes in the fatty acid pathway and resulted in increased amounts of EPA and DHA. Whilst dietary C18 PUFA stimulated fatty acid enzymes activities, desaturation of ALA was still insufficient to maintain n-3 LCPUFA status in fish fed vegetable oil diet at the same level as in fish fed the fish oil diet (149). These differences suggested that the mechanism of LCPUFA biosynthesis is complicated and seems to be highly species dependent. In the current study, the ALA level used in the diets was able to increase mRNA abundances of FADS2 and ELOVL when compared with those levels in fish fed diets containing LCPUFA, however, the extent of the increase may not be sufficient to trigger enzyme activity of Δ6 desaturase and elongase for converting dietary C18 PUFA to their corresponding longer chain metabolites. The total monoenes and PUFA levels together with deprivation of n-3 LCPUFA in these vegetable oil diets, therefore, may have played more important roles on up-regulating of FADS2 and ELOVL genes in barramundi.

Due to the observed difference in body weight in fish among treatments, the association between DHA level and body weight were further analysed, however according to the linear regression analysis, I found no significant relationship between the two factors (Figure 4.15 A-E). Thus, our data suggest that the diet composition is an environmental factor that can generate significant diversity in major physiological traits of metabolism in the barramundi, with increased intake of dietary n-3 LCPUFA leading to improved accumulation of these biologically active fatty acids in tissues of fish fed the commercial diet (Figure 4.15 F). However, with increased intake of dietary C18 ALA, the precursor fatty acid for EPA and DHA synthesis, showed increased accumulation of ALA but no further increase of its longer chain metabolites, and the DHA levels in fish fed these vegetable oil-based diet were not related to body weight (Figure 4.15 A-E). Not only were the tissue DHA level in the 5

vegetable oil diets fed fish not related to body weight, the DHA levels were not positively related to the relative FADS2 (Figure 4.16) and ELOVL (Figure 4.17) mRNA abundance. On the contrary, it shows a slightly negative correlation between liver DHA and gene expression of both FADS2 and ELOVL (Figure 4.16-4.17 A-E). Results obtained here are in agreement with the results of existing studies, for example a n-3 LCPUFA-free vegetable diets were associated with a significant increase in FADS2 and SREBP-1 mRNA expression in sea bass, however, FADS2 enzyme activity and its protein level were not up-regulated by the vegetable diets when compared with the levels in fish fed fish diet (36). The authors of this paper suggested that a post transcriptional regulation of FADS2 mRNA (or other genes involved in the fatty acid pathway) blocked the up-regulation of enzyme activity in fish fed n-3 LCPUFA diets and this may have been the cause of the poor conversion of the PUFA in vegetable oil diets to n-3 LCPUFA in this species (36). In this current study, whilst I did not measure the enzyme activities and protein expression levels of FADS2 and ELOVL enzymes, the negative correlation between fish body weight and gene expressions of FADS2/ELOVL with tissue DHA levels has led me to hypothesise that some barramundi may have a higher capacity for using the dietary short chain PUFA, ALA, as a precursor to synthesise DHA. However, the mechanism responsible for increased LCPUFA biosynthetic activity during periods of limited LCPUFA intake is not clear, seems to be species dependent and has a high degree of inter-individual variability. Thus, while there can be no doubt that dietary and tissue fatty acids exert profound effects upon the metabolism of vertebrates, further work is needed to explore the biological mechanisms for these effects and thereby explain contrasting results of different studies.

The ability to increased n-3 PUFA levels (particularly ALA) in barramundi tissues with ALA diets could improve the nutritional value of fillets (3). Nevertheless, reduced n-3 LCPUFA levels are undesirable from a human nutrition perspective. Fillet EPA and DHA levels (4.7% and 24% of total fatty acids, respectively, in the commercial diet fed fish) decreased to 2.5% and 11.3% of total fatty acids, respectively, in fish fed the vegetable oil-based diets with the highest dietary ALA content 3.2%en (Diet 5) relative to the commercial diet fed fish. However, an important observation in the present study was the significant variability within treatments in fatty acid profiles, especially n-3 LCPUFA content, between individual fish in the vegetable oil-based groups, which suggests a high degree of heterogeneity between individuals in their capacity for ALA conversion or tissue incorporation. A previous study has also shown that the individual variation within fish fed on the same dietary treatment exists in

the content of n-3 LCPUFA in the flesh of Atlantic salmon smolts, indicating that individual fish may be able to maintain high levels of n-3 LCPUFA in their tissues (283). Results presented in this chapter show remarkable inter-individual diversities in the fatty acids in fillet and liver of fish, particularly in livers, after feeding fish with vegetable oil-based diets (Figures 4.10 and 4.11). The CV% gives an indication of how significant the inter-individual variation in dietary treatments in barramundi (283). The CV% values in this study showed that the variation in individual fatty acid, particularly n-3 LCPUFA, was higher in liver than fillet in fish fed the vegetable oil-based diets (with average CV% of 61.1% versus 25.6% for EPA, 25.1% versus 19% for DPA and 50.6% versus 17.1% for DHA). Moreover, the CV% values of all n-3 LCPUFA were also much higher in fish fed on the vegetable oil diets than those in fish fed on the commercial diet, irrespective of the ALA content. In this chapter, without the inclusion of fish oil and fish meal in the 5 vegetable oil diets, the fatty acid profile of fish fed on the vegetable oil-based diets still contained EPA, DPA and DHA, and this could be suggested that may be due either to retention of these n-3 LCPUFA or conversion of dietary PUFA precursors or a combination of both of these processes (283). The variation in tissue n-3 LCPUFA contents within the vegetable oil fed group indicates that some fish may be suitable for selection for genetic improvement to increase the capacity of these fish for ALA conversion to n-3 LCPUFA. Furthermore, the natural diets of a given species change substantially during development. It has been suggested that fish at early developmental stages consume relatively simple food items and may readily convert C18 fatty acids from food sources such as insects and zooplankton to EPA and DHA whereas fish at mature stages may become more piscivorous and gradually lose their ability for LCPUFA conversion (40). Thus, a breeding program for barramundi or other species which selects for fish with the highest capacity of ALA conversion at an early developmental stage and employs specific diets to maximise n-3 LCPUFA conversion may be a strategy for reducing the reliance of barramundi aquaculture on marine resources

4.8 Summary

The findings in this study showed that juvenile barramundi fed the vegetable oil-based diets had lower growth performance compared with fish fed the commercial diet. Thus, n-3 LCPUFA are shown to be required for better growth in juvenile barramundi. The absence of fish oil and fish meal from the diet resulted in decreases of all major n-3 LCPUFA but increases in tissue ALA, LA and some other intermediate metabolites in the fatty acid pathway. Though the vegetable oil diets induced hepatic mRNA abundances of FADS2 and

ELOVL genes when compared with mRNA levels in fish fed the commercial diets, the increasing ALA level in the vegetable oil diets showed no effects on regulation of gene expression. The results presented in this chapter are consistent with the hypothesis that the desaturase/elongase activities in marine or carnivorous fish are low and also support our results presented in chapter 5 (yeast heterologous expression) which shows that limited Δ6 desaturase activity in barramundi may prevent conversion of ALA to n-3 LCPUFA. Moreover, there were no correlations between tissue DHA levels and either fish body weight or FADS2/ELOVL gene expression in any of the dietary treatments. However, it is noteworthy that I observed substantial variations between individual fish in their n-3 LCPUFA contents after exposure to the ALA diets, pointing to significant heterogeneity in the capacity of barramundi for converting dietary ALA to DHA. This raises to the possibility that this natural variation could be exploited to selective breeding programs to increase the number of fish with higher n-3 LCPUFA conversion abilities. Further study is needed to discover exactly what controls to the activity of the enzymes responsible for this conversion.

Chapter 5

Cloning and Functional Characterization of Δ6 Desaturase and Elongase in Juvenile Barramundi (*Lates calcarifer*)

5.1 Abstract

To examine the enzymatic capability for conversion of C18 ALA to n-3 LCPUFA in barramundi, I performed 5' and 3' rapid amplification of cDNA end (5'/3' RACE) to identify the full sequences of barramundi putative Δ6 desaturase and elongase and further cloned the full length open reading frames (ORF) of these two enzymes into a heterologous expression system using yeast *S. cerevisiae* to test for substrate specificities and activities of these enzymes. Functional expression of barramundi Δ6 desaturase and elongase revealed that the recombinant barramundi Δ6 desaturase was not only capable of desaturating at the position 6 ALA to 18:4 n-3, LA to 18:3 n-6 and 24:5 n-3 to 24:6 n-3 but also showed Δ8 desaturase activity, with synthesis of 20:4 n-3 and 20:3 n-6 from 20:3 n-3 and 20:2 n-6, respectively. The recombinant barramundi elongase showed a broad range of fatty acid substrate specificity from C18 to C22 PUFA with the greatest activity with n-3 C20 EPA. However, the rate of desaturation was generally lower than the rate of elongation in the heterologous expression system. In addition, the recombinant barramundi Δ6 desaturase showed a preference for n-3 substrates over n-6, whilst the elongase enzymes showed a preference for n-3 over n-6 only in the case of C18 PUFA. I also found that a significant amount of the desaturation and elongation fatty acid products could be detected in the culture medium at various time points after the addition of fatty acid substrates, and that it was important to take the levels of fatty acids in the medium into account when it came to calculating enzyme activity. These findings show that barramundi have both desaturation and elongation capacity for LCPUFA biosynthesis, and can utilise dietary ALA and LA as substrates. Moreover, the recombinant barramundi Δ6 desaturase possesses Δ8 desaturase activity, thus providing an alternate route for LCPUFA synthesis in this fish species.

5.2 Introduction

The n-3 LCPUFA, particularly EPA and DHA, are constituents of the phospholipids of vertebrate cell membranes. The LCPUFA are important for normal growth and brain development in humans (284, 285). This appears to be true also for fish where n-3 LCPUFA are required in larval development and deficiency can impair vision, and cause low survival,

poor growth rate and abnormal behaviours (286, 287). Vertebrates cannot synthesize PUFA *de novo* but rather derive them from metabolic precursor molecules, C18 PUFA such as ALA and LA, of plant origin. Alternatively, LCPUFA with C20 to C22 or more carbons can be obtained directly from animal products, particularly fish sources, in the diet.

The n-3 LCPUFA are supplied to farmed fish in fish oil and fish meal which have long been major ingredients of the diet for fish husbandry. However, being heavily reliant on declining global marine fish stocks which are expensive to purchase presents a risk to the sustainability of the aquaculture industry (288, 289). The substitution of fish oil with high n-3 PUFA vegetable oils could be a viable option for a sustainable and economical source of fat for fish feed production. However, the predominant n-3 fatty acid in vegetable oils, ALA, has C18 and 3 double bonds, whereas EPA and DHA are longer and more unsaturated with C20 and C22, and 5 and 6 double bonds, respectively. Therefore, the use of ALA in aquaculture requires farmed fish species to have the capacity to elongate and desaturate this fatty acid through the LCPUFA synthetic pathway, to increase the n-3 LCPUFA content in its tissues. The requirements of PUFA and LCPUFA vary with fish species (290). Generally, freshwater fish appear to be capable of converting both n-3 and n-6 C18 PUFA to their more unsaturated and elongated LCPUFA. On the other hand, marine fish are not efficient convertors of ALA to n-3 LCPUFA, and it is believed that this difference comes mainly from a deficiency or impairment of the Δ5 desaturase and C18 to C20 elongation activities in these marine fish (271). These limitations have been suggested to have important implications for both LCPUFA biosynthesis in aquatic animals and their prospective dietary requirements in aquaculture (290). Diadromous fish such as barramundi, which move between freshwater and marine/estuarine environments, are of particular interest in relation to LCPUFA biosynthesis in fish because of the difference in dietary PUFA requirements and enzyme capabilities of converting PUFA to LCPUFA between marine and freshwater species. In addition, barramundi are now an important aquaculture species in Australia, Indonesia, Malaysia, Philippine, Taiwan and Thailand, and significant industries for this fish already exist in these countries (259).

Desaturases and elongases are two key enzyme categories in LCPUFA synthetic pathway. The currently best known pathway for EPA and DHA biosynthesis has been described by Sprecher (89) and others (4, 127, 291-293). In the liver of mammals, a Δ6 desaturase, elongase, and a Δ5 desaturase successively convert ALA via 18:4 n-3 and 20:4 n-3 to EPA in

the n-3 pathway. In parallel, the same set of enzymes is also able to utilise the other essential fatty acid, LA, to form AA in the n-6 pathway (143). The Δ6 desaturase is required for the desaturation of C18 PUFA and also 24:5 n-3 to produce 24:6 n-3 (127, 294), the 24:6 n-3 is then transferred from the endoplasmic reticulum to the peroxisomes for one round of β-oxidative chain shortening, finally leading to the synthesis of DHA (83, 143). A labelling study has shown that this route is also present in trout hepatocytes (295), suggesting that this route for DHA biosynthesis is not restricted to mammals but also present in at least some species of fish (143).

Understanding the molecular mechanisms involved in LCPUFA biosynthesis and regulation will allow us to optimise the activity of the pathway to enable effective utilization of vegetable oil-based fish diets in aquaculture while maintaining the LCPUFA status of the farmed fish that are consumed by humans. In this chapter I present evidence that there are FADS and ELOVL genes encoding active Δ6 desaturase and elongase 5 proteins in barramundi hepatocytes. Heterologous expression and functional characterization of the enzymes showed the Δ6 desaturase in barramundi is a Δ6/Δ8 dual function desaturase and the elongase has a wide range of elongation activities towards C18 to C22 fatty acid substrates.

5.3 Design of the study

This work was designed to use the 3'/5' RACE techniques to identify the full length of ORF of the Δ6 desaturase and elongase genes from barramundi hepatic total RNA, and then construct the two genes into a yeast-*E-coli* shuttle vector. The constructed plasmids were then transfected into *S. cerevisiae* to enable functional characterisation of the two recombinant enzymes using a heterologous expression system.

5.4 Methods and Materials

5.4.1 Chemicals

Organic solvents used in this study were all analytical grades from Ajax Finechem Pty Ltd (Auckland, New Zealand) or Chem-Supply (SA, Australia). Fatty acid substrates ALA, LA, 18:4 n-3, 18:3 n-6, 20:3 n-3, 20:2 n-6, EPA, AA and DPA (all >98-99% purity) were purchased from Cayman chemical (Michigan, USA). Free fatty acid 17:0 was from Nuchek Prep Inc (Elysian, USA). The fatty acid substrate 24:5 n-3 was purchased from Larodan Fine Chemicals (Malmö, Sweden). Other chemicals and reagents were purchased from Sigma-Aldrich (Missouri, USA) unless specified otherwise.

5.4.2 Animals

All experimental procedures were performed in accordance with institutional guidelines for the use of animals and the Australian code of practice for the care and use of animal for scientific purposes. The protocol was approved by the Animal Ethics Committee, University of Adelaide (Ethics number S-28-08). Australian juvenile barramundi were obtained from a commercial supplier (W. B. A. Hatcheries, SA, Australia).

5.4.3 Isolation of RNA from barramundi liver tissue

Total RNA was isolated from 10 mg of fish liver using a Qiagen RNeasy® kit (Qiagen) following the protocol provided by the manufacturer with the tissue initially disrupted using a Tissue Lyser (Mixer MM 300, Germany). The concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm and RNA integrity was confirmed by agarose gel electrophoresis. Procedures for liver tisssue disruption, RNA isolation and RNA quality determination were as described earlier (Chapter 2).

5.4.4 Sequence analysis

Molecular biology information for all relevant nucleotide and peptide sequences were extracted from the GenBank®, the National Institutes of Health (NIH) genetic sequence database under the NCBI. Multiple sequence alignments and comparisons were carried out using Genedoc (http://www.psc.edu/biomed/genedoc). Transmembrane domains were predicted by SOSUI software (http://bp.nuap.nagoya-u.ac.jp/sosui/). The phylogenetic tree constructions were performed using Neighbor Joining (NJ) analysis with ClustalX (http://www.clustal.org/) and MEGA version4 (http://www.megasoftware.net/).

5.4.5 Primers

Allele-specific oligonucleotide primers were synthesized by GeneWorks (GeneWorks Pty. Ltd., SA, Australia). All primers in this study are presented in Table 5.1 and Table 5.2 for the desaturase and the elongase genes, respectively.

5.4.6 General molecular techniques

5.4.6.1 Polymerase chain reaction (PCR)

An Eppendorf Mastercycler® (Eppendorf South Pacific Pty Ltd, NSW, Australia) was used for all PCR amplifications. PCR in different reaction volumes (10, 25 or 50 μL) in 0.2 mL thin

wall clear flat cap tubes (Axygen, CA, USA) was used for the amplifications. The HotStar HiFidelity PCR kit (Qiagen) was used to perform all PCR amplifications for cloning and sequencing. The final concentrations of reagents used in the PCR reactions were as follows:

1) HotStar HiFidelity PCR buffer (5X): the buffer was used 1X in a reaction. For a 25 µL reaction, this meant 5 µL of the 5X buffer. This buffer contained dNTPs and 7.5 mM $MgSO_4$.
2) HotStar HiFidelity DNA polymerase (2.5 units/µL): in general 1 µL per 50 µL PCR amplification. The volume was adjusted dependent on expected PCR product length. In accordance with the manufacturer's instructions (Qiagen), 1 µL of enzyme was used when amplifying PCR products less than 2 kb and 2 µL of enzyme when amplifying PCR products between 2 and 5 kb.
3) Primer set: all primers were diluted to 100 ng/µL and 2 µL of primer was used per 50 µL PCR amplification.
4) Q-solution (5X): this was used 1X in a reaction. The Q-solution changes the melting behaviour of DNA and was used for DNA templates which have strong secondary structures.
5) RNase-free water: the volume was variable. The water was used to dilute the reaction to an appropriate volume.
6) Template DNA: the volume was variable. The volume depended on DNA concentration. The template DNA was added to the individual tubes containing the reaction mix.

PCR amplification was performed according to a standard method following the manufacturer's instruction (Qiagen). PCR amplification was begun an initial hot start enzyme activation step at 95°C for 5 min followed by 30-40 cycles of denaturation at 95°C for 1 min, annealing at 55-65°C for 0.5-1 min and extension at 72°C for 0.5-1 min and followed by a final extension at 72°C for 10 min. The second round of PCR (nested PCR) was performed if necessary. After amplification, samples were stored at -20°C until further applications.

5.4.6.2 One-step reverse transcription PCR (RT-PCR)

The OneStep RT-PCR kit (Qiagen) was used to perform reverse transcription and PCR. The two reactions were carried out sequentially in the same tube and the Eppendorf Mastercycler® was used for all PCR amplifications. All procedures were conducted according to the manufacturer's protocol. Each 50 µL reaction components for one-step RT-PCR were as follows:

1) QIAGEN OneStep RT-PCR Buffer (5X): the buffer was used 1X in all reactions.
2) dNTP Mix: the mix containing 10 mM of each dNTP. For a 50 µL reaction, 2 µl of the dNTP was added to achieve a concentration of 400 µM for each dNTP.
3) Q-solution (5X): optional. Used 1X per reaction.
4) RNase-free water: the volume was variable. The water was used to dilute the reaction to a volume of 50 µL.
5) Primer set: all primers were diluted to 100 ng/µL and 2 µL of primer was used per 50 µL RT-PCR amplification.
6) OneStep RT-PCR Enzyme Mix: 2 µL per 50 µL reaction.
7) RNase inhibitor: RNaseOUT™ (Invitrogen) was used at 5-10 units/reaction.

Template RNA: <2 µg of RNA per reaction was added to the individual PCR tubes containing the reaction mix. RT-PCR amplification was performed according to a standard method following the manufacturer's instructions (Qiagen). RT-PCR amplification was commenced with a reverse-transcription reaction at 50°C for 30 min and an initial hot start enzyme activation step at 95°C for 15 min followed by 25-40 cycles of denaturation at 94°C for 0.5-1 min, annealing at 55-65°C for 0.5-1 min and extension at 72°C for 1 min and followed by a final extension at 72°C for 10 min.

5.4.6.3 Reaction clean up

The MinElute® PCR Purification Kit, Gel Extraction Kit or MinElute® Reaction Cleanup Kit (Qiagen) was used to clean up and purify PCR reactions or enzymatic reactions, all procedures were performed following the manufacturer's protocol using an Eppendorf microcentrifuge 5415 R (Eppendorf South Pacific Pty. Ltd., NSW, Australia).

5.4.6.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed for separating and analysing DNA. The gels were visualised under UV light illumination after the addition of EtBr solution at a final concentration of 0.5 µg/mL. DNA grade agarose was used for preparation of agarose gels (1-2%) in 1X TBE buffer. The lowest percentage gels were used to separate large DNA fragments (>1 kb) and the highest percentage gels were used to analyse the smallest DNA fragments (0.1-1 kb). A Mini-Sub Cell GT Cell accompanied with a 7 x 7 cm gel tray, one 8-well and one 15-well comb (Bio-Rad Laboratories, CA, USA) were used to perform gel electrophoresis. The gels were immersed in 1X TBE buffer and the DNA samples were

loaded after being mixed with GelPilot DNA Loading Dye (5X) (Qiagen) at a 5:1 ratio. The DNA sample loading amount was decided based on the DNA concentration, typically, a band is easily visible if it contains about 20 ng of DNA. The gel was run at 100-110 volt (V) for 30-60 min depending on gel density. The visualization and documentation system used was an UVP High Performance Ultraviolet Transilluminator (UVP, CA, USA). The gels were placed on the UV transilluminator inside an EDAS 290 Imaging Cabinet and photographed using a Kodak DC 290 Zoom Digital Camera with an EDAS 290 Close-up Lens and an EDAS EB Filter (Kodak, NY, USA). The photographs were then run through Kodak ID Image Analysis Software version 3.5.2 (Kodak, NY, USA).

5.4.6.5 Nucleotide sequencing

All sequence analyses were performed by the BigDye® terminator sequencing (Applied Biosystems automated 3730 sequencer) at IMVS, SA, Australia. Purified DNA products were used in BigDye® terminator DNA sequencing reactions and nucleotide precipitation prior to automated sequence analysis. Each DNA sequencing reaction (template/primer combination) consisted of 1μL of Dye Terminator (DT) mix (BigDye® version 3, Applied Biosystems), 100 ng primer, 100-200 ng of DNA template, 2 μL (5X) buffer and ddH_2O to make up the total reaction volume to 10 μL. Sequencing PCR amplification was performed according to the standard protocol of the IMVS sequencing centre. The thermocycler program was as follows: 25 cycles of 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min. After the amplification, extension products were purified using an isopropanol precipitation protocol provided by IMVS. Briefly, 10 μL of extension product was mixed with 80 μL of freshly prepared 75% molecular grade isopropanol (Sigma-Aldrich) and vortexed briefly. The sample was incubated at room temperature for 15 min to precipitate the extension products and then spun at the maximum speed for 20 min in a bench-top microcentrifuge. The supernatant was carefully aspirated and discarded. The pellet was washed in 250 μL of 75% of isopropanol and vortexed briefly and spun for 10 min at the maximum speed. Finally, the supernatant was discarded and the pellet air-dried.

5.4.6.6 The 5' and 3' RACE strategies

1) Barramundi Δ6 desaturase gene: FADS genes in fish and other animal species in the GenBank® including Atlantic cod (mRNA: DQ054840, protein: AAY46796), Atlantic salmon (mRNA: NM_001123575, protein: NP_001117047), cherry salmon (mRNA: AB070444, protein: BAB63440), zebra fish (mRNA: NM_131645, protein: NP_571720),

human (mRNA: NM_004265, protein: NP_004256), rat (mRNA: NM_031344, protein: NP_112634) and mouse (mRNA: NM_019699, protein: NP_062673), were aligned and the partial genes of desaturase was obtained by RT-PCR (Qiagen) with primers (PSfads2-F and PSfads2-R) designed from the conserved regions of the same genes of other species. The gene specific primers were then designed based on the partial gene fragments of the putative desaturase gene. The RACE protocol was modified from methods of Frohman (296) to obtain the 3' and 5'end of the full length of barramundi desaturase gene. The cDNA for 3'RACE was reverse transcribed from total RNA by a hybrid primer (QtT) and incubated with reverse transcriptase (Omniscript® RT Kit, Qiagen) at 42°C for 1 hr to generate a 3'end partial putative FADS sequence. PCR amplification was then performed using a primer containing part of the 3'end sequence (Qo-R) and a Fads3'raceGSP1-F with the HotStar HiFidelity Polymerase Kit (Qiagen). PCR amplification was begun with an initial hot start enzyme activation step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56-60°C for 30 sec and extension at 72°C for 1 min and followed by a final extension at 72°C for 10 min. The nested PCR was performed using Fads3'raceGSP2-F and Qi-R primers to generate a more specific 3'end of FADS for BigDye® terminator sequencing. Similar methods were applied for the 5' RACE. To generate 5'end partial cDNA clones, reverse transcription was carried out using total RNA with the Fads5'raceRT-R primer to synthesize first strand products. Then, a polyA tail was appended to the cDNA template using terminal deoxynucleotidyltransferase (TdT) (Invitrogen, Victoria, Australia) and dATP (Promega). PCR amplification was then performed using the QtT-F to form the second strand of cDNA and the Qo-F primer with the Fads5'raceGSP1-R used for reverse transcription. A nested PCR was carried out by using Fads5'raceGSP2-R primer with Qi-F primer to increase specificity of barramundi desaturase gene 5'ends. Both 3' and 5' RACE product of the desaturase gene were aligned with the partial gene fragments and the overlapping regions identified.

2) Barramundi elongase: ELOVL gene in fish and other animal species in the GenBank® including seabream (mRNA: AY660879, protein: AAT81404), Atlantic salmon (mRNA: NM_001136552, protein: NP_001130024), Atlantic salmon (mRNA: NM_001123567, protein: NP_001117039), rainbow trout (mRNA: NM_001124636, protein: NP_001118108), cherry salmon (mRNA: DQ067616, protein: AAY79352), Atlantic cod (mRNA: AY660881, protein: AAT81406), zebra fish (mRNA: NM_200453, protein: NP_956747), human (mRNA: NM_021814, protein: NP_068586), mouse (mRNA:

BC022911, protein: AAH22911) and rat (mRNA: NM_134382, protein: NP_599209), were aligned and the partial gene of the elongase was obtained by one-step RT-PCR (Qiagen) with primers designed from the conserved regions of the same genes of other species. The gene specific primers were then designed based on the partial gene fragments of the putative ELOVL. The RACE protocol was modified from methods of Frohman M.A. (296) to obtain the 3' and 5'end of the full length of barramundi elongase gene. The cDNA for 3' RACE was reverse transcribed from total RNA by a hybrid primer (QtT) and incubated with reverse transcriptase (Omniscript® RT Kit, Qiagen) at 42°C for 1 hr to generate a 3' end partial putative ELOVL sequences. PCR amplification was then performed using a primer containing part of the 3' end sequence (Qo-R) and an Elovl3'raceGSP1-F with the HotStar HiFidelity Polymerase Kit (Qiagen). PCR amplification was begun with an initial hot start enzyme activation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56-60°C for 30 sec and extension at 72°C for 1 min and followed by a final extension at 72°C for 10 min. The nested PCR was performed using Elovl3'raceGSP2-F and Qi-R primer to generate a more specific 3'end of ELOVL for BigDye® terminator sequencing. Similar methods were applied in the 5' RACE. To generate 5'end partial cDNA clones, reverse transcription was carried out using total RNA with the Elovl5'raceRT-R primer to synthesize first strand products. Then, a polyA tail was appended to the cDNA template using terminal deoxynucleotidyltransferase (TdT) (Invitrogen) and dATP (Promega). PCR amplification was then performed using the QtT-F to form the second strand of cDNA and the Qo-F primer with the Elovl5'raceGSP1-R used for reverse transcription. A nested PCR was carried out by using Elovl5'raceGSP2-R primer with Qi-F primer to increase specificity of barramundi ELOVL 5'ends. Both 3' and 5' RACE product of the ELOVL were aligned with the partial gene fragments and the overlapping regions identified.

5.4.6.7 Construction of plasmid containing a target gene

Based on the RACE results, specific primers (Fads-F and Fads-R) were designed for identifying the full sequence of the barramundi desaturase gene (Figure 5.1) and FadsEcoRI-F and FadsXhoI-R were used to amplify the PCR product containing the putative desaturase gene (Figure 5.2). The putative barramundi FADS ORF was then purified, restriction digested and inserted into EcoRI and XhoI sites digested pYES2™ vector (Invitrogen, USA) to yield the constructed plasmid pYES2/BarraFADS (see section 5.4.6.8 for details). The coding region was successfully cloned (GU047383) and then sequenced by pYES2-F and pYES2-R

primers to confirm the sequence orientation and accuracy. The same procedures were also utilised for the barramundi ELOVL gene cloning. Primers (Elovl-F and Elovl-R) were also designed for determining the full nucleotide sequence of the barramundi elongase gene (Figure 5.3) and ElovlEcoRI-F and ElovlXhoI-R were used to amplifiy the PCR product containing the putative elongase gene (Figure 5.4). The putative barramundi ELOVL ORF which contained specific restriction sites was then purified, restriction digested and inserted into EcoRI and XhoI sites in the digested pYES2™ vector (Invitrogen) to yield the constructed plasmid pYES2/BarraELOVL (see section 5.4.6.8 for details). The barramundi elongase coding region was successfully cloned (GU047382) and then sequenced by pYES2-F and pYES2-R primers. The HotStar HiFidelity Polymerase Kit (Qiagen) with proofreading function was used throughout the cloning processes to minimize potential PCR errors.

5.4.6.8 Restriction enzyme (RE) digestion and ligation

Vector pYES2™ (Invitrogen) or PCR amplified full length ORF of barramundi FADS and ELOVL gene was digested using enzymes EcoRI and XhoI (Promega, NSW, Australia) for double RE digestions, according to the manufacturer's instructions. Purified PCR product or vector (1 μg/μL) was digested in a final volume of 20 μL containing sterile deionized water, RE 10X buffer 2 μL, Acetylated bovine serum albumin (BSA) 0.2 μL (10 μg/μL) and RE 0.5 μL (10 U/μL) for 1-4 hr at 37°C and subjected to gel electrophoresis as described above. RE digested PCR products were ligated into the RE digested pYES2™ vector. The ligation was performed using T4 DNA ligase with ligase buffer (10X) (Promega), according to the manufacturer's protocol. A 1:3 molar ratio of vector to insert DNA was used for cloning a fragment into the plasmid vector. The amount of insert DNA fragment required for each ligation was calculated based on the formula: [(ng of vector x kb size of insert) / kb size of vector] x molar ratio of (insert / vector) = ng of insert. Assembling vector DNA (100 ng), insert DNA, ligase buffer 1 μL, T4 DNA ligase 1 U (weiss units), nuclease-free water to final volume of 10 μL. The reaction was incubated overnight at 4°C. Ligation reactions of barramundi pYES2/BarraFADS and pYES2/BarraELOVL were checked by PCR using the primers pYES2-F and pYES2-R (Figure 5.5).

5.4.6.9 Transformation of E-coli DH5α cells

The MAX Efficiency® DH5α™ Competent Cells (Invitrogen) were transformed with purified constructed plasmid, according to the manufacturer's protocol. Briefly, the competent cells were thawed on ice and the 5-fold diluted plasmid mixed with the cells. The cells were

incubated on ice for 30 min, heat-shocked for 45 sec in a 42°C heat block and the reaction then placed on ice for 2 min. After this, 0.9 mL of room temperature super optimal broth with catabolite repression (SOC) medium (Invitrogen) was added, and the reaction shaken at 225 rpm at 37°C for 1 hr. The resulting solution was diluted, and 100-200µL spread onto on LB plates containing 100 µg/mL ampicillin (Amp), dried and the plates incubated overnight at 37°C. On the following day, the colonies which appeared were screened, and a single colony from each plate selected and transferred into a 1.5 ml microcentrifuge tube containing 0.5 mL of LB broth supplemented the antibiotics and incubated for 1-2 hrs at 37^{o}C. The cells were then spun down at 8000 rpm (6800 x g) for 5 min, the supernatant removed and discarded and the pellets mixed with 20 µL 0.02% SDS solution, incubated at 80°C for 2 min and then vortexed for 30 sec to obtain crude extracts of *E-coli* genomic DNA. These extracts were then centrifuged at 13000 rpm (18000 x g) for 2 min. Selected transformants were screened through a double RE digestion using the supernatant as the DNA template and the two specific restriction enzymes (EcoRI and XhoI) to digest the inserted target gene. Successful transformants were subjected to agarose gel electrophoresis in order to confirm that they had inserts of the correct size (Figure 5.6).

5.4.6.10 Plasmid DNA purification from E-coli DH5α cells

The successful transformants identified as described above were placed into a sterile culture tube which contained 5 mL of LB broth with added Amp. These tubes were then incubated overnight at 37°C with vigorous shaking (250 rpm). On the following day, the cells were harvested by centrifugation at 8000 rpm (6800 x g) for 3 min at room temperature and all remaining traces of supernatant were discarded by inverting the open centrifuge tube until all supernatant had drained/evaporated. Plasmid DNA was purified using QIAprep® Miniprep (Qiagen), according to the manufacturer's protocol, and the purified plasmid DNA was used for BigDye® sequencing PCR and precipitation for sequence analysis. The identity of the plasmid DNA was also confirmed by PCR using pYES2-F and pYES2-R primers (Figure 5.7)

5.4.6.11 Preparation of S. cerevisiae INVSc1 competent cells

The *S. c.* EasyComp™ transformation kit (Invitrogen) was used to prepare *S. cerevisiae* INVSc1 competent cells for yeast transformation with plasmid DNA for which the sequence had been confirmed as described above. The competent preparation was performed according to the manufacturer's protocol and following procedures previously described (Chapter 2). Briefly, after an overnight culture of wild type yeast cells, the OD_{600nm} of the culture medium

was determined. The cells were then grown in a 10 mL volume of YPD at 30°C with shaking at 250 rpm until the OD_{600nm} reached a value 0.6-1.0. The cells were then harvested and resuspended in 10 mL of washing solution (Solution I), pelleted and resuspended in 1 mL of kit provided Lithium cation solution (Solution Π). The competent cells were separated into 50 µL aliquots in 1.5 mL sterile screw-cap microcentrifuge tubes, and the tubes were wrapped in several layers of paper towel and stored at -80°C.

5.4.6.12 Transformation of S. cerevisiae INVSc1 cells

The same *S. c.* EasyComp™ transformation kit was used to transform competent *S. cerevisiae* cells. The transformation was performed according to the manufacturer's protocol and following procedures previously described (Chapter 2). Briefly, transformant yeast cells containing the pYES2™ empty vector, pYES2/BarraFADS or pYES2/BarraELOVL plasmid were grown at 30°C and transformants were selected on synthetic minimal defined medium agar plates lacking uracil (SC selective plate^{-U}) and supplemented with 2% glucose as the only carbon source. The transformant from a single colony was verified by DNA sequencing.

5.4.7 Heterlogous expression of barramundi putative Δ6 desaturase and elongase ORF in *S. cerevisiae* INVSc1 cells

An INVSc1 transformant colony containing pYES2/BarraFADS or pYES2/BarraELOVL was grown overnight at 27°C in SC selective medium^{-U} containing 2% glucose. Expression of heterologous barramundi desaturase, elongase or empty pYES2™ vector was induced by transferring log-phase yeast cells (OD_{600nm}=0.4) into SC selective medium^{-U} containing 2% galactose and 0.25% tergitol. Cultures were then supplemented with fatty acid substrates from the following: ALA, LA, 20:3 n-3, 20:2 n-6, 20:3 n-6, 24:5 n-3 and DPA for recombinant desaturase and ALA, LA, 18:4 n-3, 18:3 n-6, EPA and AA for recombinant elongase. Yeast cells transformed with pYES2/BarraFADS or pYES2/BarraELOVL plasmid without galactose induction were used as negative controls. After 24 to 48 hr incubation, yeast cells were harvested, washed with ddH_2O and total lipids extracted and analysed as described below and chapter 2. At least three independent replicates of the yeast cultures with each fatty acid substrate supplementation were performed. Yeast cells transformed with an empty pYES2™ vector were also induced as a secondary negative control.

5.4.8 Tergitol concentration range

Mild non-ionic detergent tergitol type NP-40 (70% in H_2O) was diluted to 10% (v/v) in ddH_2O and filter sterilized. Tergitol at concentrations of 0%, 0.25%, 0.5% and 1% (v/v) accompanied with galactose culture medium were used to induce yeast cells containing the pYES2™ empty vector. Fatty acid ALA and EPA at 500 µM were used as substrates to confirm the solubility of the fatty acid at different tergitol concentrations. Cells and medium were collected for fatty acid profile analyses at different time points (<1 hr, 24 hr and 48 hr). Cell absorbance at OD_{600nm}, total cell number and colony forming unit (CFU) were also measured.

5.4.9 Cell number determination

The total number of yeast cells in 10 mL of culture medium was determined using Bürker cell counting chambers (Improved Neubauer with bright-line; Blau Brand, Wertheim, Germany) of 0.1 mm depth by 0.0025 mm^2 with a 0.40 mm coverslip. Independent samples of 10 µL were placed from the diluted cell suspension at the edge of the chamber to fill both sides. The loading procedure was carried out gently to ensure the sample was drawn into the chamber by capillary action and that no air bubbles appeared. After loading, chambers were left for 30 sec to allow all the yeast cells to settle. The counter was placed on microscope stage and a 400X lens was used for counting. The centre square was subdivided into 25 smaller squares and evenly distributed cells in 5 squares in the counting grid (top left, bottom left, top right, bottom right and centre) were counted. Buds larger than approximately one half the diameter of the original cells were counted. Total cell number was calculated using the following equation:

Number of cells counted in 5 numbered squares x 5 = number of cells in total grid

Yeast cells per mL = number of cells in total grid x dilution factor x 10^4

5.4.10 Colony forming unit determination

Culture suspension (1 mL) was aseptically removed from each thoroughly mixed individual flask and serially diluted in sterile ddH_2O. A 1 mL of original yeast culture suspension was delivered into a sterile tube containing 9 mL of ddH_2O. The tube was vortexed and then 1 mL of this 10^1 dilution transferred into a second tube containing 9 mL of ddH_2O to make a 10^2 dilution. This same procedure was followed until the 10^6 dilution was reached. The OD_{600nm} was determined for the 10^1 dilution for each sample, and a 0.1 mL aliquot of the 10^6 dilution was applied to the surface of SC selective glucose plates^{-U}. The plates were then incubated at

30°C for 48 hr or until colonies appeared. The CFU was determined as: CFU per mL = CFU per plate x dilution factor x (aliquot / mL).

5.4.11 Fatty acid analysis

The yeast cells for each treatment were harvested, dried, weighed and the total lipid extracted. 1 mL of medium from each culture treatment was also collected, weighed and total lipid extracted. Free fatty acid 17:0 was added into each extraction tube as an internal standard. The extracted total lipid from cells and medium were methylated and the fatty acid composition quantified by capillary gas chromatography following procedures previously described (Chapter 2).

5.4.12 Statistical analysis

All data are expressed as group mean ± SEM. A one-way ANOVA followed by Tukey-Kramer test was used if *P* value less than 0.05 and follow Gaussian distributions. Kruskal-Wallis post test with Dunn's multiple comparison tests was applied for non-parametric analyses if data did not pass the normality test. An unpaired t-test was used to examine differences between two groups. A probability level of 0.05 ($P<0.05$) was used in all tests. Analyses were carried out with GraphPad Instat version 3.10. Detailed selection criteria for statistical methods were previously described in chapter 2.

Table 5. 1 Primers used for amplifying the barramundi FADS cDNA and ORF

Target gene	**Primer name**	**Sequence (5' to 3')**
FADS2 partial cds	PSfads2-F	GTCTACACCTGGGAAGAGGTCC
	PSfads2-R	GAGGTGTCCACTGAACCAGTCG
FADS2 3'end	QtT (cDNA synthesis)	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC T_{17}
	Fads3'raceGSP1-F	ATGAATCATCTGCCGATGGACATCG
	Qo-R	CCAGTGAGCAGAGTGACG
	Fads3'raceGSP2-F (nested PCR)	TCTCCCTGTTGGTGTTATAG
	Qi- R (nested PCR)	GAGGACTCGAGCTCAAGC
FADS2 5'end	Fads5'raceRT-R (cDNA synthesis)	GGGCTTCAGAAACTTTCGC
	QtT-F	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC T_{17}
	Qo-F	CCAGTGAGCAGAGTGACG
	Fads5'raceGSP1-R	TTTGTTCCGGTCCTGGCTTGG
	Qi- F (nested PCR)	GAGGACTCGAGCTCAAGC
	Fads5'raceGSP2-R (nested PCR)	GCATCCTCTCCAGCGTAGTGG
FADS2 full length sequence	Fads-F	AGGTGGATCAAGATCAAGGCCAG
	Fads-R	CGATTTATGATGCAGAGGAGGAG
FADS2 ORF cloning	FadsEcoRI-F	CCGGAATTCAGGATGGGAGGTGGAGGCCAG
	FadsXhoI-R	CCGCTCGAGTCATTTATGGAGATATGCATCGAGC
FADS2 ORF sequencing	pYES2-F	CTGGGGTAATTAATCAGCGAAGCG
	pYES2-R	CGTGACATAACTAATTACATGATGC

Table 5. 2 Primers used for amplifying the barramundi ELOVL cDNA and ORF

Target gene	Primer name	Sequence (5' to 3')
ELOVL partial cds	PSelovl-F	GGATGGGGCCCAAGTACATG
	PSelovl-R	GTCTGCATGTAGAAGTTTGAG
ELOVL 3'end	QtTVN (cDNA synthesis)	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC T_{17}VN
	Elovl3'raceGSP1-F	GGTCTACAATCTGGGCCTC
	Qo- R	CCAGTGAGCAGAGTGACG
	Elovl3'raceGSP2-F (nested PCR)	GACCCAGACAATGTGTGCAGTC
	Qi- R (nested PCR)	GAGGACTCGAGCTCAAGC
ELOVL 5'end	Elovl5'raceRT-R (cDNA synthesis)	CATGTAGAAGGACAAGAGCG
	QtT-F	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC T_{17}
	Qo-F	CCAGTGAGCAGAGTGACG
	Elovl5'raceGSP1-R	CCAGATTGTAGAGCACCAGGAG
	Qi- F (nested PCR)	GAGGACTCGAGCTCAAGC
	Elovl5'raceGSP2-R (nested PCR)	GGCTGCCTGTGTTTCATGTAC
ELOVL full length sequence	Elovl-F	CTCTCTCTCCCCCCGCCTC
	Elovl-R	GACCATAGTAAGCACTGTGTTG
ELOVL cloning	ElovlEcoRI-F	CCGGAATTCCAAATGGAGACCTTCAATCATAAAC
	ElovlXhoI-R	CCGCTCGAGATGTCAATCCACCCTCAGTTTC
ELOVL ORF sequencing	pYES2-F	CTGGGGTAATTAATCAGCGAAGCG
	pYES2-R	CGTGACATAACTAATTACATGATGC

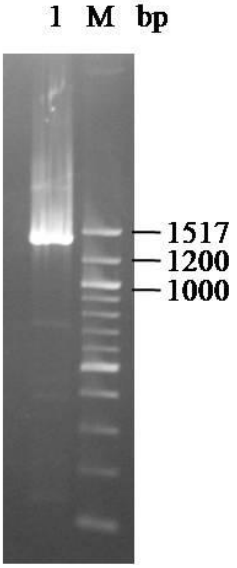


Figure 5. 1 Agarose gel image of barramundi Δ6 desaturase PCR fragments including partial 5' and 3' untranslated regions (UTR). Lane1: RT-PCR was conducted as described in 5.4.6.2 with barramundi hepatic RNA as template and with primers Fads-F and Fads-R (Table 5.1), with Q-solution to obtain a 1444 bp PCR fragment.

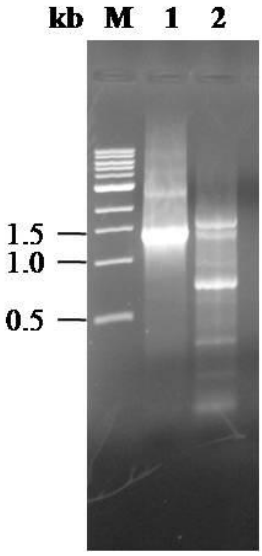


Figure 5. 2 Agarose gel image of barramundi Δ6 desaturase ORF PCR fragments. PCR was conducted as described in 5.4.6.1 with primers FadsEcoRI-F and FadsXhoI-R (Table 5.1) to obtain a 1359 bp product, including RE cutting sites of EcoRI and XhoI.

Lane 1: PCR products of barramundi Δ6 desaturase ORF, with Q-solution;

Lane 2: PCR products of barramundi Δ6 desaturase ORF, without Q-solution.

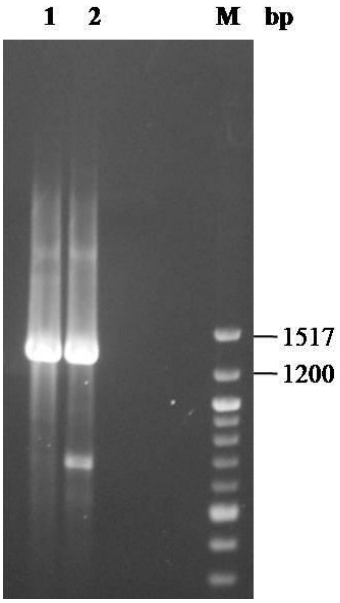


Figure 5. 3 Agarose gel image of barramundi elongase full sequence PCR fragment. PCR was conducted as described in 5.4.6.1 with primers Elovl-F and Elovl-R (Table 5.2) to obtain a 1460 bp product, including 5' and 3' UTR regions.

Lane 1: PCR products of barramundi ELOVL, with Q-solution;

Lane 2: PCR products of barramundi ELOVL, without Q-solution.

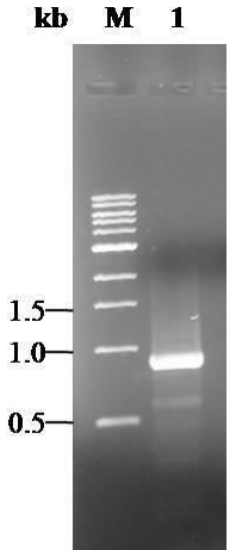


Figure 5. 4 Agarose gel image of barramundi elongase ORF PCR fragment. PCR was conducted as described in 5.4.6.1 with primers ElovlEcoRI-F and ElovlXhoI-R (Table 5.2) to obtain a 909 bp product including RE cutting sites of EcoRI and XhoI.

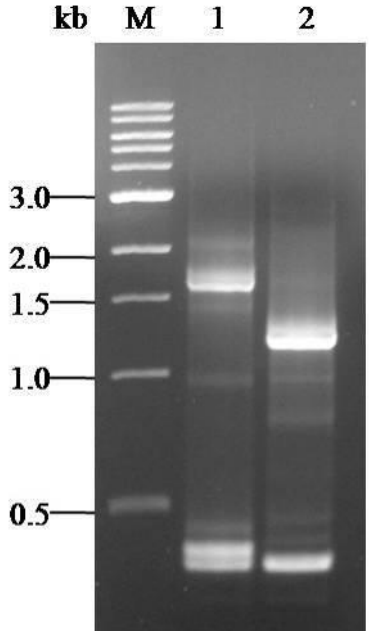


Figure 5. 5 Agarose gel image of ligation reaction check of barramundi pYES2/BarraFADS and pYES2/BarraELOVL ligation reactions. PCR was conducted as described in 5.4.6.1 with primers pYES2-F and pYES2-R (Table 5.1 and 5.2) to obtain a 1717 bp and 1267 bp products, respectively.

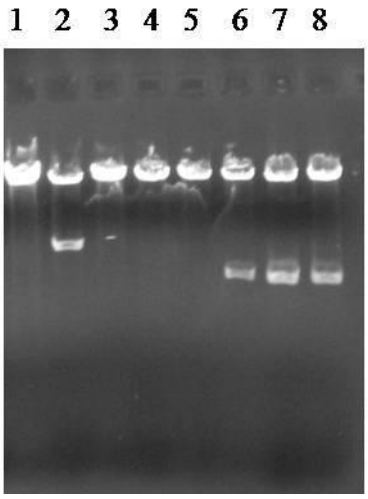


Figure 5. 6 Agarose gel image of digestion reaction of *E-coli* DH5α genomic DNA crude extracts by RE EcoRI and XhoI. The RE digestion reaction was conducted as described in 5.4.6.8.

Lane1: pYES2/ BarraFADS DH5α colony 1; Lane2: pYES2/ BarraFADS DH5α colony 2; Lane3: pYES2/ BarraFADS DH5α colony 3; Lane4: pYES2/ BarraFADS DH5α colony 4; Lane5: pYES2/BarraELOVL DH5α colony 1; Lane6: pYES2/BarraELOVL DH5α colony 2; Lane7: pYES2/BarraELOVL DH5α colony 3; Lane8: pYES2/BarraELOVL DH5α colony 4.

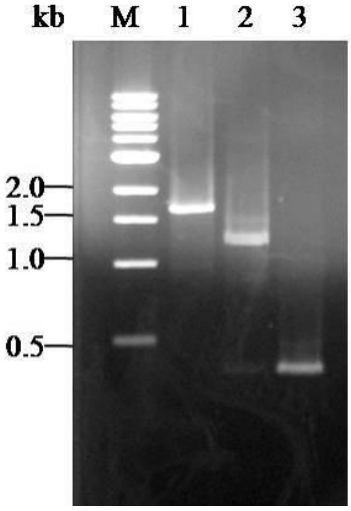


Figure 5. 7 Agarose gel image of pYES2/BarraFADS and pYES2/BarraELOVL plasmid. The PCR was conducted as described in 5.4.6.1. PCR products were amplified by pYES2-F and pYES2-R primers.

Lane1: pYES2/BarraFADS; Lane2: pYES2/BarraELOVL; Lane3: empty pYES2™ vector.

5.5 Results

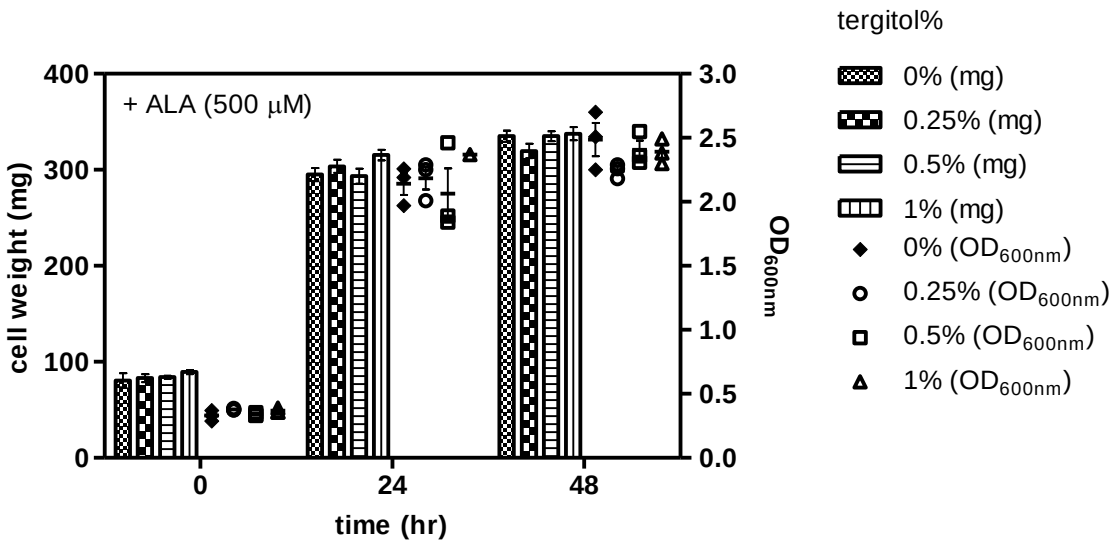
5.5.1 Effects of tergitol supplementations on yeast growth

Mild non-ionic detergent tergitol is commonly used for solubilization of lipids substrates in yeast growth media. However the possible effects of tergitol on yeast growth and hydrophobic substrate solubility in the concentration range for solubilization (0.1-1%, v/v) were not sufficiently discussed in relevant reports (119, 297). This prompted me to examine the effect of tergitol on yeast growth and fatty acid composition.

When yeast cells containing pYES2™ empty vector were supplemented with 500 μM of ALA as a substrate and the pYES2™ vector was induced by galactose in the presence of a range of tergitol concentrations from 0%, 0.25%, 0.5% to 1% (v/v) for up to 2 days, no significant differences were observed for cell mass and OD_{600nm} between yeast cells treated with different tergitol concentrations (Figure 5.8 A). The cell mass and OD_{600nm} were also examined for EPA treatments under the same culture and induction conditions. After EPA was added into the induction medium with a cell density of OD_{600nm}=0.4, the cells were harvested immediately (<1 hr), after 24 and 48 hr. No significant differences was observed for cell weight and OD_{600nm} at either the <1 hr or 24 hr time points, within the range of tergitol used here. After 48hr, the EPA treatment resulted in a significant difference in cell weight between the 0.25% and 1% tergitol groups (P<0.05), however, there was no significant difference in the OD_{600nm} (Figure 5.8 B).

The yeast total cell number and viable count from ALA treatment were measured and is shown in Figure 5.9. No significant differences were observed in the total cell number (Figure 5.9 A) and viable cell count (CFU) (Figure 5.9 B) between the tergitol concentrations examined in this experiment. Likewise, no significant differences in the total and viable cell counts were observed when the cells were supplemented with EPA as the substrate and cultured in different tergitol concentrations (Figure 5.10 A and B). These results suggest that the tergitol concentration up to 1% showed no significant effects on cell growth compared with no tergitol supplementation.

A



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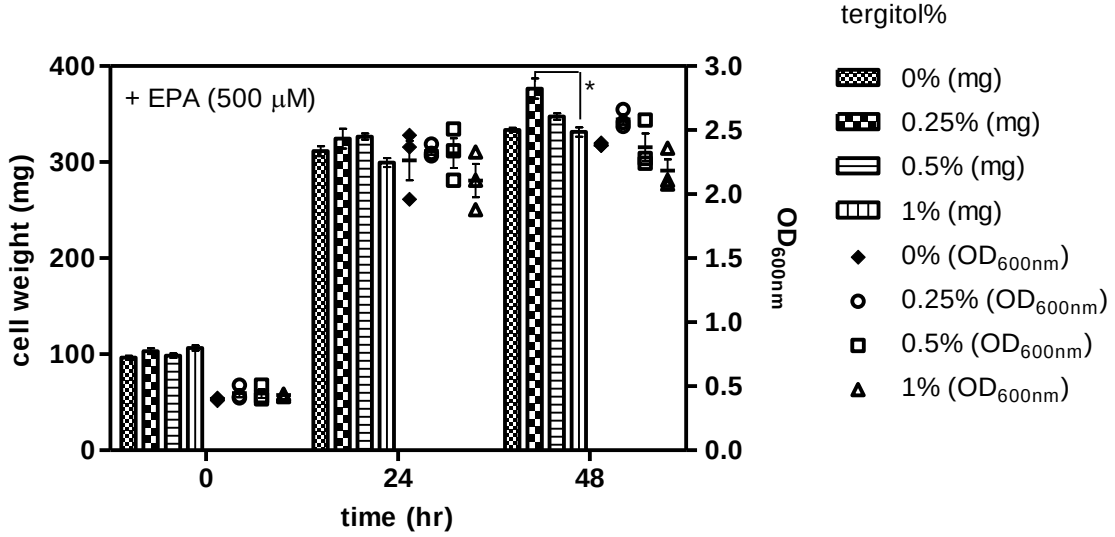
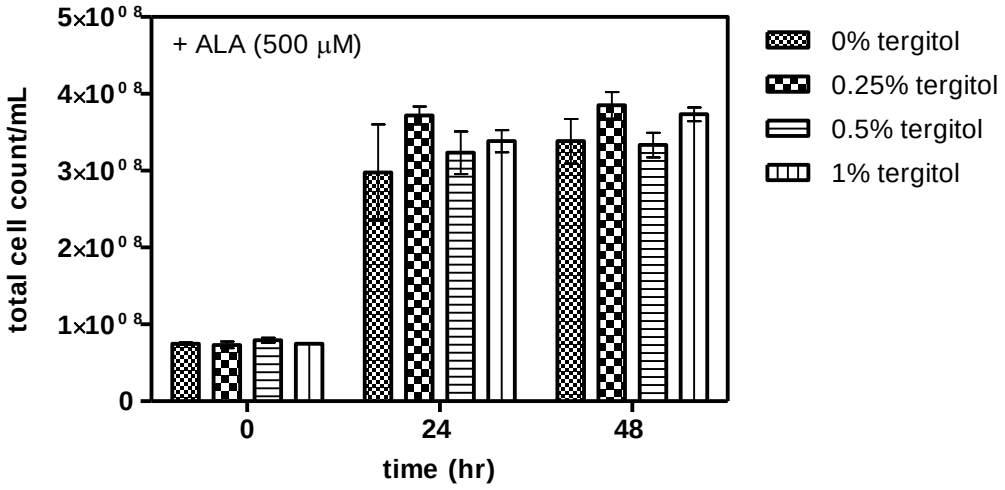


Figure 5. 8 Cell masses and OD_{600nm} values of *S. cerevisiae* yeast cells containing pYES2™ empty vectors cultured in the presence of different tergitol concentrations with 500 μM ALA (A) and EPA (B) supplementation. Each bar represents the cell mass (mg) and the dot plot refers to $OD600_{nm}$ values. All values are means ± SEM, n=3 (technical replicates). Statistical analysis for fatty acids was determined by one-way ANOVA with Kruskal-Wallis post test and Dunn's multiple comparison test. Values with an asterisk superscript are significantly different from each other ($^{*}P<0.05$).

A



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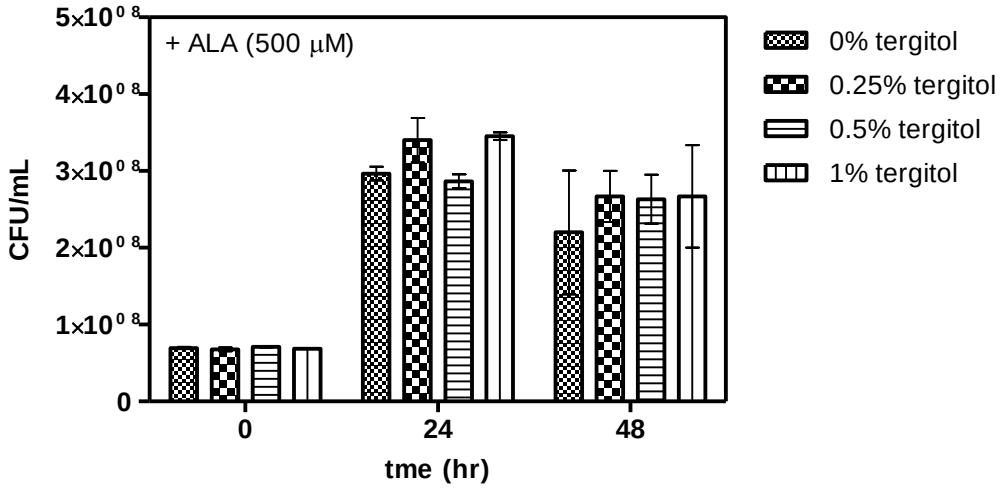
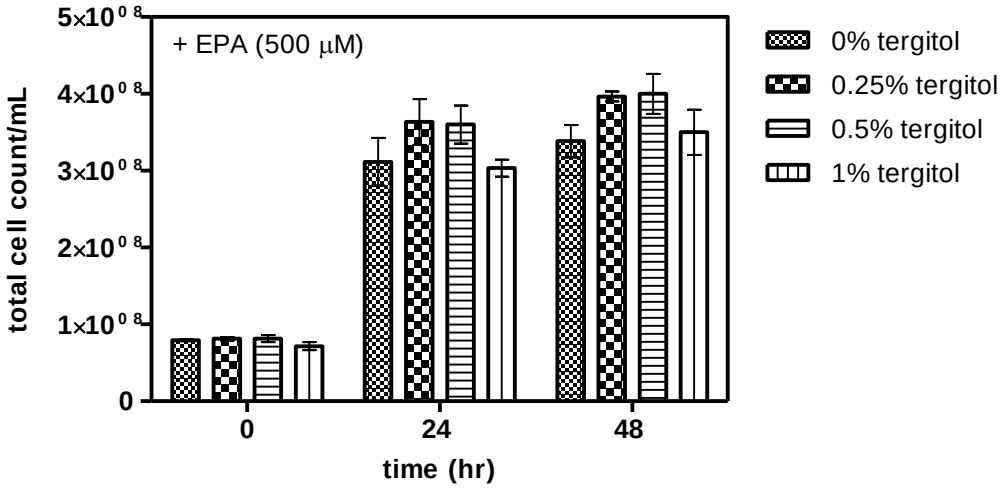


Figure 5. 9 Total cell count (A) and viable cell count (CFU/mL) (B) of *S. cerevisiae* yeast containing pYES2™ empty vectors when culturing at different tergitol concentrations with 500 μM of ALA supplementation. The cells and medium were harvested after <1 hr, 24 and 48 hr of substrate supplementation. All values are means ± SEM, n=3 (technical replicates). Statistical analysis for fatty acids was determined by one-way ANOVA with Kruskal-Wallis post test and Dunn's multiple comparison test. No significant differences were observed in the total cell number and CFU in the four tergitol concentrations.

A



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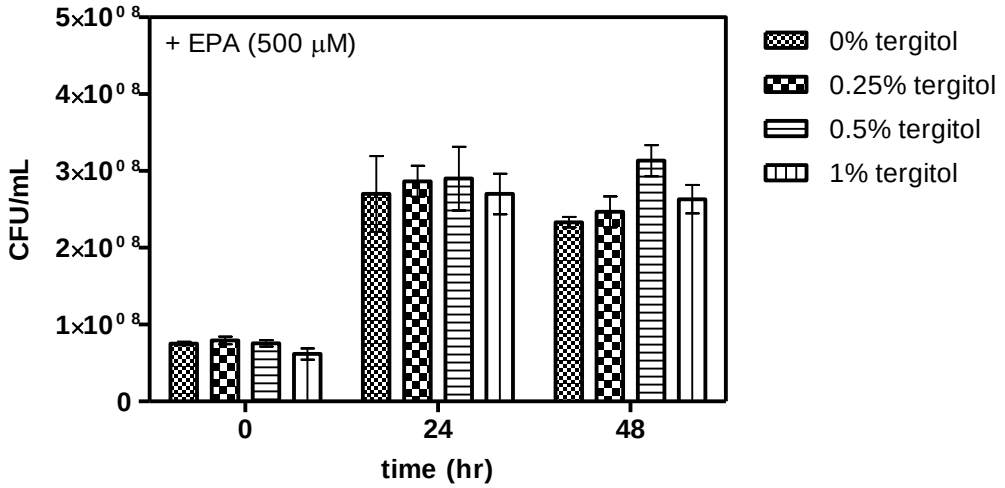


Figure 5. 10 Total cell count (A) and viable cell count (CFU/mL) (B) of *S. cerevisiae* yeast containing pYES2™ empty vectors cultured at different tergitol concentrations with 500 μM EPA supplementation. The cells and medium were harvested after <1 hr, 24 and 48 hr of substrate supplementation. All values are means ± SEM, n=3 (technical replicates). Statistical analysis for fatty acids was determined by one-way ANOVA with Kruskal-Wallis post test and Dunn's multiple comparison test. No significant differences were observed in the total cell number and CFU in the four tergitol concentrations.

5.5.2 Effects of tergitol supplementations on yeast fatty acid profile and substrate solubility

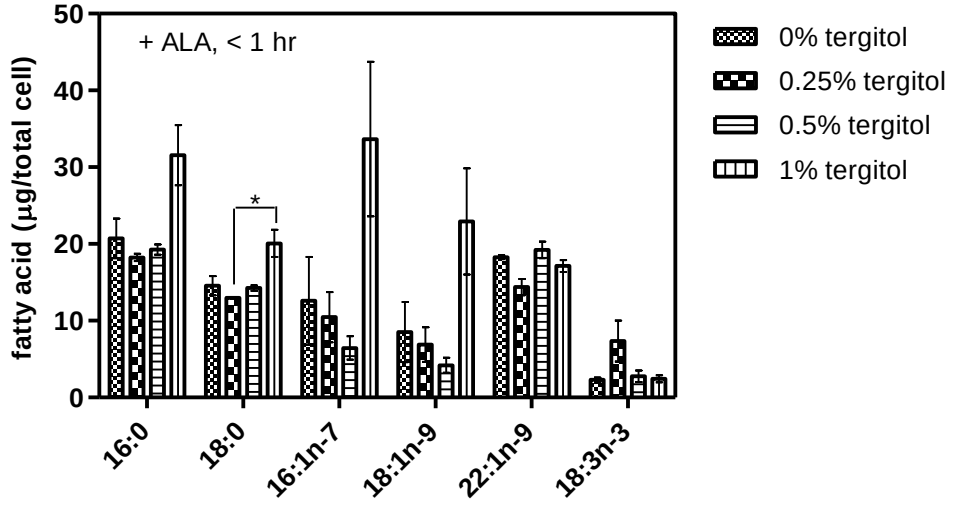
Yeast cells containing pYES2™ empty vector were supplemented with 500 μM of ALA or EPA as substrates and then the pYES2™ vector was induced by galactose in the presence of tergitol within the concentration range described above. Cells and medium from each treatment were collected for fatty acid extraction and GC analysis. For the ALA treatment, there was a significant increase in yeast endogenous 18:0 level in cells within 1 hr in the cells treated with 1% tergitol (Figure 5.11 A). Whilst no significant differences were observed for other endogenous fatty acids, the tergitol at 1% seemed to result in a certain degree of disturbance to the fatty acid levels for 16:0, 16:1 n-7 and 18:1 n-9. Low levels of ALA substrates could also be detected in cellular total fatty acid extracts less than 1 hr after substrate treatment. At the 0.25% tergitol level, the substrate incorporation tended to be increased when compared to no tergitol addition and other tergitol concentrations (Figure 5.11 A). Fatty acid profiles of the culture medium were also examined. The results of the fatty acid concentrations in the culture medium from the ALA treatment at <1 hr under various tergitol concentrations is shown in Figure 5.11 B. Fatty acids 16:0, 18:0 and 22:1 n-9 were the 3 major lipid components in the culture medium. No significant differences were observed in the concentrations of any of the original fatty acid in the culture medium between the different tergitol levels. However, higher tergitol levels resulted in an increased solubility of ALA compared to the no tergitol group, and a significant increase in ALA levels in the medium was observed between the no tergitol and 1% tergitol treatments. However, tergitol at 0.25% resulted in 25 fold increase of measurable ALA levels in the culture medium and likewise the 0.5 and 1% tergitol concentrations resulted in 24 and 37 fold increases in medium ALA levels, respectively (Figure 5.11 B).

The results of ALA substrate supplementation at 24 and 48 hr after supplementation are shown in Figure 5.12 and 5.13. No significant differences were observed for any of the endogenous fatty acid or ALA levels in cells (Figure 5.12 A), however, in the medium (Figure 5.12 B), the levels of 16:1 n-7, 18: 1n-9 and ALA increased gradually with increasing tergitol concentration after 24 hr ALA supplementation. Similar distributions for cells (Figure 5.13 A) and medium (Figure 5.13 B) fatty acids were also observed after 48 hr ALA supplementation, although the significant difference was not found in the ALA levels in the medium between different tergitol concentrations (Figure 5.13 B).

EPA was chosen to examine the effects of tergitol on solubility of a C20 fatty acid. For cells treated with EPA, treatment with a tergitol level at 0.25% resulted in the presence of more EPA in cellular lipids compared with the cellular ALA at either no tergitol or higher tergitol levels (Figure 5.14 A). Similar to the fatty acid distribution in the medium for the ALA supplementation studies, the fatty acid profiles in the medium for the EPA treatments indicated that higher tergitol concentrations resulted in higher measurable EPA levels (Figure 5.14 B). There was a 17, 21 and 23 fold increase in EPA levels in the culture medium with 0.25%, 0.5% and 1% tergitol concentrations, respectively compared with no tergitol treatment (Figure 5.14 B). No significant differences in fatty acid levels were observed between the no tergitol and tergitol treatment groups after 24 and 48 hr of EPA supplementation (Figure 5.15 A and Figure 5.16 A). Once again, similar to ALA supplementation, the levels of 16:1 n-7, 18:1 n-9 and EPA in the medium increased with increasing tergitol concentrations (Figure 5.15 B and Figure 5.16 B). The level of 16:0 in the medium after 48 hr of EPA treatment was also significantly elevated in the 1% tergitol compared to the no tergitol groups (Figure 5.16 B).

In summary, tergitol levels at 0.25% or above (up to 1%), resulted in an increase in extractable C18 and C20 substrate levels in the culture mediun after <1 hr of substrate supplementation. No significant differences were observed in cellular substrate levels after 24 or 48 hr of substrate supplementation.

A



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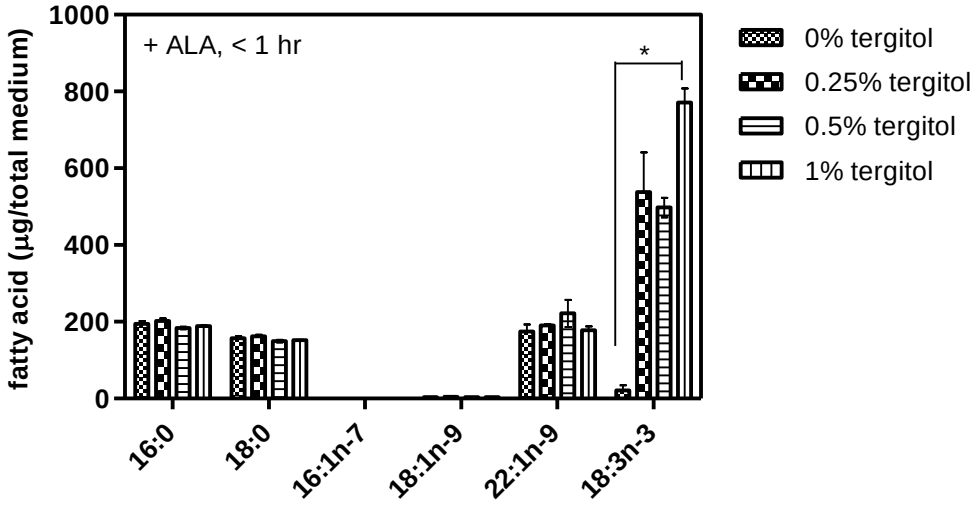
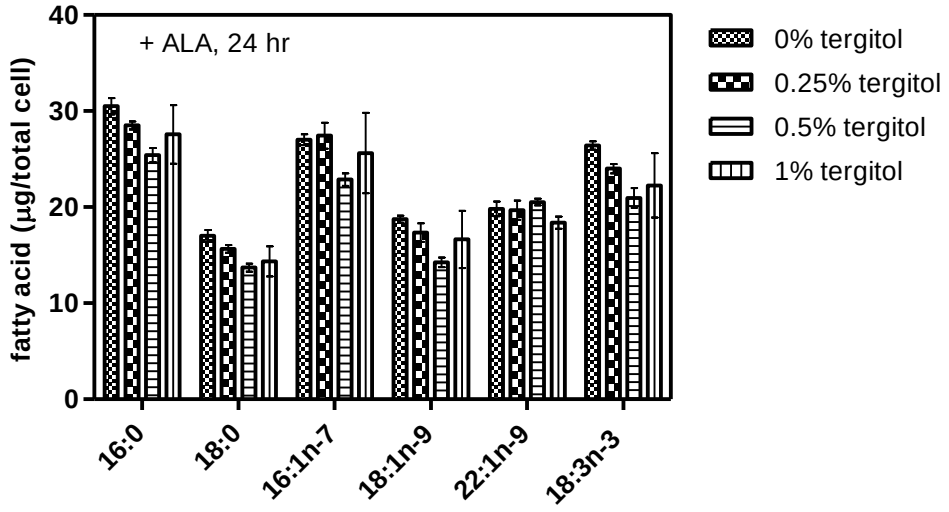


Figure 5. 11 Fatty acid levels of total cells (total mass of cells) (A) and medium (B) of *S. cerevisiae* yeast containing pYES2™ empty vectors cultured at various tergitol concentrations with 500 µM ALA supplementation. The cells and medium were harvested after <1 hr of substrate supplementation. All values are means ± SEM, n=3 (technical replicates). Statistical analysis for fatty acids was determined by one-way ANOVA with Kruskal-Wallis post test and Dunn's multiple comparison test. Values with an asterisk superscript are significantly different from each other ($^{*}P<0.05$). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

A



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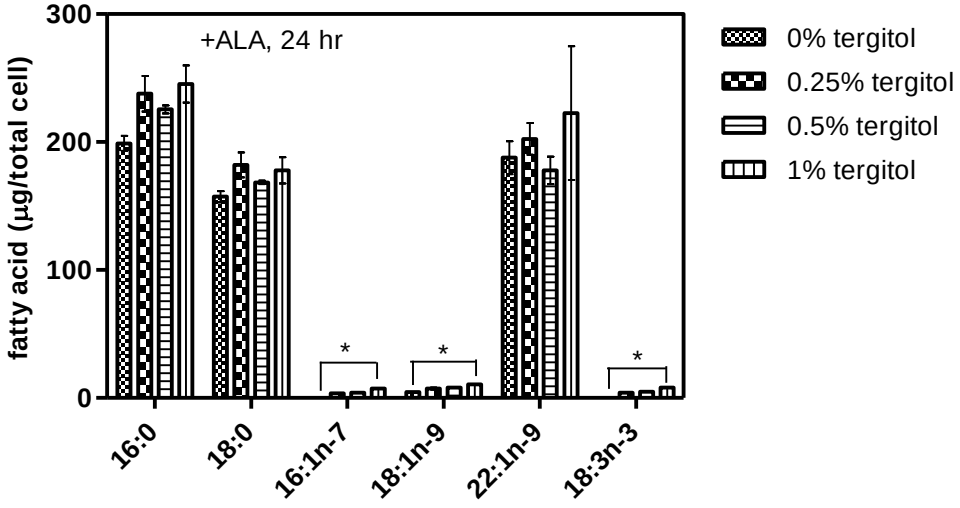
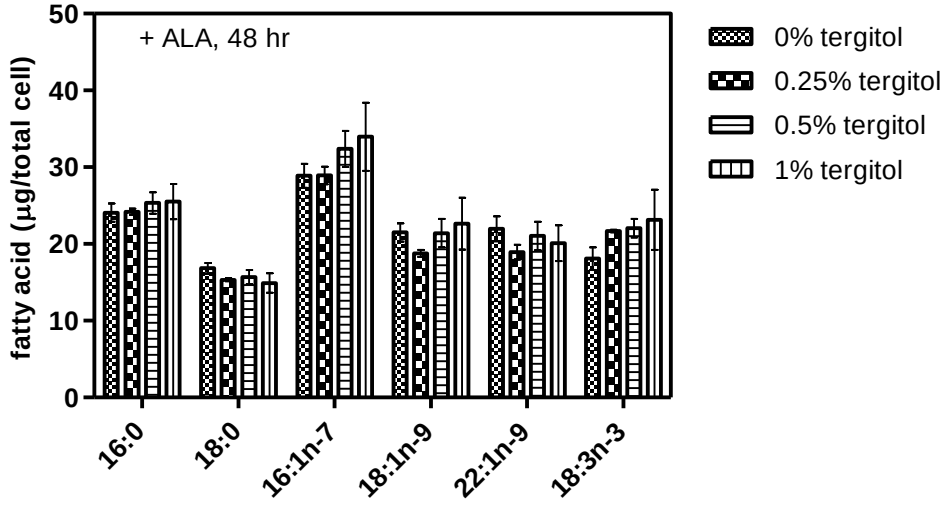


Figure 5. 12 Fatty acid levels of total cells (total mass of cells) (A) and medium (B) of *S. cerevisiae* yeast containing pYES2™ empty vectors cultured at various tergitol concentrations with 500 µM ALA supplementation. The cells and medium were harvested after 24 hr of substrate supplementation. All values are means ± SEM, n=3 (technical replicates). Statistical analysis for fatty acids was determined by one-way ANOVA with Kruskal-Wallis post test and Dunn's multiple comparison test. Values with an asterisk superscript are significantly different from each other ($^{*}P<0.05$). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

A



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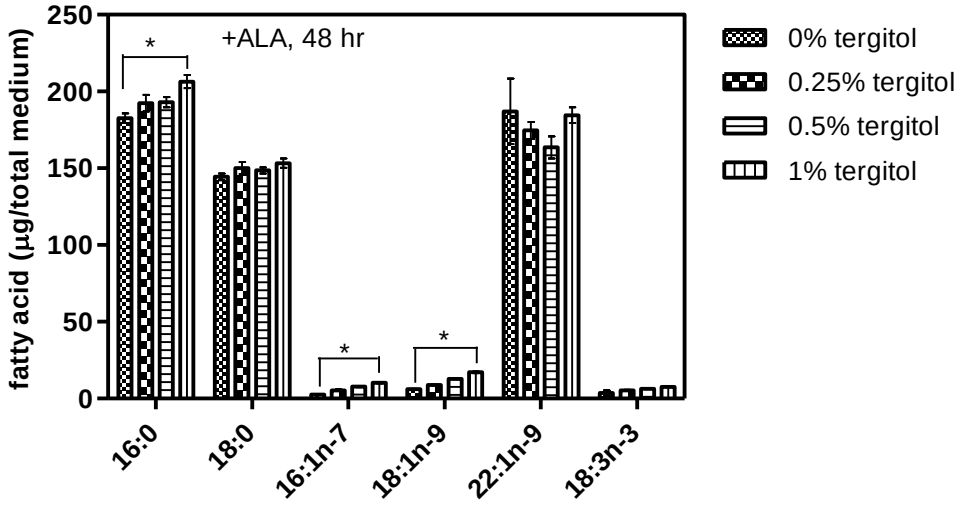
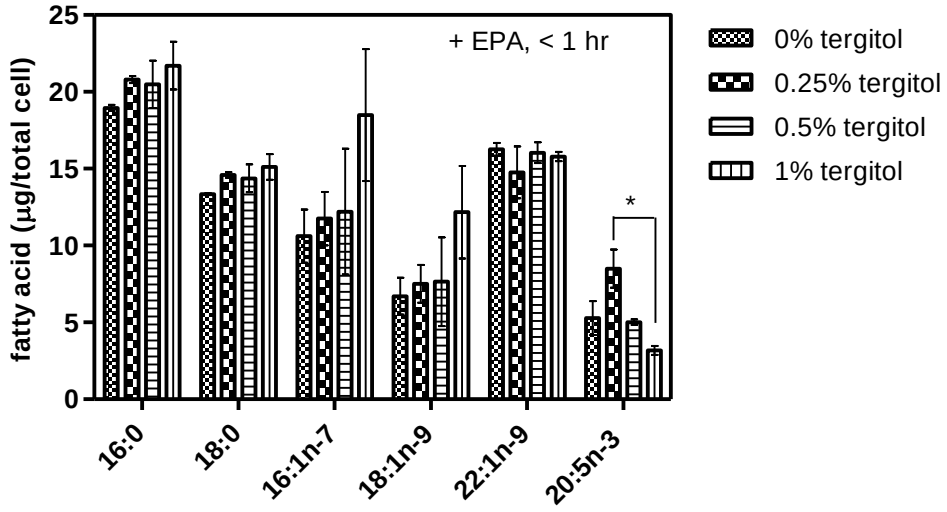


Figure 5. 13 Fatty acid levels of total cells (total mass of cells) (A) and medium (B) of *S. cerevisiae* yeast containing pYES2™ empty vectors cultured at various tergitol concentrations with 500 µM ALA supplementation. The cells and medium were harvested after 48 hr of substrate supplementation. All values are means ± SEM, n=3 (technical replicates). Statistical analysis for fatty acids was determined by one-way ANOVA with Kruskal-Wallis post test and Dunn's multiple comparison test. Values with an asterisk superscript are significantly different from each other ($^{*}P<0.05$). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

A



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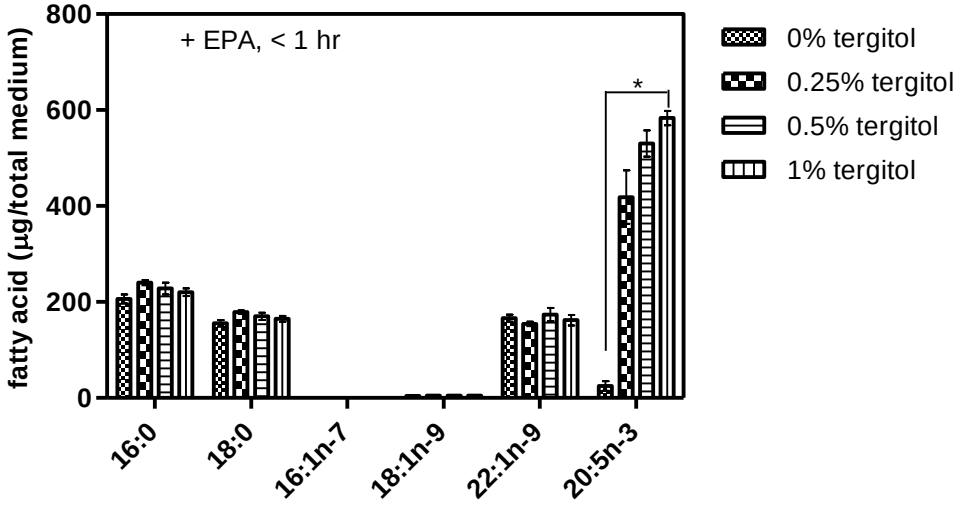
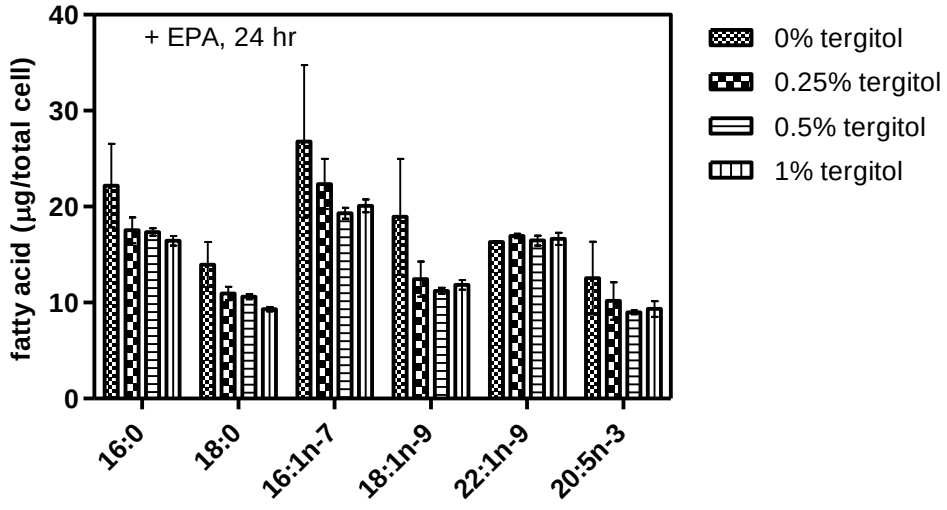


Figure 5. 14 Fatty acid levels of total cells (total mass of cells) (A) and medium (B) of *S. cerevisiae* yeast containing pYES2™ empty vectors cultured at different tergitol concentrations with 500 µM EPA supplementation. The cells and medium were harvested after <1 hr of substrate supplementation. All values are means ± SEM, n=3 (technical replicates). Statistical analysis for fatty acids was determined by one-way ANOVA with Kruskal-Wallis post test and Dunn's multiple comparison test. Values with an asterisk superscript are significantly different from each other ($^{*}P<0.05$). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

A



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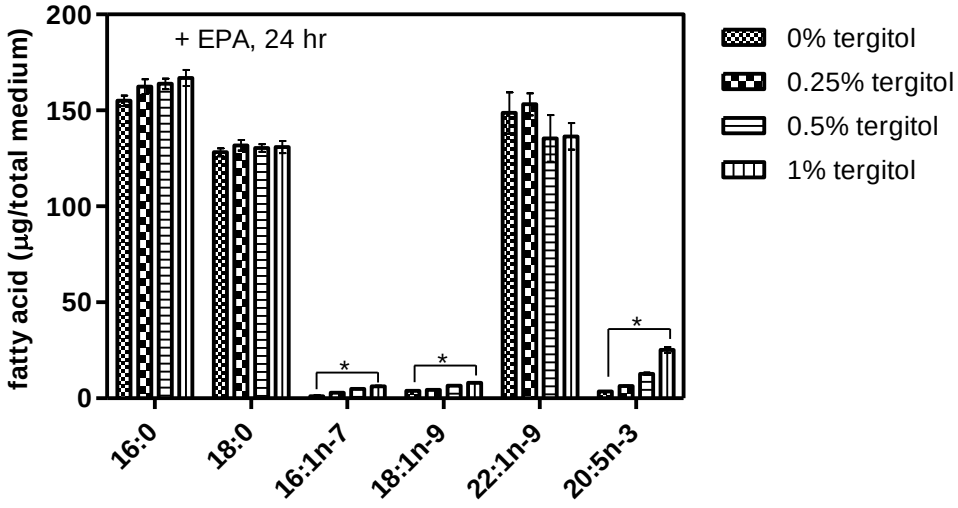
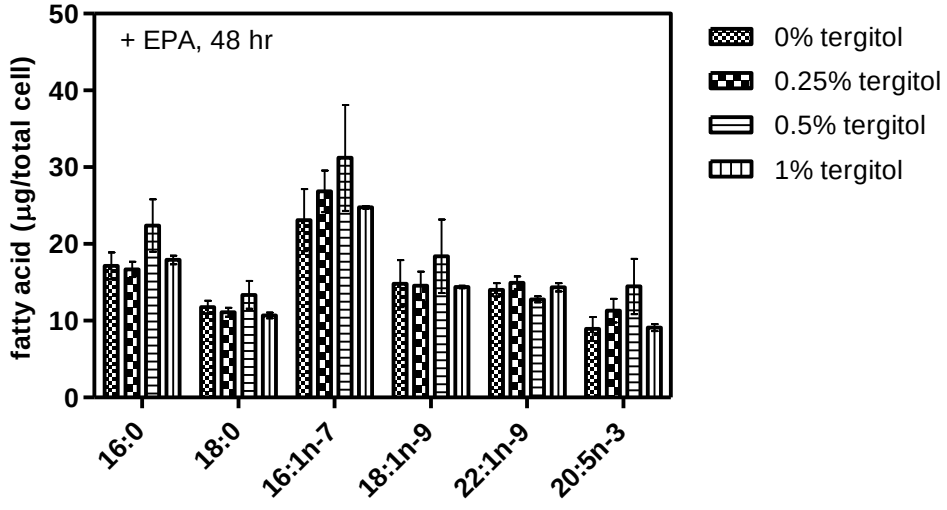


Figure 5. 15 Fatty acid levels of total cells (total mass of cells) (A) and medium (B) of *S. cerevisiae* yeast containing pYES2™ empty vectors cultured at different tergitol concentrations with 500 µM EPA supplementation. The cells and medium were harvested after 24 hr of substrate supplementation. All values are means ± SEM, n=3 (technical replicates). Statistical analysis for fatty acids was determined by one-way ANOVA with Kruskal-Wallis post test and Dunn's multiple comparison test. Values with an asterisk superscript are significantly different from each other ($^{*}P<0.05$). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

A



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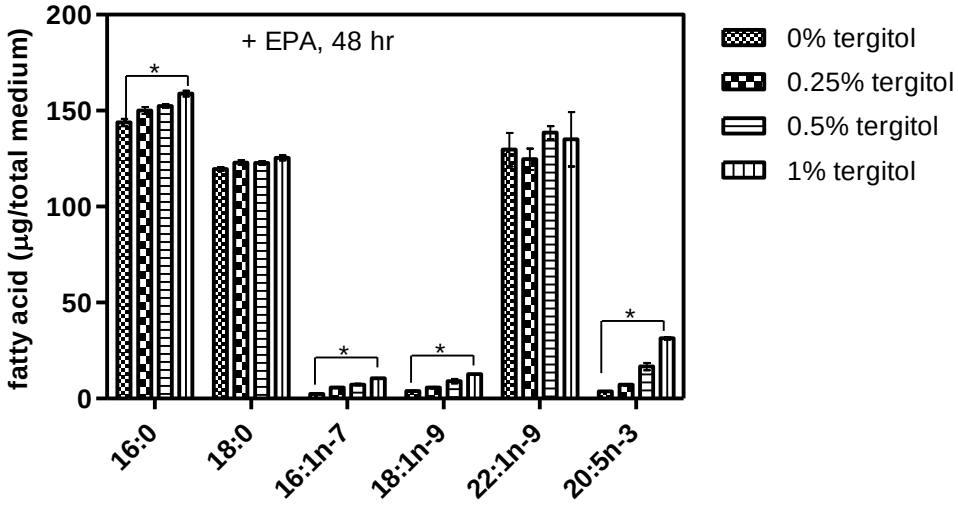


Figure 5. 16 Fatty acid levels of total cells (total mass of cells) (A) and medium (B) of *S. cerevisiae* yeast containing pYES2™ empty vectors cultured at different tergitol concentrations with 500 µM EPA supplementation. The cells and medium were harvested after 48 hr of substrate supplementation. All values are means ± SEM, n=3 (technical replicates). Statistical analysis for fatty acids was determined by one-way ANOVA with Kruskal-Wallis post test and Dunn's multiple comparison test. Values with an asterisk superscript are significantly different from each other ($^{*}P<0.05$). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

5.5.3 Barramundi FADS

5.5.3.1 Sequence analysis

Overlapping fragments of a FADS cDNA were amplified from juvenile barramundi hepatic RNA using one-step RT-PCR and 3'/5' RACE. The fragments were assembled to give a nucleotide sequence of 1991 bp (excluding a 21 bp external added polyT-tail) as a putative complete barramundi FADS mRNA. The 1991 bp sequence included an ORF of 1338 bp and a 178 bp 5' UTR and a 475 bp 3' UTR plus a polyA-tail (Figure 5.17). The 1338 bp length of the FADS ORF encoded a protein of 445 amino acids. Parallel alignment of the putative barramundi desaturase ORF peptide sequence with Δ5 and Δ6 desaturase ORF peptide sequences (Figure 5.18) from human, baboon, rat, zebra fish and Atlantic salmon indicated that the barramundi putative desaturase gene contained elements typical of a membrane-bound desaturase. These included a HPGG heme binding motif in an N-terminal cytochrome b5-like domain, three histidine rich sequences (His boxes) comprised of a group of eight conserved histidines: HPGG, HDXGH, HFQHH and QIEHH and four transmembrane regions: N_{130}-RPLFFCLHLGHIVLLEALAWLMI-C_{152}, N_{154}-LWGTNWILTSLCAVMLATAQSQ-C_{175}, N_{267}-FFLVGPPLLIPVFFHIQIMHTMI-C_{289} and N_{309}-SCYIPLYGLFGSLALISFVRFLE-C_{331} (Figure 5.18). The barramundi putative desaturase had higher sequence identity to human (*Homo sapiens*) (65%) and rat (*Rattus norvegicus*) (64%) Δ6 desaturase (FADS2) than human (51%) and rat (57%) Δ5 desaturase (FADS1). The putative barramundi desaturase also showed 65% sequence identity to a baboon (*Papio Anubis*) Δ6 desaturase. When comparing the peptide sequence homology (Table 5.3) with Atlantic salmon (*Salmon salar*), the barramundi putative desaturase was found to share 77% identity with FADS1 and 76% with FADS2. Presumably it relates to very small difference between the FADS1 and FADS2 peptide sequences. In addition, alignment of the barramundi putative desaturase peptide sequence in this study with another recently reported barramundi putative Δ6 desaturase (GQ214179) showed 98% homology and identity in the peptide sequence, but differed in 6 amino acids throughout the coding region (Figure 5.18).

The predicted peptide sequence of FADS in this study had a molecular weight of 51.8 kilodaltons (kDa) which was predicted by Science Gateway Protein Molecular Weight Calculator (http://www.sciencegateway.org/tools/proteinmw.htm). A phylogenetic tree analysis of peptide sequence of barramundi desaturase aligned with Δ5 and Δ6 desaturase gene family previously characterized in other fish species and animals and analysed by NJ

algorithm by ClustalX and MEGA version 4 (298) suggested that barramundi putative FADS corresponds to the vertebrate FADS2 family and is most closely related to FADS2 sequences of marine fish cobia (*Rachycentron canadum*) (ACJ65149) and turbot (*Psetta maxima*) (AAS49163). The FADS2 sequences of the diadromous fish European seabass (*Dicentrarchus labrax*) (ACD10793) and the marine fish nibe croaker (*Nibea mitsukurii*) (ACX54437) FADS2 sequences were in the same cluster, which was closely related to the sequence cluster of barramundi, cobia and turbot (Figure 5.19).

5.5.3.2 The recombinant barramundi desaturase enzyme has both Δ6 and Δ8 activity

The barramundi putative desaturase gene was functionally characterized by expression in transformed *S. cerevisiae* containing plasmid pYES2/BarraFADS and induced by SC selective medium^{-U} containing 2% of galactose. Addition of ALA resulted in a time-dependent increase in synthesis of 18:4 n-3, indicating Δ6 desaturase activity (Figure 5.20). This Δ6 desaturase activity was also observed with 24:5 n-3 and with the n-6 substrate, LA (Table 5.4). The rate of conversion of LA was about half that of the equivalent n-3 substrate, ALA (Table 5.4 and Table 5.6). Other potential desaturase activities were also investigated by adding the appropriate fatty acids. Δ8 desaturase activity was observed with both n-3 (20:3) and n-6 (20:2) substrates (Table 5.4 and Figure 5.21). No Δ4 or Δ5 desaturase activity was observed following addition of the relevant substrates (Table 5.6).

The characterisation studies revealed that there were two n-3 Δ6 desaturase substrates, ALA and 24:5 n-3, having 2.3% and 1.2% product conversion in the yeast cells under identical culture conditions despite the final concentration was 500 μM for ALA and 100 μM for 24:5 n-3. Also, there was the n-6 substrate, LA, having ~1% product conversion (Table 5.6). The recombinant barramundi desaturase enzyme had both Δ6 and Δ8 desaturase activities. Supplementation of Δ8 desaturase substrates 20:3 n-3 and 20:2 n-6 showed 1.3% and 0.4% product conversion in the yeast cells, respectively.

5.5.3.3 Desaturation products in the culture medium

For the fatty acids with C18, only trace amounts of LA and ALA substrates was present in the medium after 48 hr of culture (Table 5.5), and neither of the desaturation products 18:3 n-6 and 18:4 n-3 were detected in the medium (Table 5.5). In contrast, significant amounts of 20:2 n-6, 20:3 n-3 and 24:5 n-3 substrates were still present in the culture medium after 48 hr (Table 5.5). In addition, the desaturation products 20:4 n-3 and 24:6 n-3 from both n-3

substrates (20:3 n-3 and 24:5 n-3) were detected in the culture medium (Table 5.5). Therefore the desaturation product concentrations present in the culture medium, as well as those present in the cells, were taken into account for calculating the apparent conversion rate (Table 5.6). When this was done, the conversion of ALA to 18:4 n-3, LA to 18:3 n-6 and 20:2 n-6 to 20:3 n-6 remained unchanged but the conversion of both 20:3 n-3 to 20:4 n-3 and 24:5 n-3 to 24:6 n-3 increased 6.9 fold.

5.5.3.4 Dose-response of ALA on the effect of the desaturase activity

Although accumulation of ALA in cells was concentration dependent (Figure 5.22 A), the 18:4 n-3 amounts detected still only represented a small percentage (2%-6%) of the total ALA added to the culture (Figure 5.22 B). The accumulation of 18:4 n-3 was not strictly related to cellular ALA levels but followed a bell shape-like curve.

5.5.3.5 No desaturation activity towards saturates and monoenes

Without supplementation of any exogenous fatty acid substrates, culturing the yeast cells containing barramundi FADS2 ORF in the induced (galactose) or uninduced (glucose) media, did not result in changes in the major endogenous fatty acid levels in yeast cells after 48 hr (Figure 5.23). These results indicated that the recombinant barramundi desaturase had no desaturation activity towards saturates and monoenes.

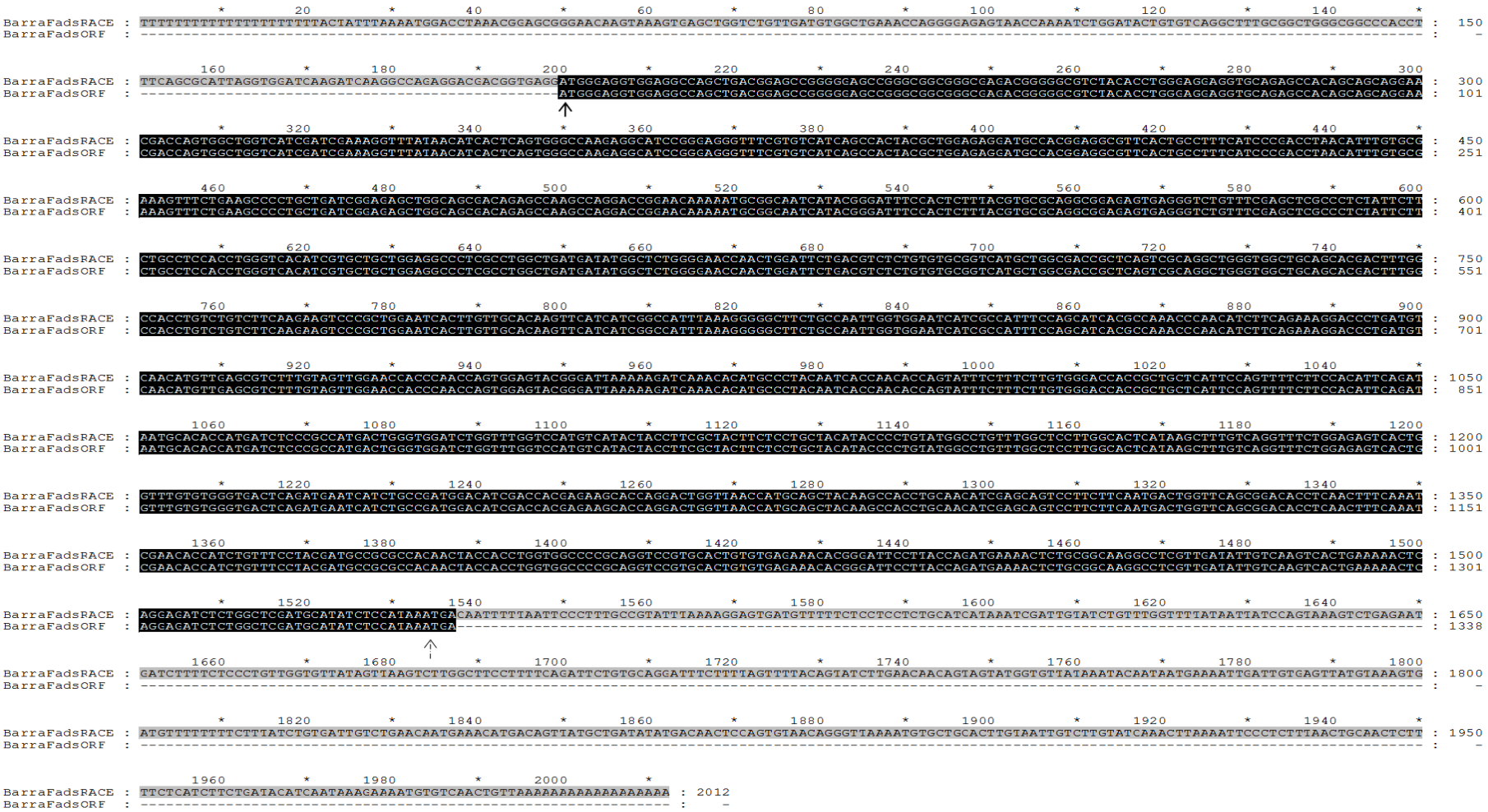


Figure 5. 17 Alignment of barramundi putative FADS cDNA (include 5’ and 3’ UTR) with the FADS ORF nucleotide sequence. The complete FADS nucleotide sequence was determined by combining sequence information from 3’/5’ RACE PCR products overlapped with the partial FADS cDNA

fragments. The FADS ORF nucleotide sequence was determined by sequencing of PCR products, which were amplified by using Fads-F and Fads-R primers to obtain a fragment containing the partial 5' and 3' UTR regions. The ATG start codon is indicated by a solid arrow sign and the TGA stop codon is indicated by a dashed arrow sign.

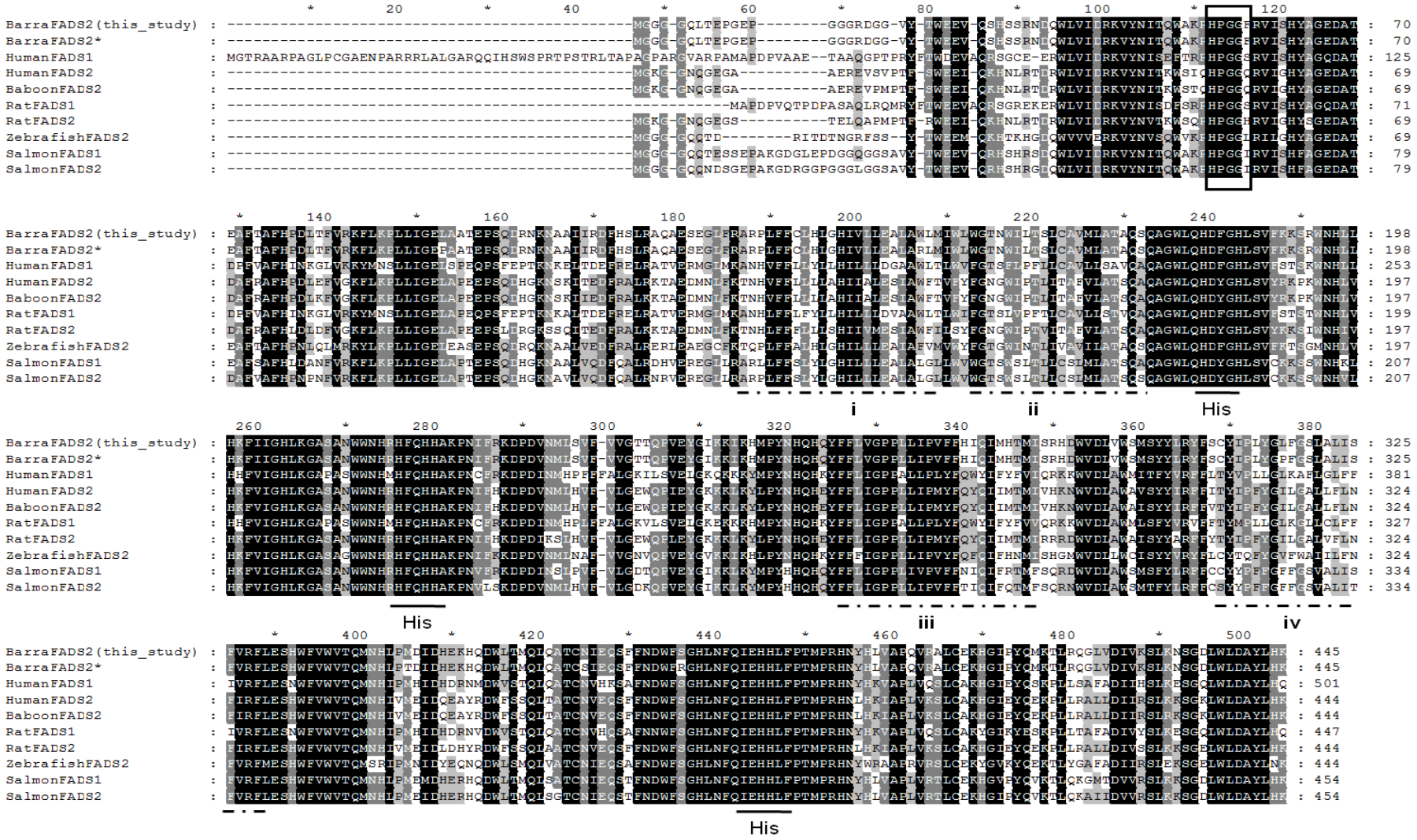

BarraFADS2(this_study)
BarraFADS2*
HumanFADS1
HumanFADS2
BaboonFADS2
RatFADS1
RatFADS2
ZebrafishFADS2
SalmonFADS1
SalmonFADS2
His
i
ii
His
iii
iv
His

Figure 5. 18 Alignment of the predicted amino acid sequences of the putative barramundi desaturase (GU047383, this study) with desaturases in human, baboon, rat, zebra fish, and salmon. Identical amino acids are in black, the heme binding motif in cytochrome b_5-like domain is circled by a square; three histidine-rich domains are underlined. Four putative transmembrane regions (dashed line) are predicted by SOSUI software (http://bp.nuap.nagoya-u.ac.jp/sosui/). *Putative barramundi Δ6 desaturase (GQ214179) (124). The GenBank® accession numbers for other sequences are listed in Table 5.3.

Table 5. 3 Identity comparison of desaturase peptide sequences of barramundi and other species

	Lates calcarifer FADS2 (GQ214179)	*Homo sapiens* FADS1 (NP_037534)	*Homo sapiens* FADS2 (AAG23121)	*Papio anubis* FADS2 (ACI46980)	*Rattus norvegicus* FADS1 (BAB69054)	*Rattus norvegicus* FADS2 (BAA75496)	*Danio rerio* FADS2 (AAH49438)	*Salmon salar* FADS1 (AAL82631)	*Salmon salar* FADS2 (AAR21624)
***Lates calcarifer* FADS2 (this study) (GU047383)**	98%[1]	51%	65%	65%	57%	64%	69%	77%	76%
Lates calcarifer FADS2 (GQ214179)		50%	64%	64%	56%	63%	68%	76%	75%
Homo sapiens FADS1 (NP_037534)			53%	53%	78%	52%	50%	52%	53%
Homo sapiens FADS2 (AAG23121)				97%	57%	88%	64%	63%	64%
Papio anubis FADS2 (ACI46980)					57%	88%	64%	62%	64%
Rattus norvegicus FADS1 (BAB69054)						57%	55%	57%	57%
Rattus norvegicus FADS2 (BAA75496)							62%	63%	62%
Danio rerio FADS2 (AAH49438)								64%	65%
Salmon salar FADS1 (AAL82631)									91%

[1]The peptide sequences were derived from coding sequences deposited at the NCBI Genbank® database. Sequences were aligned and the identity was computed using the Genedoc.

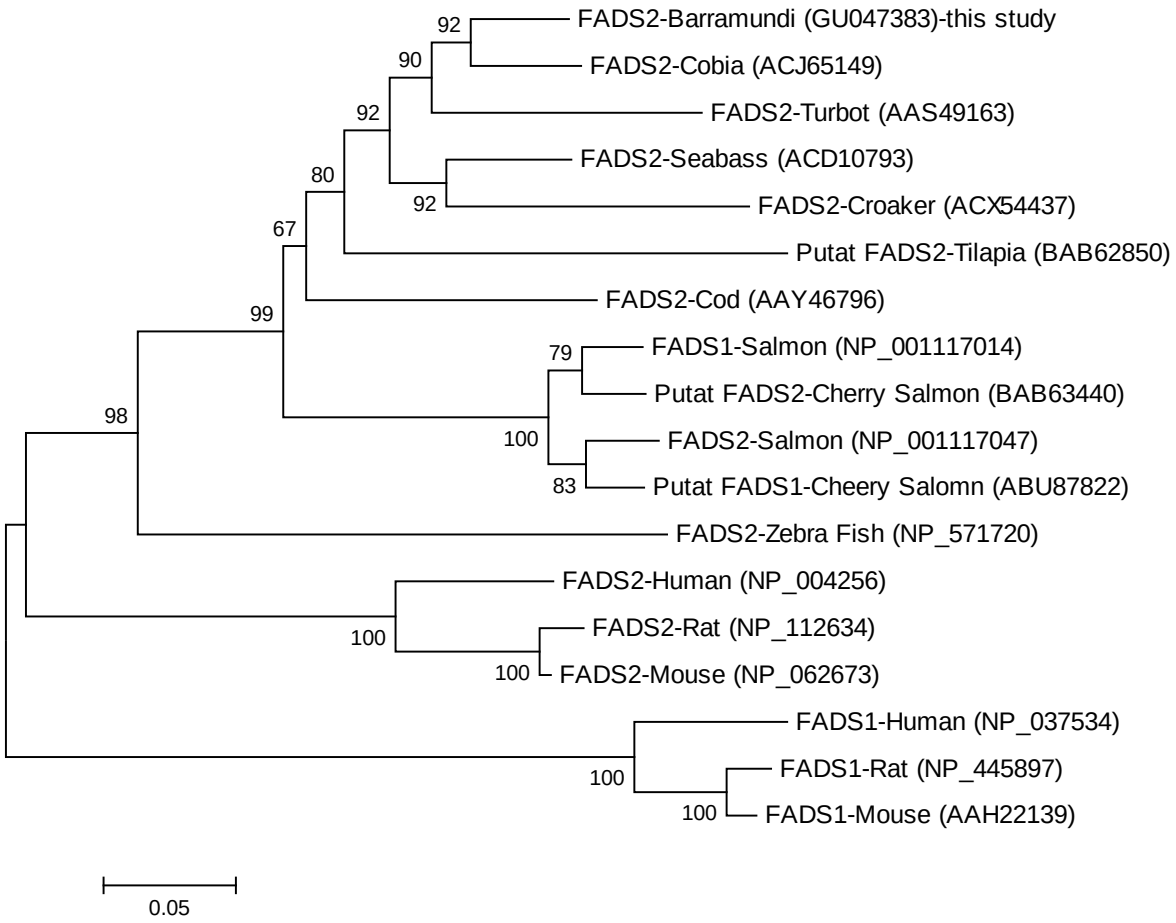
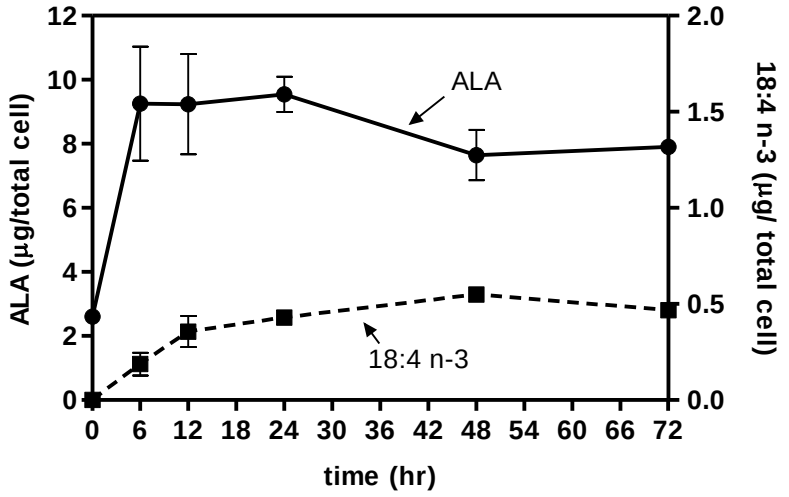


Figure 5. 19 A phylogenetic tree comparing peptide sequences of the barramundi desaturase and 17 available animal Δ5 and Δ6 desaturases. The accession numbers of peptide sequences were derived from NCBI GenBank® database. The distance tree was constructed using NJ algorithm analysis with ClustalX and MEGA version 4. The numbers on the branches indicate bootstrapping value for the node of the frequencies with which the tree topology presented was replicated after 1,000 iterations.

A



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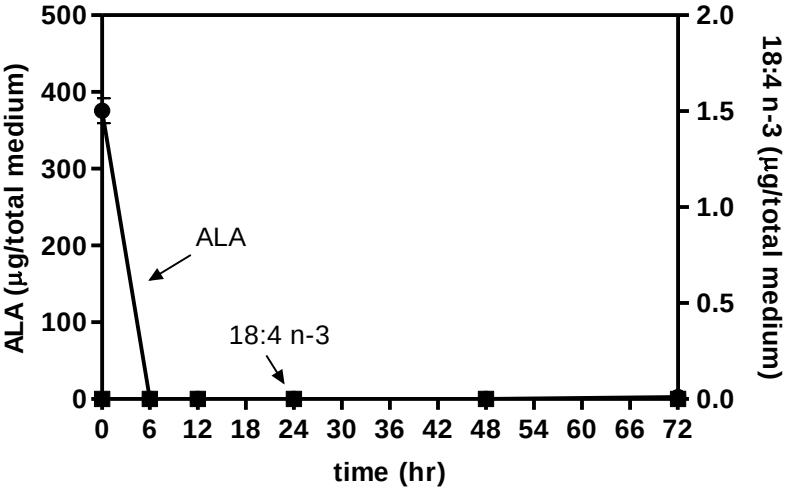

Figure 5. 20 Time course for 18:4 n-3 production after supplementation of ALA (250 µM) in transformed yeast cells (total mass of cells) (A) and medium (B) after expressing barramundi FADS ORF. All values are means ± SEM, n=3 (technical replicates). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

Table 5. 4 Fatty acid composition of yeast expressing barramundi FADS2 ORF

Barramundi desaturase										
µg/total cell	18:2 n-6 (LA) (500 µM)		18:3 n-3 (ALA) (500 µM)		20:2 n-6 (500 µM)		20:3 n-3 (500 µM)		24:5 n-3 (100 µM)	
Fatty acid	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced
16:0	27.1±4.7[1]	25.9±0.7	16.6±2.4	29.6±1.7	32.1±7.1	29.4±2.5	25.4±1.9	25.2±1.0	15.1±0.4	31.2±12.0
18:0	12.1±1.9	11.7±0.3	8.6±1.1	14.05±0.5	13.8±2.5	12.9±0.9	12.5±1.5	11.4±0.3	8.6±0.4	15.6±4.9
16:1n-7	32.6±7.7	25.8±0.2	17.0±3.4	27.2±1.5	53.8±16.0	39.0±4.9	32.4±0.1	31.2±2.9	25.4±0.6	54.2±24.7
18:1n-9	16.8±4.8	14.4±0.3	8.4±2.1	19.9±1.3	31.0±8.2	22.6±2.5	20.1±0.3	19.6±1.4	12.9±0.5	32.8±15.7
18:2n-6	24.2±5.2	19.2±0.1	-	-	-	-	-	-	-	-
18:3n-3	-	-	11.3±2.6	21.3±1.1	-	-	-	-	-	-
18:3n-6	-	0.2±0.0	-	-	-	-	-	-	-	-
18:4n-3	-	-	-	0.5±0.1	-	-	-	-	-	-
20:2n-6	-	-	-	-	29.3±6.3	31.1±1.7	-	-	-	-
20:3n-6	-	-	-	-	-	0.1±0.0	-	-	-	-
20:3n-3	-	-	-	-	-	-	12.4±0.5	19.2±1.6	-	-
20:4n-3	-	-	-	-	-	-	-	0.3±0.0	-	-
22:1n-9	15.6±0.6	15.1±1.2	13.5±0.6	15.0±1.1	11.7±3.0	9.2±2.2	7.7±0.7	13.6±2.3	15.6±0.8	16.1±1.4
24:5n-3	-	-	-	-	-	-	-	-	7.3±0.3	16.7±0.6
24:6n-3	-	-	-	-	-	-	-	-	-	0.2±0.0

[1]Desaturation products were quantitatively computed as µg of fatty acids of total cells (total mass of cells) per culture. Mean ± SEM, n≥3 (technical replicates).

Table 5. 5 Fatty acid composition of remaining medium in yeast expressing barramundi FADS2 ORF

Barramundi desaturase										
µg/total medium	18:2 n-6 (LA) (500 µM)		18:3 n-3 (ALA) (500 µM)		20:2 n-6 (500 µM)		20:3 n-3 (500 µM)		24:5 n-3 (100 µM)	
Fatty acid	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced
16:0	107.2±2.2[1]	174.7±3.5	160.4±6.0	147.3±5.5	154.6±1.3	154.9±9.1	143.1±5.3	146.3±5.9	104.3±3.3	112.1±0.2
18:0	96.9±1.9	144±2.5	132.7±4.3	122.6±3.9	125.6±0.8	125.1±5.9	120.2±3.6	119.8±3.8	93.6±2.3	99.5±0.3
18:1n-9	4.5±0.1	7.5±0.5	3.3±0.1	7.7±0.6	6.8±0.2	8.7±0.8	5.4±0.3	7.1±0.4	5.8±0.0	11.3±0.3
18:2n-6	3.7±0.2	5.8±0.8	-	-	-	-	-	-	-	-
18:3n-3	-	-	-	5.1±0.3	-	-	-	-	-	-
18:3n-6	-	-	-	-	-	-	-	-	-	-
18:4n-3	-	-	-	-	-	-	-	-	-	-
20:2n-6	-	-	-	-	290.5.±3.4	412.6±15.5	-	-	-	-
20:3n-6	-	-	-	-	-	-	-	-	-	-
20:3n-3	-	-	-	-	-	-	26.7±2.8	109.9±4.2	-	-
20:4n-3	-	-	-	-	-	-	-	1.6±0.1	-	-
22:1n-9	158.3±7.9	130.3±7.6	142.2±4.4	110.6±14.7	145.4±18.2	155.6±8.6	111.4±12.8	112.4±13.2	159.1±3.7	156.7±4.4
24:5n-3	-	-	-	-	-	-	-	-	136.1±5.4	189.5±4.6
24:6n-3	-	-	-	-	-	-	-	-	-	1.3±0.1

[1]Desaturation products were quantitatively computed as µg of fatty acids of total medium per culture. Mean ± SEM, n≥3 (technical replicates).

Table 5. 6 Substrate specificity and enzyme activity of barramundi FADS

Fatty acid substrate[1]	Desaturastion product	Conversion (%)		Enzyme activity
n-3 PUFA		**Cell only[2]**	**Cell + medium[3]**	
18:3 n-3 (ALA)	18:4 n-3	2.3±0.2	2.3±0.2	Δ6 desaturase
20:3 n-3	20:4 n-3	1.3±0.1	9.0±0.0	Δ8 desaturase
22:5 n-3 (DPA)	22:6 n-3 (DHA)	N.D.	N.D.	Δ4 desaturase
24:5 n-3	24:6 n-3	1.2±0.1	8.3±0.6	Δ6 desaturase
n-6 PUFA				
18:2 n-6 (LA)	18:3 n-6	1.1±0.1	1.1±0.1	Δ6 desaturase
20:3 n-6	20:4 n-6 (AA)	N.D.	N.D.	Δ5 desaturase
20:2 n-6	20:3 n-6	0.4±0.1	0.4±0.1	Δ8 desaturase

[1]Fatty acids ALA, 20:3 n-3, LA, 20:3 n-6 and 20:2 n-6 were supplemented 500 μM, DPA was supplemented 250 μM and 24:5 n-3 was supplemented 100 μM into yeast culture medium as a final concentration.

[2]Fatty acid in yeast cells only were used for calculating the conversion (%). Data are means ± SEM of n≥3 (technical replicates).

Conversion (%) = [product / (substrate + product) x 100]; all fatty acids were calculated based on fatty acid values listed on Table 5.4.

[3]Desaturated fatty acid products in medium were taken into account for calculating the conversion (%). Data are means ± SEM of n≥3. Conversion (%) = [product / (substrate + product) x 100]; all fatty acids were calculated based on fatty acid values listed on Table 5.4 and Table 5.5.

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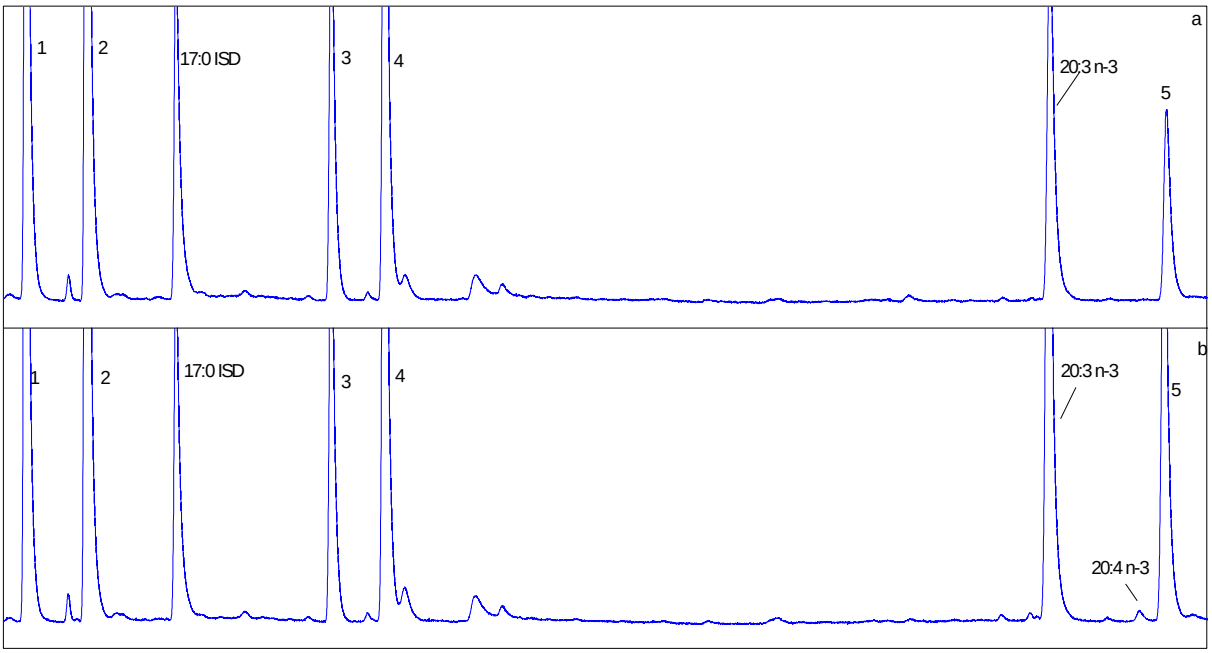

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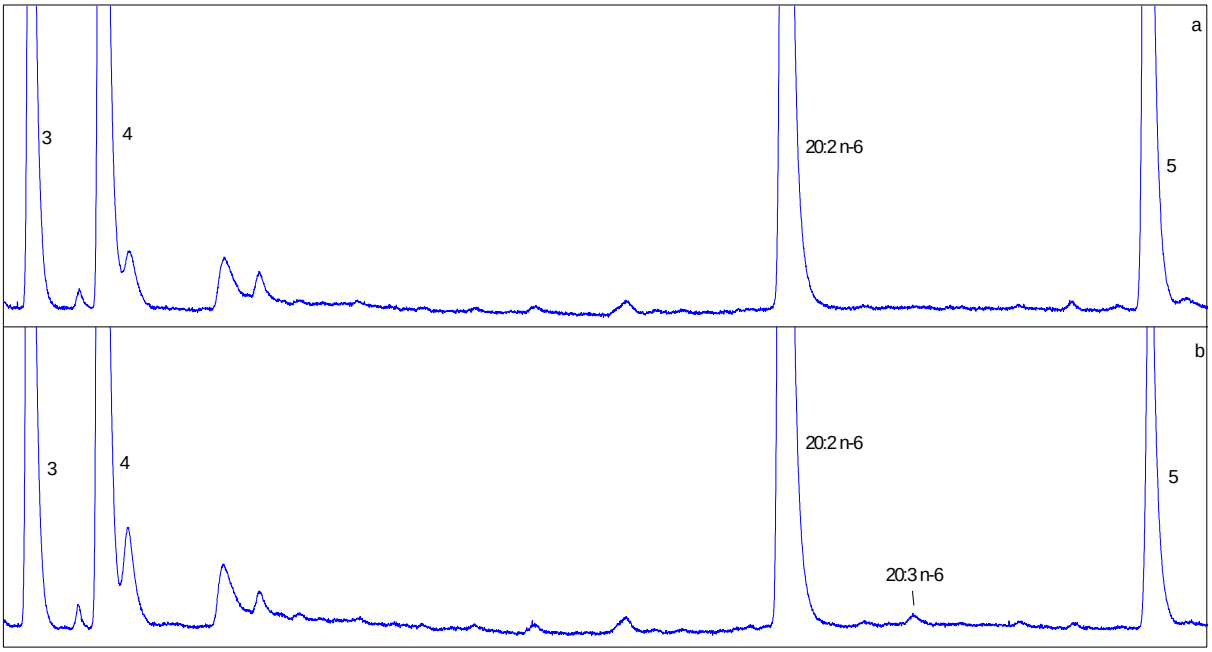
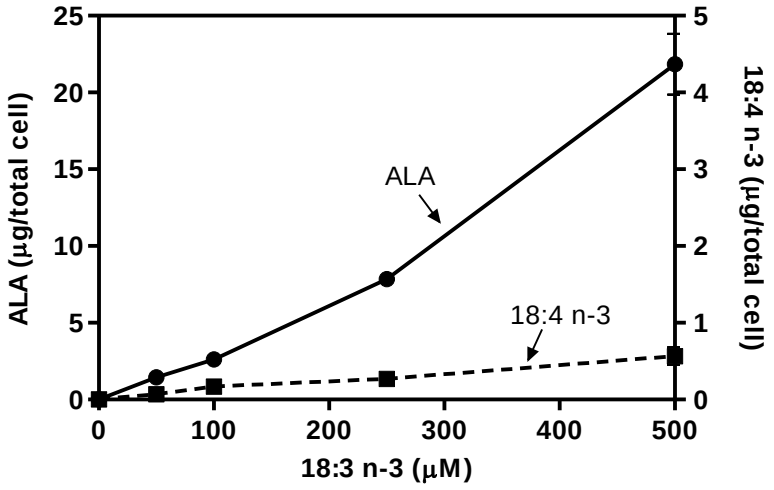


Figure 5. 21 Chromatograms of fatty acids from yeast transformed with barramundi FADS ORF was exogenous supplemented with substrate 20:3 n-3 (A) and 20:2 n-6 (B) and then cultured at uninduced (A-a, B-a) and induced (A-b, B-b) media for 48 hr. Identities of peaks 1 (16:0) (not shown), 2 (16:1 n-7) (not shown), 3 (18:0) 4 (18:1 n-9) and 5 (22:1 n-9) in all panels are the endogenous fatty acids of the yeast cells. Two additional peaks are observed in A-b and B-b panels. The peaks were identified as 20:4 n-3 and 20:3 n-6.

A



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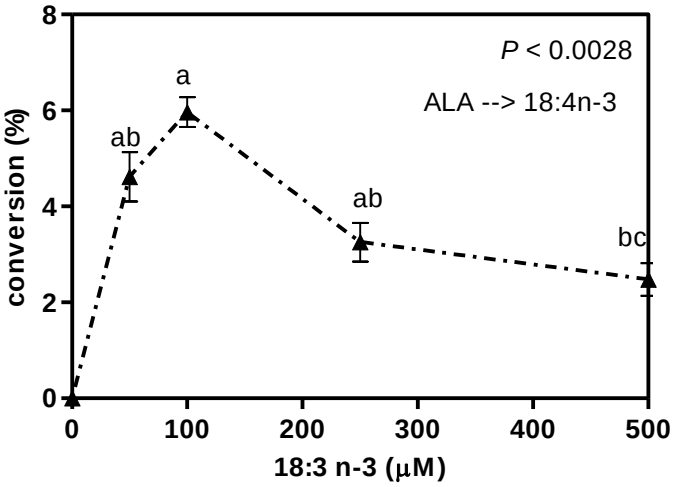

Figure 5. 22 Dose-response of ALA on effects of barramundi desaturase activity. Effects of increasing ALA at concentrations up to 500 µM on synthesis of 18:4 n-3 (A) and on the conversion (%) of ALA to 18:4 n-3 (B). Desaturation products were quantitatively computed as µg of fatty acids of total cells per culture. Conversion (%) was calculated by conversion (%) = [product / (substrate + product) x 100]. Mean ± SEM, n=3 (technical replicates). The total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture. Values with different superscripts are significantly different from each other (P<0.05).

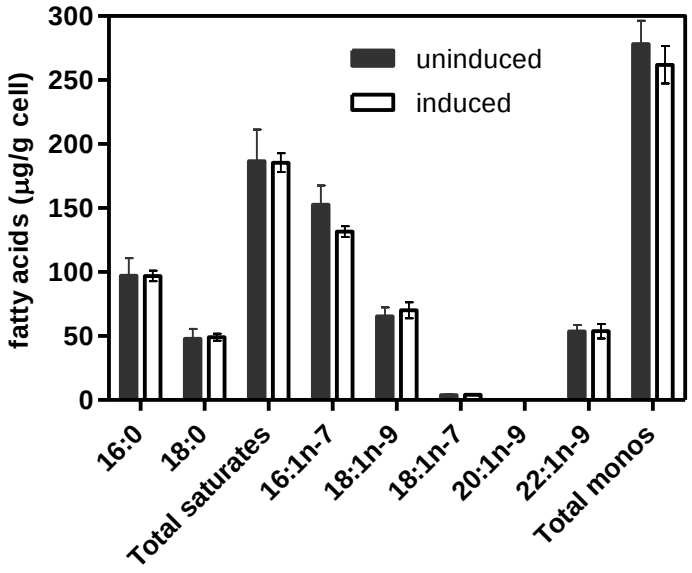

Figure 5. 23 Fatty acid profiles of total lipid in uninduced (solid bar) and induced (open bar) yeast cells containing barramundi FADS ORF. Fatty acid amounts were quantitatively calculated based on peak area of an internal standard 17:0. Data were represented as mean ± SEM, n=6 (technical replicates). The total amount of fatty acids in the yeast is calculated as µg/g cells.

5.5.4 Barramundi ELOVL

5.5.4.1 Sequence analysis

Sequence results confirmed that a 1473 bp (excluding a 23 bp external added polyT-tail) full length cDNA of barramundi putative fatty acid elongase was obtained using 3' and 5' RACE. The cDNA which was isolated from juvenile barramundi using 3'/5' RACE by degenerate and gene specific primers had overlapping regions of a given cDNA fragment and the deduced putative full length ORF sequence was confirmed by nucleotide sequencing (Figure 5.24). The putative barramundi elongase gene included an ORF of 885 bp nucleotides encoding a protein of 294 amino acids (Figure 5.25). The predicted peptide sequence of ELOVL in this study has a molecular weight of 35.1 kilodaltons calculated by Protein Molecular Weight Calculator online program from Science Gateway (http://www.sciencegateway.org/tools/proteinmw.htm). Parallel alignment of barramundi putative elongase with elongase 2 and 5 sequences from human (*Homo sapiens*), zebra fish (*Danio rerio*) and Atlantic salmon (*Salmon salsa*) (Figure 5.25) indicated that the barramundi ELOVL contained a histidine rich sequence (His box) comprised of a group of three conserved histidines HXYHH and four transmembrane regions: N_{31}-NYPPTFALTVMYLLIVWMGPKY-C_{52}, N_{64}-LLVLYNLGLTLLSFYMFYELVTA-C_{86}, N_{204}-QLQLIQFFLTVTQTMCAVIWPCG-C_{226} and N_{231}-WLYFQISYMVTLIILFSNFYIQ-C_{252}, typical of a membrane-bound elongase. It showed high sequence homology to human, zebra fish and Atlantic salmon elongase 5 (Table 5.7). Moreover, alignment of the barramundi putative elongase sequence from this study with a recently reported barramundi elongase 5 (GQ214180) (124) showed one nucleotide difference in the coding region (Figure 5.26) but 100% amino acid sequence identity (Figure 5.25). There were also differences in cDNA nucleotides and length of the 3' and 5' UTR between the two barramundi (Figure 5.26).

A phylogenetic tree analysis of the peptide sequence of barramundi elongase aligned with elongase 2 and 5 gene family previously characterized in other fish species and vertebrates and analysed by NJ algorithm (Figure 5.27) suggests that barramundi putative ELOVL closely corresponds to the mammalian ELOVL5 family and is most closely related to ELOVL sequences of the marine teleosts, southern bluefin tuna (*Thunnus maccoyii*) (ACZ55930), gilthead seabream (*Sparus aurata*) (AAT81404) and nibe croaker (*Nibea mitsukurii*) (ACR47973) cluster as well as the marine fish cobia (*Rachycentron canadum*) (ACJ65150) and turbot (*Psetta maxima*) (AF465520) cluster.

5.5.4.2 Elongation activity of the barramundi putative ELOVL

The elongation activity was examined with approaches of time course, dose dependence, and substrate preferences.

Time course

The C18 n-3 substrate, 18:4 n-3 was added to transformed yeast cells expressing the barramundi ELOVL gene under 27°C culture conditions for 72 hr. After addition of the C18 n-3 substrate 18:4 n-3 to transformed yeast cells expressing the barramundi ELOVL gene, there was an initial increase in 20:4 n-3, the first elongation product, in the yeast lipids at 6 hr, followed by a decrease to a lower level which remained constant at all other time points. At the same time, there was a linear increase in the next elongation product in the fatty acid pathway 22:4 n-3 in the yeast cells over the 72 hr of the incubation (Figure 5.28 A). The concentration of extracellular elongation products (i.e. those in the culture medium) were also examined and similar time course patterns were seen for 20:4 n-3 and 22:4 n-3, indicating secretion of these fatty acids from yeast lipids to the medium (Figure 5.28 B). Elongation of EPA showed a similar time course in transformed yeast, except that there was very limited conversion of the first elongation product, DPA to 24:5 n-3 (Figure 5.29).

Dose dependence

In a 24 hr incubation with increasing concentrations of 18:4 n-3, the production of 20:4 n-3 and 22:4 n-3 in yeast cells increased linearly up to 500 μM of 18:4 n-3 (Figure 5.30 A) and a significant amount of 22:4 n-3 was observed in the medium (Figure 5.30 B). When the total mass (yeast cell + medium) was calculated, it was clear that the second elongation reaction, that of 20:4 n-3 to 22:4 n-3, occurred at a greater rate than the first elongation step, that of 18:4 n-3 to 20:4 n-3 (Figure 5.30 C and Table 5.8 A). With the addition of EPA up to 500 μM there was an increase in the concentration of the first elongation product, DPA, in both cells and incubation medium but only low amounts of the second elongation product, 24:5 n-3, in either the yeast cells (Figure 5.31 A) or the medium (Figure 5.31 B) suggesting limited ability of the transformed yeast cells to produce 24:5 n-3. When taking the fatty acids in both the yeast cells and the medium into account for calculating the conversion rates, more than 90% of EPA was elongated into DPA (Figure 5.31 C and Table 5.8 B).

In addition to the examination of C18 (18:4 n-3), C20 (20:4 n-3 and EPA) and C22 (DPA) substrates, which are considered as the main pathway for conversion of ALA to DHA, I also examined the ability of the transformed yeast cells to elongate ALA to 20:3 n-3 (Figure 5.32). ALA is normally a substrate for Δ6 desaturase activity, resulting in the formation of 18:4 n-3 in the conventional LCPUFA pathway. After the addition of ALA to the transformed yeast

cells, the elongation products 20:3 n-3 plus a small amount of the next elongation product, 22:3 n-3 were detected in the yeast cell fatty acids (Figure 5.32 A). Elongation products 20:3 and 22:3 n-3 were also detected in the culture medium (Figure 5.32 B) and when the amount of all substrates and products in the yeast cells and medium were calculated, the conversion efficiency of ALA to 20:3 n-3 was in the range of 43%-67% (Figure 5.32 C and Table 5.8 C).

Substrate preferences

The fatty acid compositions of yeast cultures supplemented with various C18 and C20, n-3 and n-6 fatty acid substrates are shown in Table 5.9 and Table 5.10. The recombinant barramundi ELOVL enzyme showed a broad range of elongation activities towards fatty acid substrates including LA, ALA, 18:3 n-6, 18:4 n-3, AA and EPA. Before and after taking medium elongation product levels into account for computing the enzymatic conversion, the relative values changed accordingly (Table 5.11). There was little difference in n-3/n-6 preference for EPA versus 20:4 n-6, however, the recombinant ELOVL had a higher affinity for 18:4 n-3 than 18:3 n-6 and ALA had considerably more activity as a substrate compared to LA (Table 5.10). It is interesting that the uninduced cells, those containing pYES2/BarraELOVL plasmid cultured in glucose medium to suppress the gene expression, still showed low degrees of production of elongated products from C18 and C20 fatty acids, suggesting basal levels of expression (leaky expression) from a pYES2 GAL-1 promoter (Table 5.9 and 5.10).

There was little difference in n-3/n-6 preference for EPA versus AA. However, ALA and 18:4 n-3 were considerably more preferred by the recombinant barramundi ELOVL as substrates compared with LA and 18:3 n-6, respectively (Table 5.11). Moreover, the recombinant barramundi ELOVL showed higher elongation activity for elongating C20 than C18 fatty acids. More than 90% of EPA and AA were elongated to C22 elongation products DPA and 22:4 n-6, respectively, when the elongation products were taken into account for the conversion % (Table 5.11).

The effect of induction of the barramundi ELOVL on endogenous yeast fatty acids, which are mainly saturates and monoenes, was examined. Induction of the elongase enzyme increased the peak areas and overall amounts of 18:1 n-9, 18:1 n-7 and 20:1 n-9 (Figure 5.33 and Figure 5.34). The levels of 18:1 n-9, 18:1 n-7 and 20:1 n-9 traces in the induced cells were 1.6, 5.6 and 14.5 fold, respectively, higher than the traces in the uninduced cells, which resulted in increase in the total monoenes, however, there was no effect on the saturates (Figure 5.34). Moreover, a putative 20:1 n-7 peak which appeared next to the peak of 20:1 n-9 was also

observed on the GC chromatogram in the galactose induced yeasts containing the barramundi ELOVL gene (Figure 5.33).

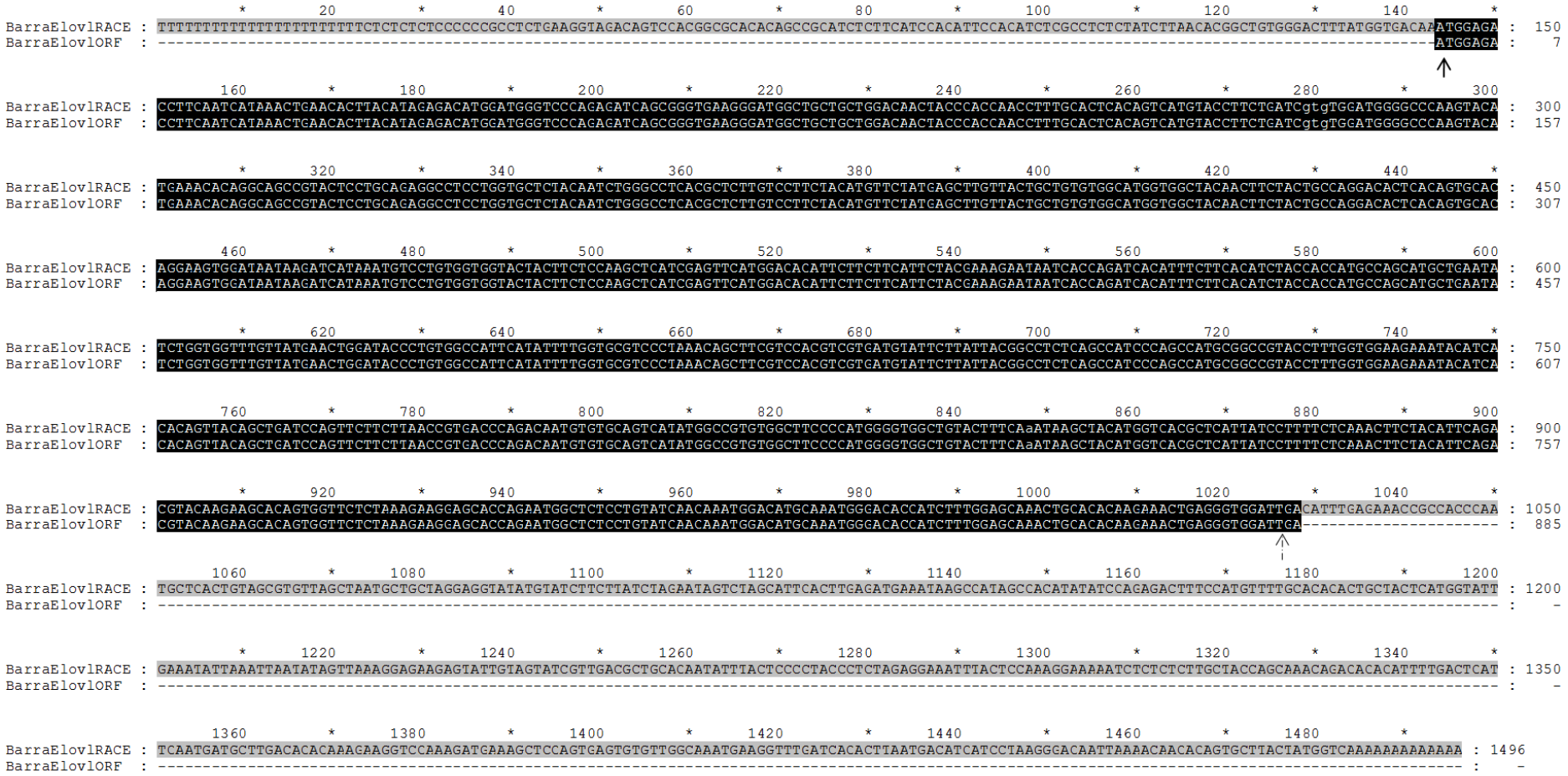

Figure 5. 24 Alignment of barramundi putative complete ELOVL cDNA with the ELOVL ORF nucleotide sequence. The complete ELOVL nucleotide sequence was determined by combining sequence information from 3'/5' RACE PCR products overlapped with the partial ELOVL cDNA fragments. The ELOVL ORF nucleotide sequence was determined by sequencing of PCR products, which were amplified by using Elovl-F and Elovl-R primers to obtain a

fragment containing the partial 5’ and 3’ UTR regions. The ATG start codon is indicated by a solid arrow sign and the TGA stop codon is indicated by a dashed arrow sign.

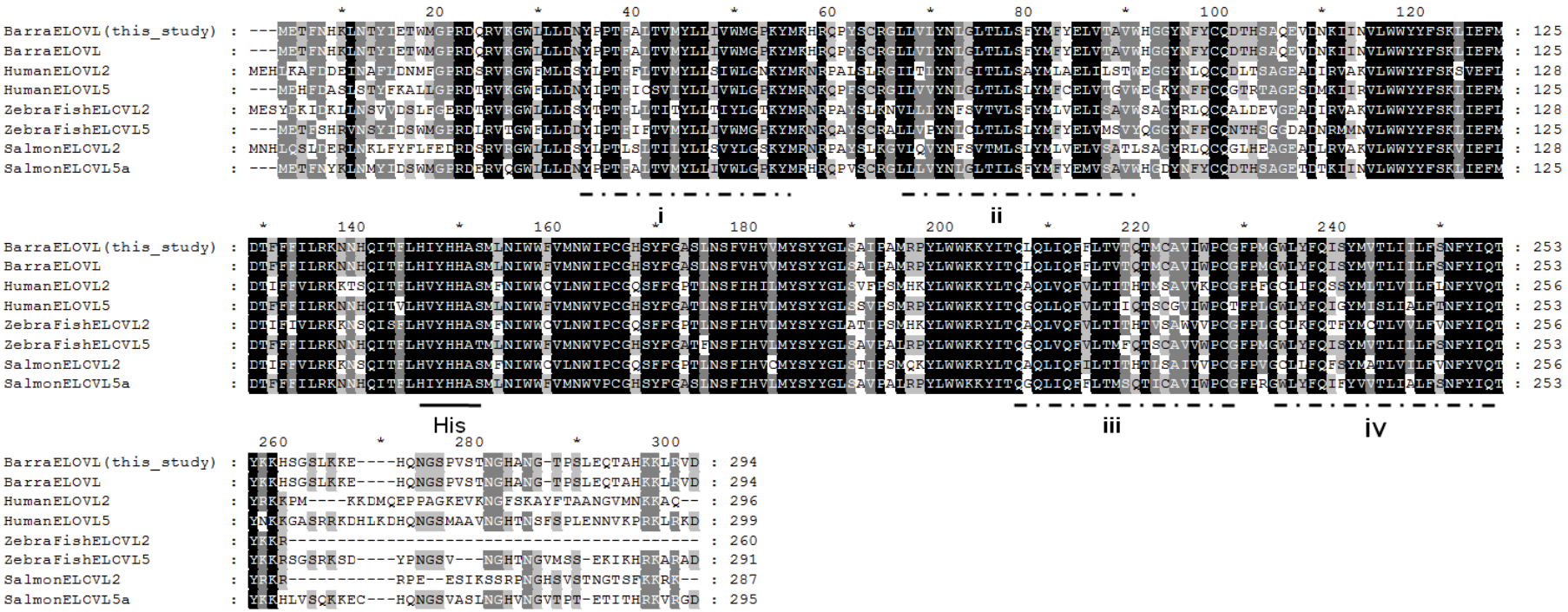

Figure 5. 25 Alignment of the predicted amino acid sequences of the barramundi elongase with elongases in human, zebra fish, and salmon. The GenBank® accession numbers for all sequences are listed in Table 5.7. Identical amino acids are in black, one histidine-rich domain is underlined. Four putative transmembrane regions (dashed line) are predicted by SOSUI software (http://bp.nuap.nagoya-u.ac.jp/sosui/) (299).

Table 5. 7 Identity comparison of elongase peptide sequences of barramundi and other species

	Lates calcarifer Elovl *(GQ214180)*	*Homo sapiens* Elovl2-like (NP_060240)	*Homo sapiens* Elovl5-like (NP_068586)	*Danio rerio* Elovl2-like (NP_001035452)	*Danio rerio* Elovl5-like (NP_956747)	*Salmon salar* Elovl2 (NP_001130025)	*Salmon salar* Elovl5a (AAO13175)
***Lates calcarifer* Elovl (this study) (GU047382)**	100%[1]	54%	70%	51%	76%	53%	83%
Lates calcarifer Elovl (GQ214180)		54%	70%	51%	76%	53%	83%
Homo sapiens Elovl2-like (NP_060240)			53%	64%	55%	67%	52%
Homo sapiens Elovl5-like (NP_068586)				50%	69%	51%	71%
Danio rerio Elovl2-like (NP_001035452)					52%	72%	51%
Danio rerio Elovl5-like (NP_956747)						52%	75%
Salmo salar Elovl2 (NP_001130025)							51%

[1]The peptide sequences were derived from coding sequences deposited at the NCBI GenBank® database. Sequences were aligned and the identity was computed using the Genedoc.

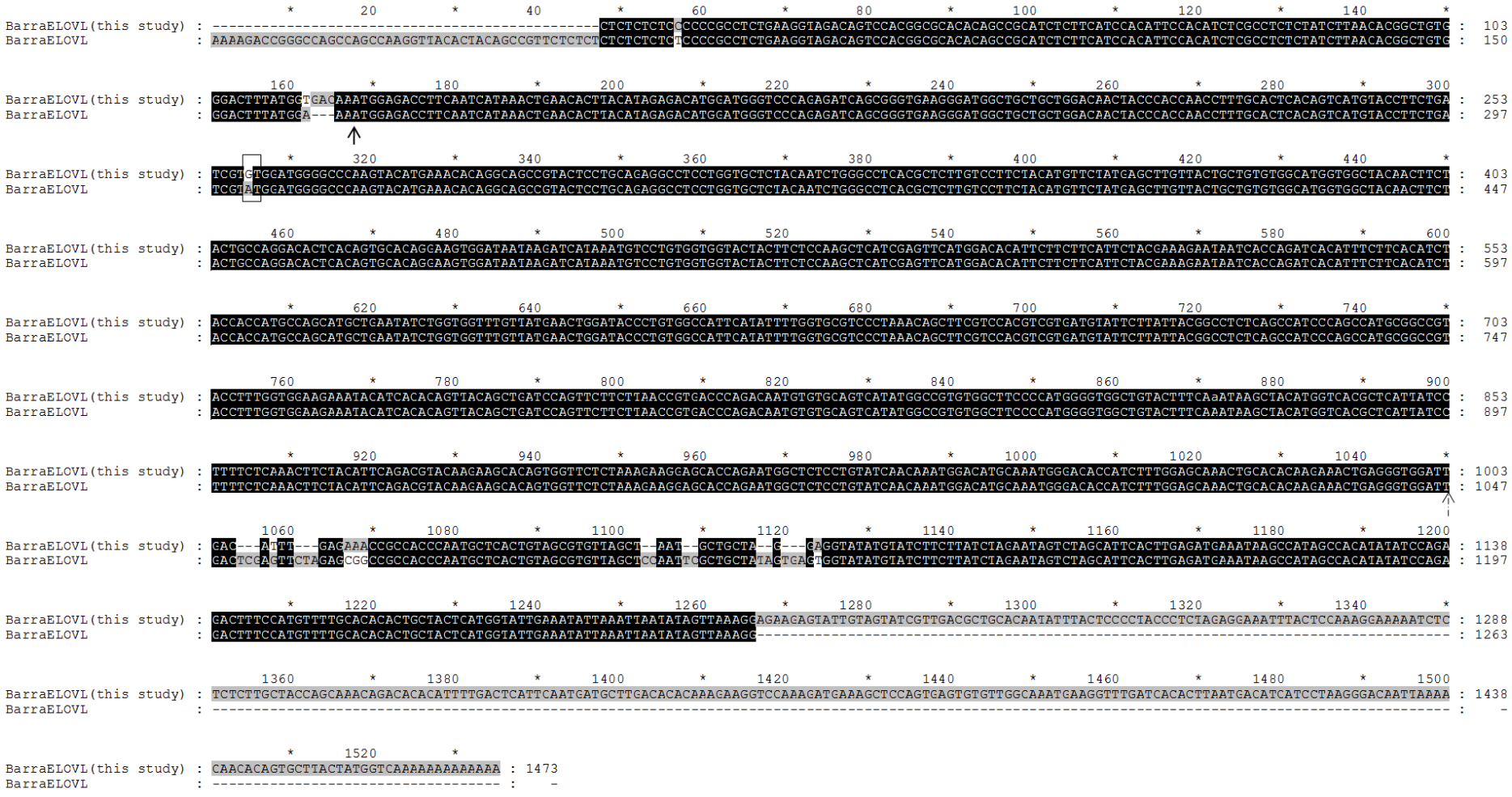
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Figure 5. 26 Alignmen of the nucleotide sequences of the barramundi elongase in this study (GU047382) and the sequence from Mohd-Yusof *et. al.* (GQ214180). Identical nucleotids are in black, the ATG start codon is pointed by a solid arrow sign and the TGA stop codon is pointed by a dashed arrow sign. One nucleotide difference within the ORF is circled by a square. The 77% identity between the two nucleotides was computed using the Gendoc.

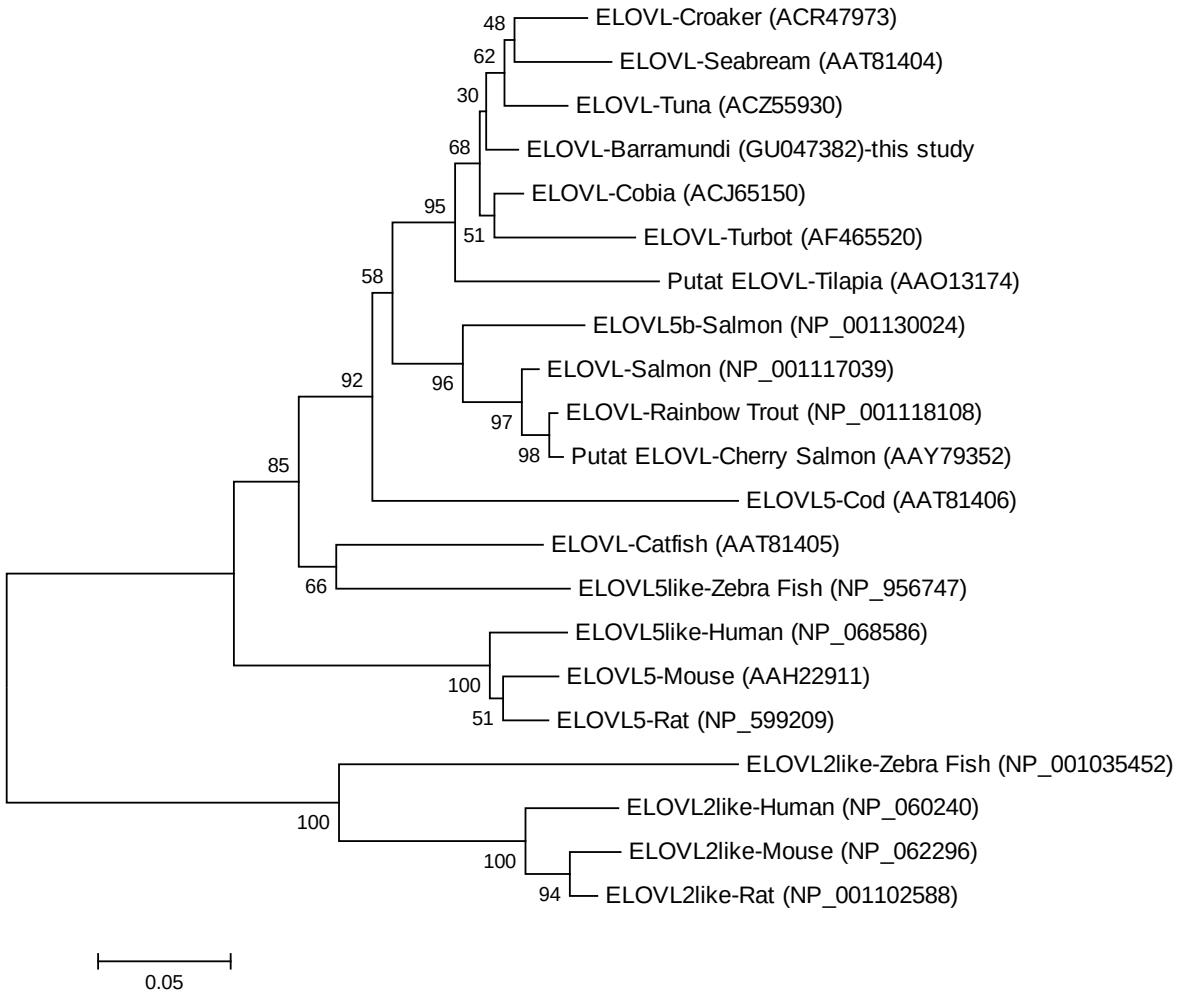
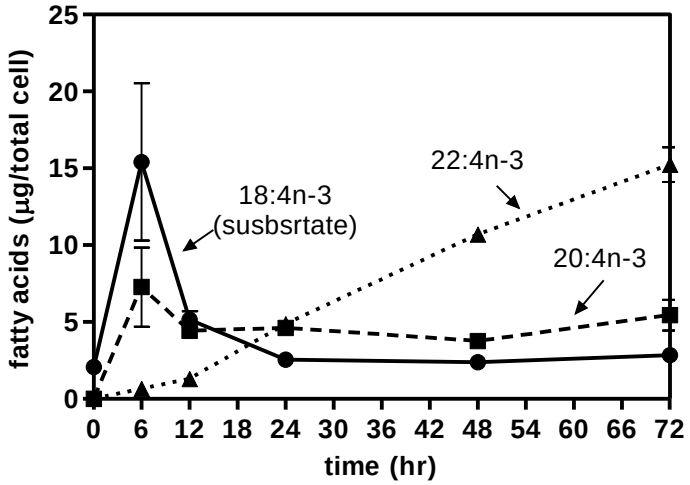


Figure 5. 27 A phylogenetic tree comparing peptide sequences of the barramundi elongase and 20 available animal elongases. The accession numbers of peptide sequences were derived from NCBI GenBank® database. The distance tree was constructed using NJ analysis with ClustalX and MEGA version4. The numbers on the branches indicate bootstrapping value for the node of the frequencies with which the tree topology presented was replicated after 1,000 iterations.

A



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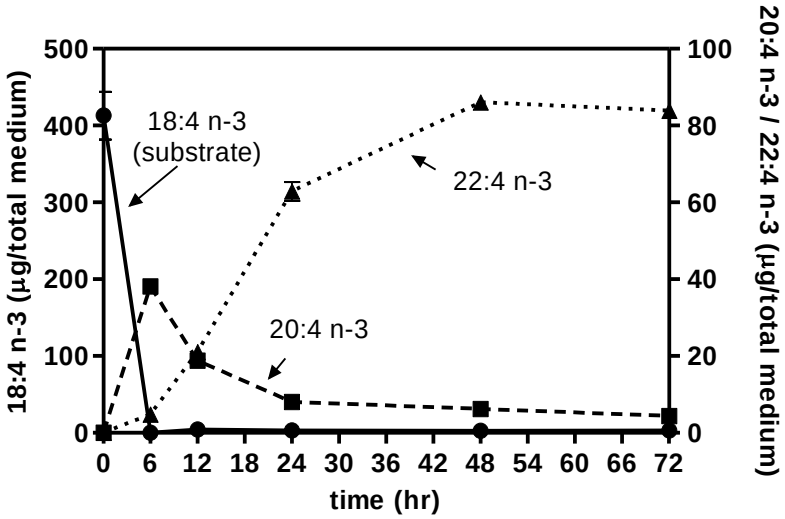
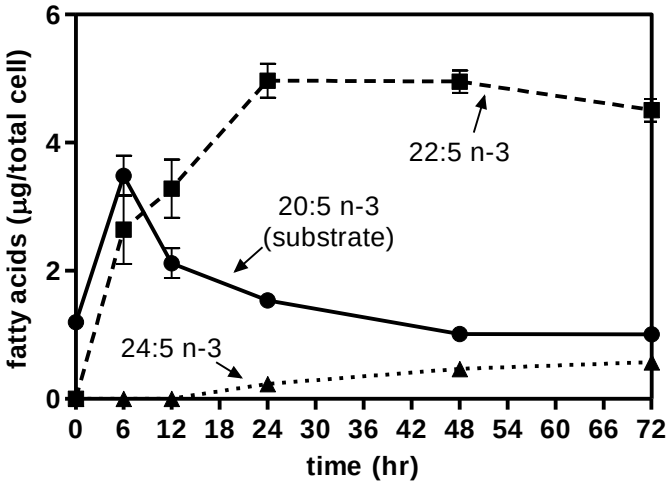


Figure 5. 28 Time course of changes in fatty acid levels of 20:4 n-3 and 22:4 n-3, after addition of 18:4 n-3 at 250 µM in transformed yeast cells (total mass of cells) (A) and medium (B) after expressing barramundi elongase. Mean ± SEM, n=3 (technical replicates). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

A



B

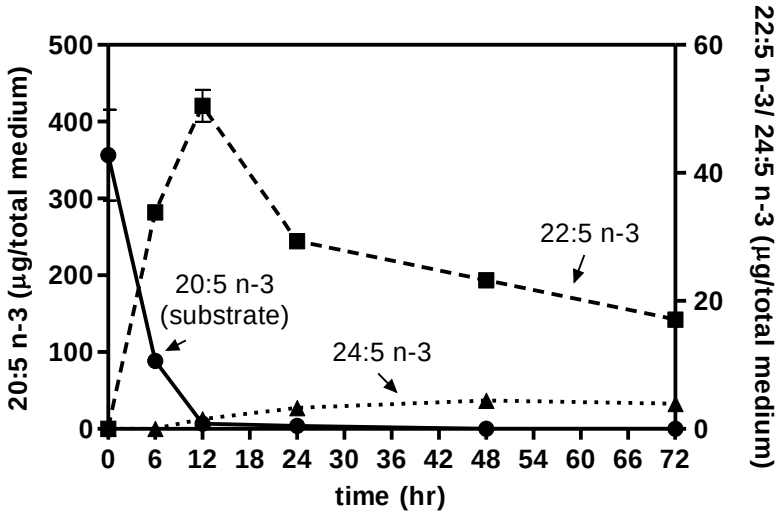


Figure 5. 29 Time course of changes in fatty acid levels of 22:5 n-3 and 24:5 n-3, after addition of EPA (20:5 n-3) at 250 µM in transformed yeast cells (total mass of cells) (A) and medium (B) after expressing barramundi elongase. Mean ± SEM, n=3 (technical replicates). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

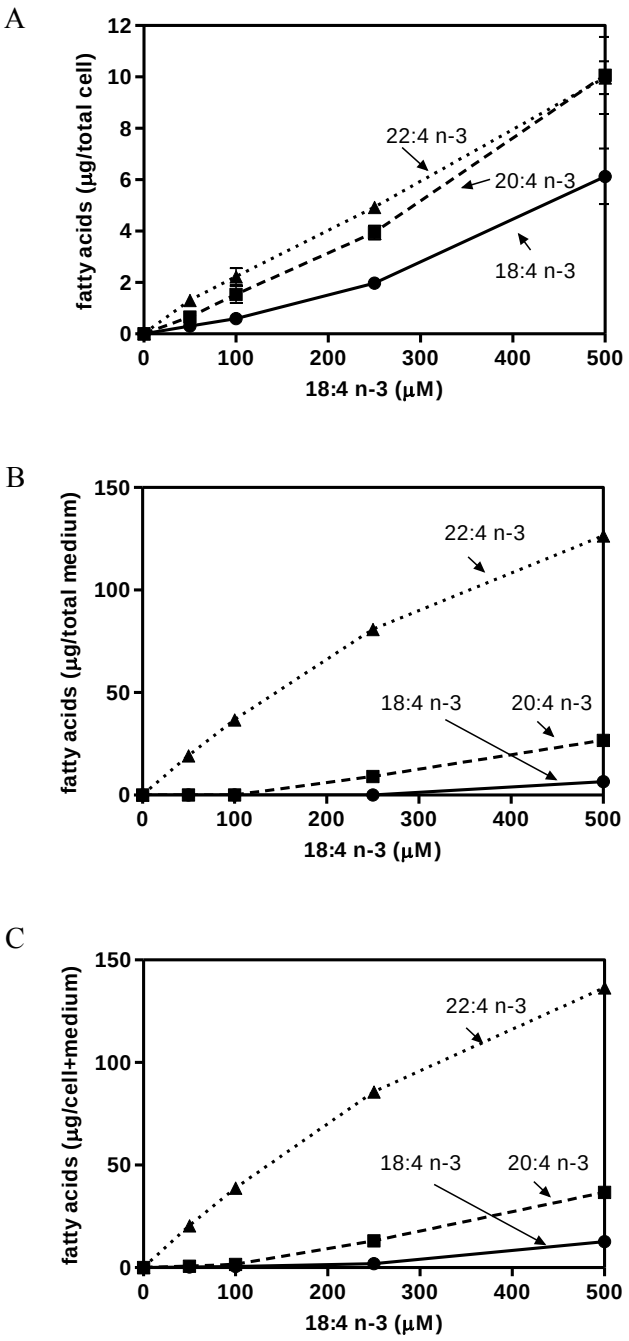


Figure 5. 30 Effect of 18:4 n-3 at 0-500 µM on biosynthesis of 20:4 n-3 and 22:4 n-3 in cells (total mass of cells) (A) and in remaining culture medium (B). Fatty acids in cells and medium were both taken into account for calculating overall production of 20:4 n-3 and 22:4 n-3 (C). Elongated products were computed as µg of fatty acids for total cells, medium or total cell + medium in a 10 mL culture. Mean ± SEM, n=3 (technical replicates). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

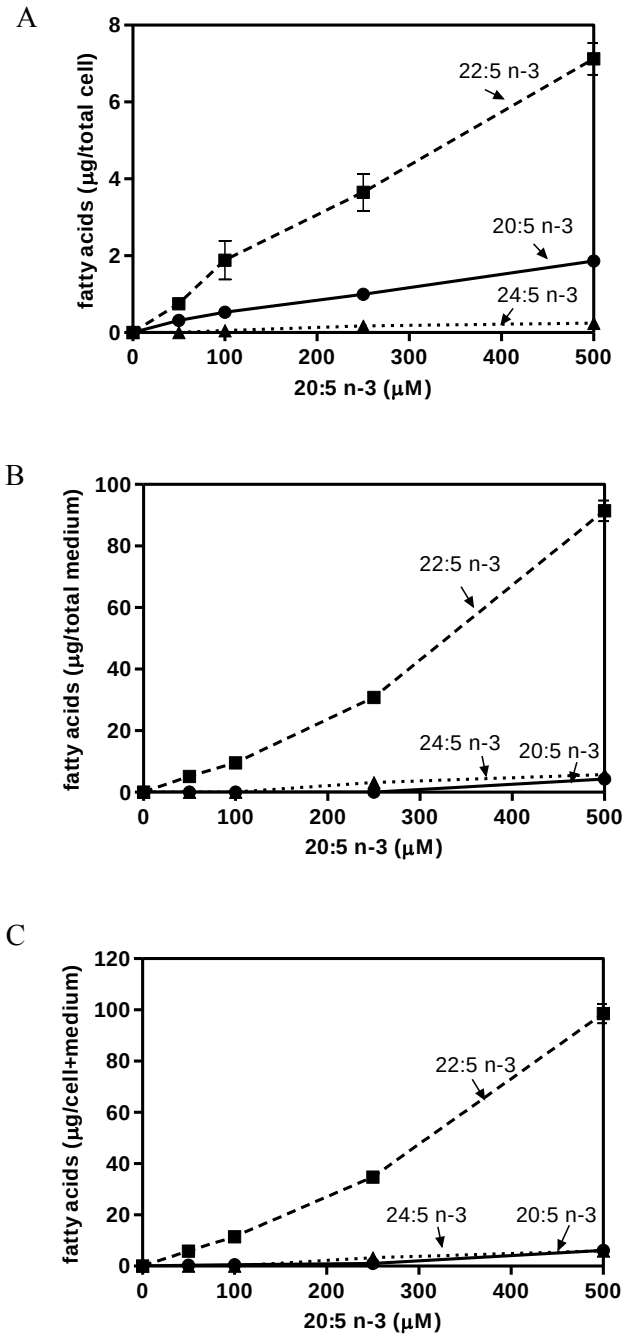


Figure 5. 31 Effect of EPA (20:5 n-3) at 0-500 µM on synthesis of DPA (22:5 n-3) and 24:5 n-3 in cells (total mass of cells) (A) and in remaining culture medium (B). Fatty acids in cells and medium were both taken into account for calculating overall production of DPA and 24:5 n-3 (C). Elongated products were quantitatively computed as µg of fatty acids per total cells, medium or total cell + medium. Mean ± SEM, n=3 (technical replicates). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

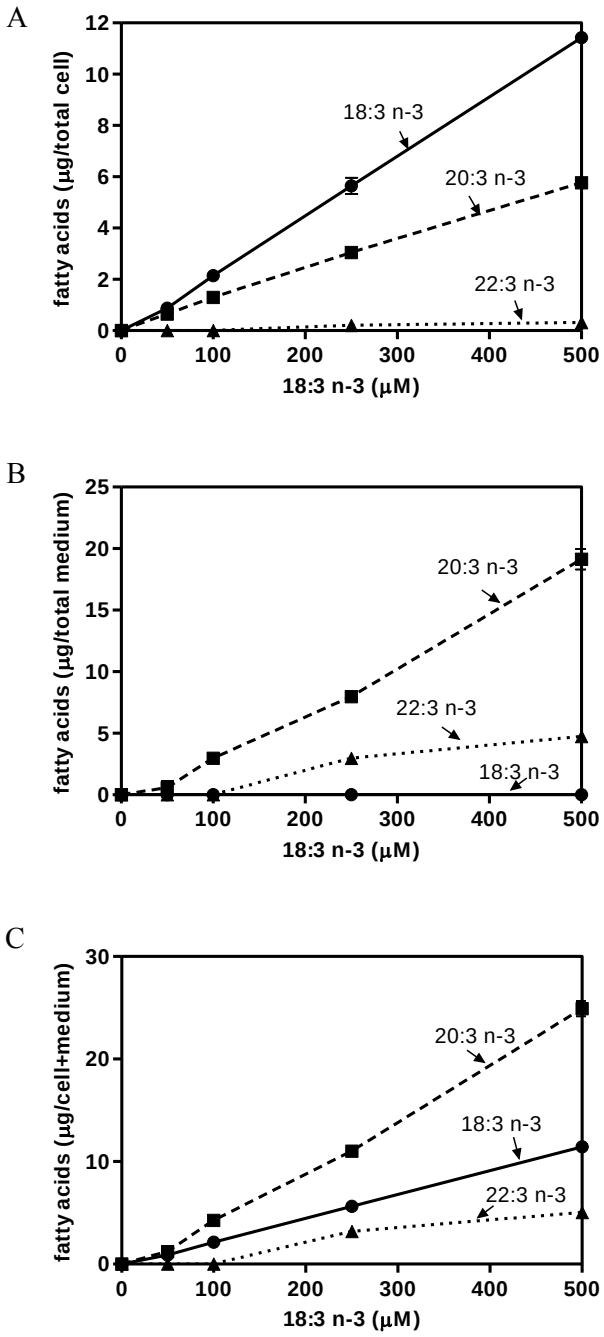


Figure 5. 32 Effect of ALA (18:3 n-3) at 0-500 µM on synthesis of 20:3 n-3 and 22:3 n-3 in cells (total mass of cells) (A) and in remaining culture medium (B). Fatty acids in cells and medium were both taken into account for calculating overall production of 20:3 n-3 and 22:3 n-3 (C). Elongated products were quantitatively computed as µg of fatty acids per total cells, medium or total cell + medium. Mean ± SEM, n=3 (technical replicates). Total amount of fatty acids in the yeast is calculated as µg/total cells per culture to compare with the total amount of fatty acids in the medium per culture.

Table 5. 8 Comparison of cell only and cell + medium conversion values

A. Dose-response of 18:4 n-3

Substrate	**Conversion (%)**[1]			
18:4 n-3 (μM)	18:4 n-3 → 20:4 n-3		20:4 n-3 → 22:4 n-3	
	Cell only	Cell + medium	Cell only	Cell + medium
0	0	0	0	0
50	28.7 ± 1.7[a]	3.0 ± 0.2[a]	57.6 ± 1.2[a]	95.5 ± 0.2
100	34.8 ± 1.3[ab]	3.7 ± 0.6[ab]	51.5 ± 1.3[ab]	94.9 ± 0.8
250	36.3 ± 1.0[ab]	12.9 ± 0.4[ab]	45.5 ± 1.9[ab]	85.1 ± 0.6
500	38.3 ± 0.9[bc]	19.7 ±0.8[bc]	38.6 ± 2.1[bc]	76.2 ± 1.3

B. Dose-response of 20:5 n-3

Substrate	**Conversion (%)**[1]			
20:5 n-3 (μM)	20:5 n-3 → 22:5 n-3		22:5 n-3 → 24:5 n-3	
	Cell only	Cell + medium	Cell only	Cell + medium
0	0	0	0	0
50	70.9 ± 5.2	94.8 ± 1.4	0	0
100	76.3 ± 2.5	95.3 ± 1.0	1.3 ± 1.3	0.4 ± 0.4[a]
250	75.8 ± 0.4	88.9 ± 1.7	3.5 ± 0.1	8.3 ± 1.3[b]
500	77.1 ± 1.0	92.6 ±0.7	2.6 ± 0.1	5.6 ± 0.6[ab]

C. Dose-response of 18:3 n-3

Substrate	**Conversion (%)**[1]			
ALA (18:3 n-3) (μM)	ALA (18:3 n-3) → 20:3 n-3		20:3 n-3 → 22:3 n-3	
	Cell only	Cell + medium	Cell only	Cell + medium
0	0	0	0	0
50	42.4 ± 0.5[a]	42.9 ± 1.0[a]	0	0
100	37.6 ± 0.3[ab]	66.7 ± 1.9[b]	0	0
250	34.2 ± 0.3[ab]	55.5 ± 1.0[ab]	2.4 ± 0.1	16.1 ±1.2
500	33.0 ± 0.1[bc]	60.2 ±0.8[ab]	1.8 ± 0.1	12.2 ± 0.1

[1]Elongated products were quantitatively computed as μg of fatty acids per total cell, total medium or total cell + medium. Conversion were calculated by conversion (%) = [product / (substrate + product) x 100]. Mean ± SEM, n=3 (technical replicates). Means in the same column superscripted with different letters indicate statistical difference (P<0.05), according to Kruskal-Wallis post test with Dunn's multiple comparison test.

Table 5. 9 Fatty acid composition of yeast cells expressing barramundi ELOVL ORF

Barramundi elongase

µg/total cell	LA (18:2 n-6) (500 µM)		ALA (18:3 n-3) (500 µM)		18:3 n-6 (500 µM)		18:4 n-3 (500 µM)		AA (20:4 n-6) (500 µM)		EPA (20:5 n-3) (500 µM)	
Fatty acid	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced
16:0	27.2±1.0[1]	27.6±2.7	27.1±1.0	25.9±0.9	35.7±7.0	26.5±1.7	34.6±2.9	30.9±2.5	19.9±0.7	23.3±0.7	18.5±0.6	24.0±1.1
18:0	12.9±0.9	15.1±1.1	12.3±0.3	14.4±0.5	16.3±2.1	13.7±1.0	17.5±.1.8	15.7±1.3	7.8±0.3	9.0±0.3	7.6±0.3	11.6±1.1
16:1n-7	19.4±0.8	19.0±5.0	20.9±1.0	18.2±1.6	25.0±9.2	18.6±0.9	19.6±1.5	19.9±2.3	22.4±0.2	24.7±1.2	23.9±0.5	21.6±2.0
18:1n-9	8.6±0.5	10.9±3.5	10.5±0.6	11.0±1.3	12.6±5.7	9.9±0.6	8.6±1.1	11.8±1.6	8.3±0.2	13.0±0.8	9.6±0.4	12.2±1.3
18:1n-7	0.8±0.0	3.5±1.2	1.0±0.1	3.6±0.3	1.0±0.4	3.0±0.2	0.6±0.1	3.8±0.5	0.6±0.0	3.7±0.3	0.7±0.0	3.2±0.2
18:2n-6	16.3±0.6	22.3±8.1	-	-	-	-	-	-	-	-	-	-
18:3n-3	-	-	17.5±0.7	14.8±2.1	-	-	-	-	-	-	-	-
18:3n-6	-	-	-	-	18.3±7.4	7.9±0.5	-	-	-	-	-	-
18:4n-3	-	-	-	-	-	-	14.1±1.3	6.1±1.1	-	-	-	-
20:2n-6	0.1±0.1	3.1±1.5	-	-	-	-	-	-	-	-	-	-
20:3n-6	-	-	-	-	3.2±1.8	7.7±0.5	-	-	-	-	-	-
20:3n-3	-	-	1.0±0.1	6.8±0.8	-	-	-	-	-	-	-	-
20:4n-6	-	-	-	-	-	-	-	-	6.0±0.3	9.3±0.6	-	-
20:4n-3	-	-	-	-	-	-	3.0±0.6	10.1±1.5	-	-	-	-
20:5n-3	-	-	-	-	-	-	-	-	-	-	3.9±0.1	2.7±0.4
22:1n-9	11.6±0.5	13.8±0.6	12.7±0.2	11.8±1.2	13.6±0.8	12.0±0.2	8.5±0.1	9.8±1.3	14.9±0.9	14.3±0.1	15.0±1.2	13.6±1.3
22:2n-6	-	0.5±0.1	-	-	-	-	-	-	-	-	-	-
22:3n-6	-	-	-	-	-	1.2±0.1	-	-	-	-	-	-
22:3n-3												
22:4n-6	-	-	-	0.4±0.0	-	-	-	-	0.6±0.0	8.0±0.5	-	-
22:4n-3	-	-	-	-	-	-	0.4±0.1	10.0±0.6	-	-	-	-
22:5n-3	-	-	-	-	-	-	-	-	-	-	0.6±0.0	7.4±0.3
24:5n-3	-	-	-	-	-	-	-	-	-	-	-	0.2±0.0

[1]Elongation products were quantitatively computed as µg of fatty acids of total cells (total mass of cells) per culture. Mean ± SEM, n≥3 (technical replicates).

Table 5. 10 Fatty acid composition of remaining medium by yeast expressing barramundi ELOVL ORF

Barramundi elongase												
µg/total medium	LA (18:2 n-6) (500 µM)		ALA (18:3 n-3) (500 µM)		18:3 n-6 (500 µM)		18:4 n-3 (500 µM)		AA(20:4 n-6) (500 µM)		EPA (20:5 n-3) (500 µM)	
Fatty acid	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced	uninduced	induced
16:0	237.6±2.8[1]	263.6±20.9	245.8±1.1	219.2±9.3	258.0±13.6	252.9±6.7	239.6±20.1	309.5±3.7	198.7±8.7	199.3±1.8	198.5±4.2	262.1±29.9
18:0	179.9±1.9	194.9±13.4	184.0±0.9	168.9±5.2	190.9±9.7	186.5±5.0	191.0±15.1	219.9±2.9	158.0±6.0	154.7±1.2	157.2±3.3	193.7±18.1
18:1n-9	4.8±0.1	5.1±0.6	4.4±0.0	6.3±0.4	4.8±0.1	6.1±0.3	7.6±0.7	6.2±0.2	3.9±0.2	6.4±0.1	3.6±0.1	6.6±0.1
18:2n-6	3.0±0.6	2.8±0.4	-	-	-	-	-	-	-	-	-	-
18:3n-3	-	-	-	1.0±0.4	-	-	-	-	-	-	-	-
18:3n-6	-	-	-	-	1.7±0.5	2.2±0.4	-	-	-	-	-	-
18:4n-3	-	-	-	-	-	-	8.6±0.2	6.7±0.0	-	-	-	-
20:2n-6	-	3.2±0.2	-	-	-	-	-	-	-	-	-	-
20:3n-6	-	-	-	-	2.6±0.3	8.2±0.5	-	-	-	-	-	-
20:3n-3	-	-	0.9±0.5	13.3±2.6	-	-	-	-	-	-	-	-
20:4n-6	-	-	-	-	-	-	-	-	15.3±1.1	55.9±11.8	-	-
20:4n-3	-	-	-	-	-	-	6.8±0.3	26.6±1.0	-	-	-	-
20:5n-3	-	-	-	-	-	-	-	-	-	-	5.5±0.1	6.9±1.3
22:1n-9	110.7±1.5	120.9±3.5	116.0±3.7	98.4±5.9	143.9±18.3	118.1±5.2	101.8±8.5	110.6±16.0	147.9±9.3	135.3±10.3	125.7±5.9	156.5±6.6
22:2n-6	-	-	-	-	-		-	-	-	-	-	-
22:3n-6	-	-	-	-	-	11.8±0.3	-	-	-	-	-	-
22:3n-3						-		-	-			
22:4n-6	-	-	0.1±0.0	3.9±0.4	-	-	-	-	9.4±0.5	92.1±6.0	-	-
22:4n-3	-	-	-	-	-	-	5.0±0.2	126.4±1.0	-	-	-	-
22:5n-3	-	-	-	-	-	-	-	-	-	-	5.6±0.5	77.4±6.6
24:5n-3	-	-	-	-	-	-	-	-	-	-	-	4.4±0.6

[1]Elongation products were quantitatively computed as µg of fatty acids of total medium per culture. Mean ± SEM, n≥3 (technical replicates).

Table 5. 11 Substrate specificity and enzyme activity of barramundi ELOVL

Fatty acid substrate[1]	Elongation product	Conversion (%)		Enzyme activity
n-3 PUFA		**Cell only[2]**	**Cell + medium[3]**	
18:3 n-3 (ALA)	20:3 n-3	31.2±0.8	50.5±1.8	Elongase5
	22:3 n-3	1.7±0.1	11.3±0.6	
18:4 n-3	20:4 n-3	38.3±0.9	20.4±0.8	Elongase5
	22:4 n-3	38.6±2.1	76.2±1.3	
20:5 n-3 (EPA)	22:5 n-3 (DPA)	72.1±2.3	91.9±0.5	Elongase5/2
	24:5 n-3	2.1±0.2	4.9±0.5	
n-6 PUFA				
18:2 n-6 (LA)	20:2 n-6	11.2±1.2	23.2±2.2	Elongase5
	22:2 n-6	N.D.	N.D.	
18:3 n-6	20:3 n-6	45.7±0.5	43.1±0.7	Elongase5
	22:3 n-6	7.3±0.4	35.4±0.6	
20:4 n-6 (AA)	22:4 n-6	46.0±1.6	91.5±0.3	Elongase5/2
	24:4 n-6	N.D.	N.D.	

[1]All fatty acids were supplemented 500 μM into yeast culture medium as a final concentration.
[2]Fatty acid in yeast cells only were used for calculating the conversion (%). Data are means ± SEM of n≥3 (technical replicates).
Conversion (%) = [product / (substrate + product) x 100]; all fatty acids were calculated based on fatty acid amount listed on Table 5.9.
[3]Elongated fatty acid products in medium were taken into account for calculating the conversion (%). Data are means ± SEM of n≥3 (technical replicates). Conversion (%) = [product / (substrate + product) x 100]; all fatty acids were calculated based on fatty acid amount listed on Table 5.9 and Table 5.10.

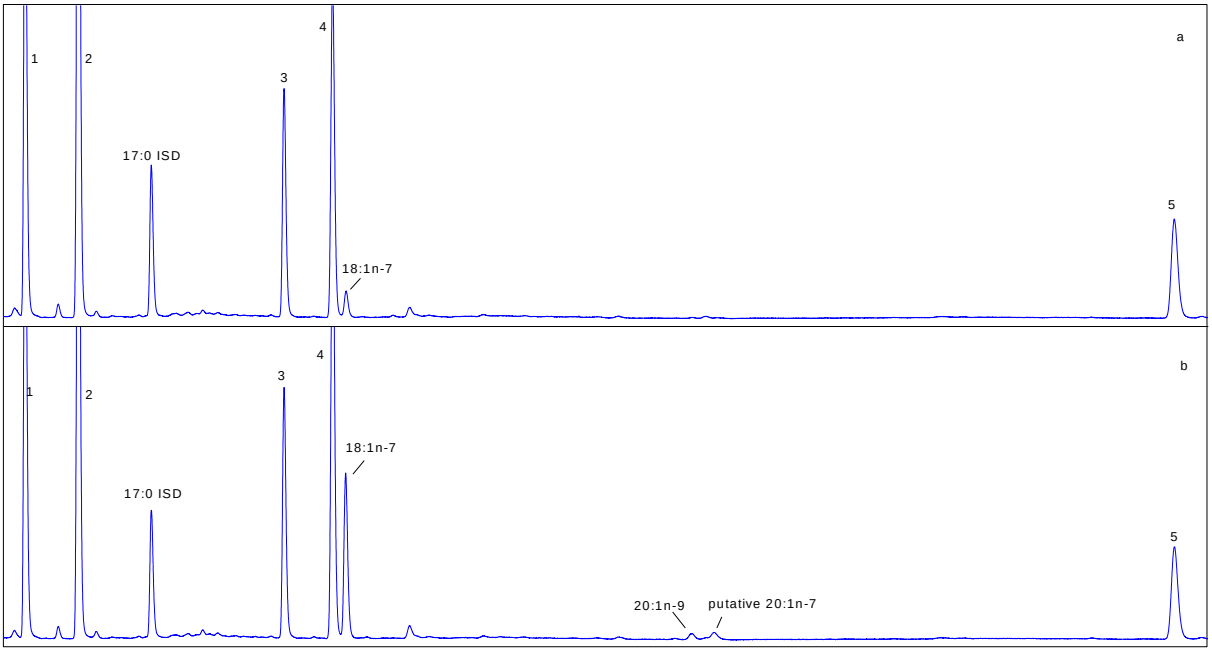


Figure 5. 33 GC chromatograms of fatty acids from the uninduced (a) and induced (b) yeast cells containing barramundi ELOVL ORF without exogenously fatty acid substrate supplementation. Identities of the peaks 1 (16:0), 2 (16:1 n-7), 3 (18:0), 4 (18:1 n-9), 5 (22:1 n-9), 18:1 n-7 and 20:1 n-9 in all panels are the endogenous fatty acids of the yeast cells and they were determined by comparing their retention time with those of authentic standards. An internal standard 17:0 was added into harvested cells. Panel b shows a significant increased peak area of peak 4, 18:1 n-7 and two additional peaks, a 20:1 n-9 and a putative 20:1 n-7.

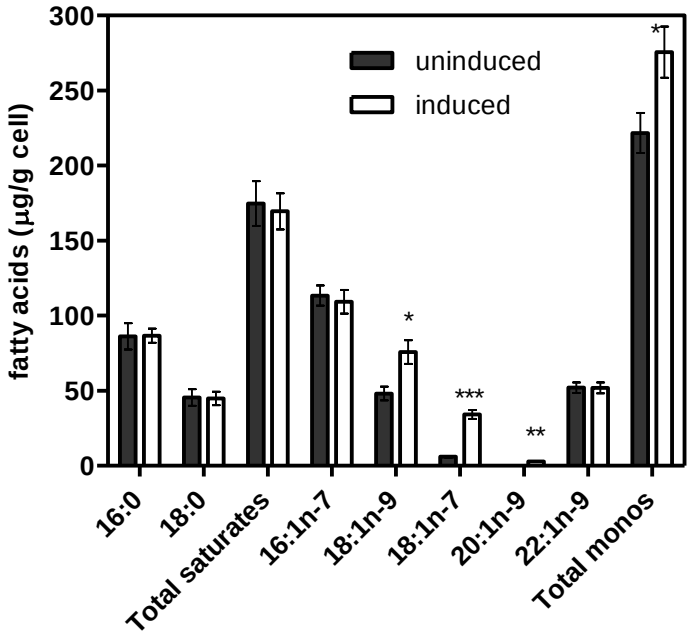


Figure 5. 34 Fatty acid levels of total lipid in uninduced (solid bar) and induced (open bar) yeast cells containing barramundi ELOVL ORF. Fatty acid amounts were quantitatively calculated based on peak area of an internal standard 17:0. Data were represented as mean ± SEM, n=6 (technical replicates). Values with different superscripts are significantly different from each other ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$). Total amount of fatty acids in the yeast is calculated as µg/g cells.

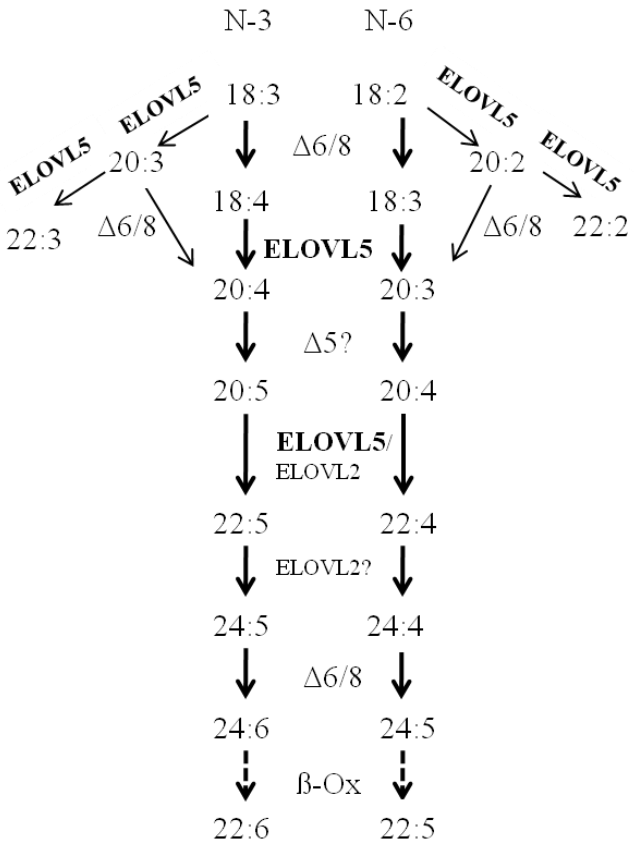


Figure 5. 35 A postulated n-3 and n-6 LCPUFA synthetic pathway in barramundi.

5.6 Discussion

5.6.1 FADS

Conversion of the plant derived ALA to produce 18:4 n-3, generally recognised as an intermediate in n-3 LCPUFA synthesis, requires Δ6 desaturase activity. This study has successfully isolated a Δ6 desaturase in barramundi that is able to utilise C18 n-3 and n-6 PUFA and C24 n-3 LCPUFA as substrates. The ability to desaturate 24:5 n-3 is important because this fatty acid is an intermediate in the synthesis of DHA via an indirect or shunt pathway, at least in rat and human tissues (90). An apparent direct pathway to DHA synthesis from EPA would involve elongation to DPA followed by Δ4 desaturation to DHA. A recent report indicated that the FADS1 and FADS2 genes from another marine species, *Siganus canaliculatus,* displayed bifunctional Δ4/Δ5 and Δ6/Δ5 desaturase activities, respectively (99). Another Δ4 desaturase which can convert DPA directly to DHA has also been identified in *Thraustochytrium sp*., a common marine fungus which produces DHA (105, 300). However, Δ4 desaturase activity has not been found in rats and humans and the barramundi FADS with Δ6/Δ8 desaturase described in this study showed no Δ4 activity. In rats and humans there is evidence that DPA is elongated to 24:5 n-3 which is converted by Δ6 desaturation to 24:6 n-3 and then chain-shortened by β-oxidation to DHA (90). There is evidence that this may also occur in at least one fish species, gilthead sea bream, in which radio-labelled 24:5 n-3 and 24:6 n-3 were found following administration of radio-labelled ALA or EPA (301). The fact that the Δ6 desaturase isolated in this study is capable of desaturating 24:5 n-3, suggests that the pathway identified in mammalian tissue, and suggested to be present in gilthead sea bream, could also be active in barramundi.

The dual n-3 C18/C24 activity and Δ6/Δ8 desaturase activity of the desaturase enzyme in barramundi raises the possibility of competition between ALA and 24:5 n-3, as well as Δ6/Δ8 substrates. In a previous study, increasing ALA concentrations in HepG2 cells resulted in an initial increase in DHA concentrations followed by a subsequent decrease in DHA production, suggesting competition between n-3 substrates for Δ6 desaturase at higher concentrations of ALA (53).

Δ6 Desaturase activity has been identified in other fish species, and an enzyme in zebra fish exhibited both Δ5 and Δ6 desaturase activity (119). However, the recombinant barramundi desaturase in this study had no detectable Δ5 desaturase activity. Δ6 desaturases from carp, turbot (97), rainbow trout (120) and gilthead sea bream (121) also had little or no Δ5

desaturase activity (97). Δ6 and Δ5 desaturase activities reside in separate genes; namely, FADS2 and FADS1, respectively. To date, the only unifunctional Δ5 desaturase in fish has been characterized in Altlantic salmon (125). In this study, degenerate primer sets were designed from consensus regions of known Δ5 desaturase cDNA in mammalian animals and Salmonoids to probe for barramundi Δ5 desaturase cDNA but without success. The partial nucleotide sequence (~850 bp) (data not shown) that has been obtained from the barramundi cDNA pool using Δ5 desaturase primers was 100% matched with the partial sequence of the desaturase that has been isolated and characterized in this chapter. There was a 91% homology in peptide sequences between Atlantic salmon Δ5 and Δ6 desaturases (Table 5.3). If this is also the case with barramundi enzymes, the primers designed for Δ5 desaturase may have preferentially recognised Δ6 desaturase cDNA if Δ5 desaturase mRNA levels were very low relative to those of Δ6 desaturase. Therefore, it is still unclear whether the Δ5 desaturase is expressed at very low levels in barramundi, or whether it is completely absent. What is clear, however, is that if this gene is present, it is expressed at much lower levels than the Δ6 desaturase.

The barramundi Δ6/Δ8 desaturase was able to desaturate both n-6 and n-3 substrates. The enzyme exhibited approximately twice the desaturation activity with C18 n-3, compared with C18 n-6 as a substrate, suggesting that n-3 LCPUFA synthesis could proceed efficiently even in the presence of equal concentrations of homologous n-6 substrates. However, the results of the supplementation of yeast cells with increasing concentrations of ALA indicate that the degree of incorporation of the substrate ALA and desaturation product 18:4 n-3 was not equivalent (Figure 5.22), and may be a result of saturation of the enzyme. Thus, different apparent conversion rates will be present at different substrate concentrations. The curvilinear relationship between the ALA level and that of its product 18:4 n-3 is similar to the relationship between ALA and DHA levels reported in rats (302) and piglets (4) with increasing dietary ALA levels.

Although the metabolism of ALA by Δ6 desaturase is considered the principal first reaction in its conversion to EPA via 18:4 n-3 and then 20:4 n-3, there is some evidence pointing to the existence of an alternate or bypass pathway involving Δ8 desaturation. This reaction has been shown to exist in rat and human testicular tissues, but not rat liver (303, 304). Baboon hepatic Δ6 desaturase expressed in yeast also has Δ8 desaturase activity; although it was 23 fold and 7 fold lower than Δ6 desaturase activity for n-3 and n-6 substrates, respectively (5). Apart from the present barramundi enzyme, which I observed to have Δ8 desaturase activity with n-3 and

n-6 substrates, none of the reported fish Δ6 desaturases have been examined for Δ8 desaturase activity. The Δ8 desaturase activity of the barramundi Δ6/Δ8 dual functional enzyme was 3.9 fold higher than the Δ6 desaturase activity with ALA but 2.5 fold lower than LA substrates. The fold difference was derived from the conversion (%) which has taken the extracellular product levels into account. Thus, barramundi desaturase appeared to have a greater Δ8 activity than Δ6 activity when encountering the 20:3 n-3 substrate (Table 5.6).

Another recombinant barramundi FADS gene has recently been isolated with a conversion rate of 32% from ALA to 18:4 n-3 and 28% from LA to 18:3 n-6 (124). This desaturase activity is substantially higher than the activity that I have reported here. However, direct comparisons with the other barramundi enzyme are difficult as fatty acid substrate concentrations were not stated, only conversion values and no mass data were reported, and the incubations were for 3 days (122, 124) rather than 2 days as I used here. If the differences in enzyme activities are real, they may be due to the 6 amino acid difference between the full ORF of the two genes. The origin of barramundi may be the cause of variation in FADS sequence. According to DNA barcoding analysis, the sequencing of the mitochondrial cytochrome c oxidase I (COI) gene strongly suggests that all Australian barramundi had an identical COI barcode sequence. Furthermore, this was very similar to the specimen from Singapore but all specimens from Myanmar were deeply divergent from the Australian/Singapore clade. Moreover, barramundi cytochrome b sequence comparisons show that the cluster of barramundi from Malaysia, Singapore and Australia exhibited a 1.8% divergence (305). Since the fish for the present study were sourced from Adelaide, South Australia and that for the previous study (124) were sourced in Kelantan, Malaysia, the difference in the results of these two studies suggest there may be polymorphisms in FADS gene which are differently expressed in the two barramundi populations, due to either the presence of different FADS2 isoforms or regional variations in barramundi species.

One of the major findings of this study has been to highlight the limitation of the yeast cell culture system for quantitatively assessing enzyme activity. Two key difficulties have been identified in accurately evaluating enzyme activity using this method. Namely, 1) the rapid loss of some fatty acid substrates and products, presumably by β-oxidation and 2) the significant release of some desaturation products to the culture medium. For example, C18 fatty acids had all been utilised by 6 hrs, indicating that C18 PUFA were more rapidly metabolised by cells and only limited amounts of C18 PUFA were actually used as substrates for desaturation/elongation by barramundi enzymes in this study and that the conversion rate

that I obtained may not represent the actual enzyme activity. Moreover, C20-C24 fatty acids were detected in the medium at the end of incubation period which indicates, once again, a differential capacity of uptake of the fatty acids by the yeast cells is dependent on fatty acid chain length and saturation status. As a result, it is important that any desaturation products which were present in the medium are taken into account in any calculation of enzyme activity. When the total amount of desaturation products in both the cells and medium were added together, the conversion rates of 20:3 n-3 to 20:4 n-3 and 24:5 n-3 to 24:6 n-3 were changed accordingly (Table 5.6). As mentioned above, care must be taken when comparing enzyme activities of enzymes such as desaturases in response to supplementation with different fatty acids, because C18 fatty acids, including ALA and LA, appear to be more favoured by yeast expression system than C20 and C24 substrates (Table 5.5). The less efficient incorporation of fatty acids with a longer chain (C20, C22 and C24) into yeast cells than that of fatty acids with a shorter chain (C18) has also been reported by van Roermund and others (99, 306).

5.6.2 ELOVL

The peptide sequence alignment of the barramundi ELOVL with ELOVL2 and ELOVL5 in human, zebra fish and Atlantic salmon indicated a high degree of homology with ELOVL5 (Figure 5.25 and Table 5.7). This agrees with another report of a barramundi ELOVL gene showing ELOVL5 peptide sequence homology in pair-wise comparisons with a number of fish species (124). Despite there being a difference in one nucleotide in the coding region and differences in length and nucleotide content of the 3' and 5' UTR between the ELOVL in this study and the previous report, there was 100% agreement in the amino acid sequences (124). As the same as the amino acid difference in barramundi FADS gene in the two studies, since the fish for this study and those for the other study were sourced from different regions, the differences in nucleotide sequence may be a polymorphism in ELOVL gene in the two barramundi populations, due to either the presence of the ELOVL isoforms or regional variation of barramundi species, as described above.

When I used yeast as a heterologous expression model to characterize the substrate specificities of recombinant barramundi elongase, the results indicated that the putative barramundi ELOVL gene had a wide range of elongation capabilities and was able to utilise monoenes as well as C18 and C20 (LC) PUFA as substrates. The C18 n-3 PUFA were preferred substrates over the C18 n-6 PUFA, but there was no significant n-3/n-6 preference

for the C20 substrates (Table 5.11). This differs from a previous report of barramundi ELOVL activity, which found little or no difference in n-3/n-6 preference for C18 PUFA (124). However, that study employed a 72 hr incubation period without showing the time course, did not report the concentration of substrates added and did not perform yeast cell + medium mass balance calculations. If the time courses were similar to those in our study, it is unlikely that the measurements were made in the linear range of time or concentration curves, and thus differences between n-3/n-6 values and C18/C20/C22 values may have been underestimated (124).

Studies with human and mouse ELOVL suggest that ELOVL5 elongates C18 to C20 and C22 n-3 PUFA (117, 130, 140) whereas ELOVL2 elongates C20 to C22 and C24 n-3 LCPUFA (137). Thus, the barramundi ELOVL in the present study showed substrate preferences most similar to mammalian ELOVL5. In addition, I found that this gene is able to elongate 18:4 n-3 and 18:3 n-6 as well as essential fatty acids, ALA and LA. Consistent with other reports of ELOVL5 function in mammals (117, 136, 137) and other fish species (122, 125, 141, 143, 307), the barramundi ELOVL5 showed elongation preferences for utilising C18 to 20 LCPUFA as substrates to produce C20 and C22 products and had the highest activity with EPA, with only weak activity towards C22 LCPUFA, such as DPA. The barramundi ELOVL5 in this study also showed higher activity with 18:4 n-3 and 18:3 n-6 than with ALA and LA. Nevertheless, the elongation products 20:3 n-3 and 20:2 n-6, from ALA and LA, respectively, may be important in the synthesis of LCPUFA. They require a Δ8 desaturase for conversion to 20:4 n-3 and 20:3 n-6. No Δ8 desaturase had been found in mammals or other vertebrates, until the recent report that the baboon FADS2 gene has both Δ6 and Δ8 desaturase activities (5), thereby providing a pathway to LCPUFA synthesis that does not necessarily involve Δ6 desaturase activity at the first step. This alternative may also exist in barramundi where the FADS2 isolated from barramundi has dual Δ6/Δ8 desaturase activity. Thus, dietary ALA and LA can be elongated by barramundi ELOVL5 and then desaturated by FADS2 to 20:4 n-3 and 20:3 n-6. Although the Δ5 desaturase has not been cloned from barramundi, the potential LCPUFA pathway is shown in Figure 5.35.

The recombinant barramundi elongase also showed activity toward endogenous monoenes, but not saturates, in yeast cells. Similar findings have been reported in marine and freshwater teleost fish species including zebra fish, catfish, tilapia, salmon, turbot, seabream and cod by heterologous expression of ELOVL ORF in yeast, showing that their elongases have

elongation activities towards C18 to C22 PUFA and monoenes (308). In mammals, elongation activity with monoenes is associated with ELOVL1 (117, 136).

All of the elongated products were initially absent in the medium, indicating that these products in the medium are most likely released from the yeast cells, and that the levels/concentrations of these fatty acids in the medium also need to be taken into account when evaluating enzyme activities. The percentage conversion results here have demonstrated that calculations which only use the values for the fatty acids present in the yeast cells were very different from those which were obtained when the fatty acids contained in the medium were also included in the calculation (Table 5.6, Table 5.8 and Table 5.11). Once again, findings from barramundi FADS2 and ELOVL5 heterologous expression using yeast cells highlight the importance of taking into account fatty acids released from cells into the culture medium, as well as those in the yeast cells themselves, when calculating conversion efficiencies using cell culture systems.

5.7 Summary

Heterologous expression of barramundi FADS2 gene in yeast demonstrates that this enzyme shows dual Δ6/Δ8 functionality and exhibits a preference for n-3 over n-6 fatty acid substrates. The barramundi ELOVL gene had a capacity for conversion of C18 to C20 to C22 PUFA and LCPUFA, and the highest elongation activities toward C20 EPA and AA. The availability of substrates for Δ8 desaturase activity depends on the presence of the C20 fatty acid substrates in the food chain or the occurrence of an elongase that could convert ALA to 20:3 n-3 or LA to 20:2 n-6. The barramundi elongase here is capable of elongating ALA and LA to 20:3 n-3and 20:2 n-6, respectively. As a result, I postulate that the LCPUFA production from C18 precursors in barramundi can be processed through either Δ6 or Δ8 desaturation pathways (Figure 5.35). However, it is still unclear whether barramundi has a Δ5 desaturase for desaturation of 20:4 n-3 and 20:3 n-6. If the Δ5 desaturase is expressed, it is likely to be at much lower levels than the Δ6 desaturase. Moreover, in the whole animal where all substrates can be present at the same time, there is the potential for n-3/n-3 and n-3/n-6 substrate competition which would affect pathway flux.

The yeast heterologous expression is a fast and convenient system for identifying characteristics of foreign enzymes. However, findings from this study suggest that there is a need to exercise caution when using this system for assessing the quantitative aspects of

enzyme kinetics, and also highlight the importance of taking extracellular fatty acid levels into account when calculating enzyme activity.

Chapter 6
Conclusions and Future Perspectives

There have been numerous feeding studies to increase the level of n-3 LCPUFA in the tissues of animals, particularly food animals, by increasing dietary ALA, but most have met with limited success, mainly resulting in high levels of ALA in tissues and only modest conversion into n-3 LCPUFA. From such feeding experiments, animals such as rats (302), chickens (178), pigs (4) and freshwater fish (176, 216) appear to able to produce substantial n-3 LCPUFA from the ALA found in plant sources while other animals (guinea pigs (309), hamsters (310), marine fish (154) and humans (47, 71) appear to have a very limited capacity for conversion of ALA to EPA and even less to DHA. In part, results reported in this thesis provide further insights into the discrepancy between EPA and DHA accumulation. In a series of rat experiments covering a range of dietary ALA levels (or total PUFA) (Chapter 3) I have determined that while the relationship between dietary ALA and EPA levels in plasma, erythrocytes and other tissues is linear, the correlation between dietary ALA and DHA is curvilinear, such that poor accumulation of DHA occurs at very low and very high levels of ALA in the diet but the biological explanation for these results is still unclear.

Many studies have inferred that the low LCPUFA accumulation rate in organisms fed plant-based oils is due to the apparent limited conversion of ALA or LA through to other LCPUFA caused by either by excessive amounts of dietary PUFA acting to inhibit expression of the gene for the Δ6 desaturase (encoded by FADS2) or by competitive competition between the two EFA, LA and ALA. There has been a lack of data on the correlation between tissue fatty acid compositions and the expression of genes involved in the fatty acid pathway and PUFA levels which are within a range that could reasonably be expected in normal human and animal diets. In other words, for people eating normal Western diets that are rich in PUFA, is the reason for the low n-3 LCPUFA status due to an imbalance in the ratio of LA to ALA or the fact that expression of the rate limiting enzyme, the Δ6 desaturase, is limited by the imbalanced dietary PUFA. When I tested the hypothesis in rats as described in chapter 3, I found that while high n-3 PUFA diets consistently produced higher levels of n-3 LCPUFA, particularly EPA and DPA, in rat tissues than low n-3 PUFA diets, expression of the Δ6 desaturase and elongase 2 genes were increased only in animals fed the low PUFA reference diet. Furthermore, an increased level of dietary ALA ranging from 0.2-2.9%en *per se* influenced the fatty acid composition of the blood and major tissue phospholipids, despite the

fact that no regulatory effects of the dietary ALA on the mRNA levels of desaturases (FADS1 and FADS2), elongases (ELOVL2 and ELOVL5) or transcription factors (SREBP1-c and PPARα) was observed. Thus, it appeared that there was no relationship between dietary ALA and gene expression of the key enzymes (FADS1, FADS2, ELOVL2 and ELOVL5) in the pathway when dietary PUFA levels were in the range normally present in animal and human diets. The mechanism of conversion regulation must be clarified to make evidence-based dietary recommendations of PUFA to food manufacturers, the animal production industry and general populations.

The fundamental aspects of the fatty acid pathway appears to be well conserved between species, however each species utilises dietary PUFA differently. Thus, one of the main strategies of developing sustainable n-3 rich food sources is to provide food animals with an optimal PUFA level and n-3:n-6 ratio to increase their capacity to synthesizing their own n-3 LCPUFA, thereby reducing the reliance on fish oil and fish meal in their diets. This is particularly important in the aquaculture arena where massive amounts of wild catch fish are processed for use in aquaculture ponds. In chapter 4, diets with varying ALA levels and LA:ALA ratios were applied to barramundi to examine the ability of this species to convert dietary ALA to n-3 LCPUFA. I found that dietary ALA ranging from 0.1%en to 3.2%en resulted in a dose-dependent increase in tissue ALA but levels of all n-3 LCPUFA remained unchanged. Furthermore, increasing ALA in diets had no effect on mRNA abundance of FADS2 and ELOVL genes among fish fed on these ALA containing feeds. However, the two genes were up-regulated approximately 10 fold for FADS2 and 3 fold for ELOVL in animals fed on vegetable oil-based diets, relative to those fed fish oil-based diets. Therefore, in line with studies in other species, the results described in chapter 3 and chapter 4 demonstrated that expression of desaturase and elongase genes are up-regulated in the presence of low PUFA/LCPUFA content in the diet. However, there was no positive correlation between mRNA abundance of these genes and their corresponding LCPUFA concentrations, in particular DHA, in the tissues. Clearly barramundi have a limited capacity for n-3 LCPUFA synthesis.

A major finding of chapter 4 also was the substantial variability between individual fish fed on basal diets that contained only PUFA and no LCPUFA. The percentage of DHA found in fish ranged from ~5 to 30% of total fatty acids in liver phospholipids that either indicated that some fish retain pre-existing DHA for long periods or that some individual fish are capable of synthetising DHA from ALA. This issue deserves to be addressed. Because fish in the wild

are always eating other fish that are rich in n-3 LCPUFA, there is no selection pressure on the genes for fatty acid synthesis. Thus, a case could be made for screening wild fish for their ability to convert ALA to n-3 LCPUFA for a breeding program.

In attempting to take this work further, a yeast heterologous expression system was applied to identify the functions of barramundi FADS2 and ELOVL genes. In chapter 5 of this thesis, cloning of the barramundi two key genes in the fatty acid pathway in yeast cells revealed that the conversion rate of hepatic desaturation in barramundi was generally lower than the rate of elongation. I demonstrated that the activity of barramundi Δ6 desaturase was very low, but that the enzyme also had Δ8 desaturase activity, thus providing an alternative route for ALA conversion to DHA. To the best of our knowledge the studies undertaken in this thesis are among the first to report Δ8 desaturase activity in a fish species. In addition, the recombinant barramundi Δ6 desaturase showed a preference for n-3 substrates over n-6, but this preference only applied to the elongation of C18 PUFA, particularly ALA and LA. The alternative route of the LCPUFA and its relative capability of desaturating PUFA precursors in barramundi emphasised that the enzymes involved in the fatty acid pathway in different species have not been fully explored.

In the course of these experiments I also discovered the limited suitability of the heterologous expression system using *S. cerevisiae* yeast. Though the yeast system is a well accepted and a convenient model to characterize enzyme function, the system is not completely suited to study the quantitative aspects of functional expression due to the cellular metabolism of yeast cells (311). As a result of the fact that yeast cells used fatty acids as an energy source, an extremely high percentage of the C18 substrates added to the yeast cell system was rapidly metabolised by yeast cells and only a small amount were thus available to be used for desaturation or elongation. In yeast, β-oxidation is confined to peroxisomes, and yeasts like *S. cerevisiae* are capable of utilising different types of fatty acids, including PUFA, as an energy source. This implies that β-oxidation of supplemented fatty acid substrates by the yeast cells needs to be considered carefully when undertaking fatty acid studies in this system. Another finding was that there was significant release of some desaturation and elongation products into the culture medium. All of the desaturation and elongation products were initially absent in the medium, indicating that they are most likely released from the yeast cells and need to be taken into account for evaluating overall enzyme activities. The percentage conversion results obtained when the fatty acids contained in the culture medium were also included in the calculation were very different from those where only the values for the fatty acids present

in the yeast cells were used. For all these reasons, it is not possible to make firm conclusions about the quantitative aspects of enzyme kinetics, instead we can only characterise the function of the enzymes.

The studies presented in this thesis suggest that when dietary PUFA level was changed modestly, LCPUFA synthesis is regulated more by substrate competition for existing enzymes than by an increase in their mRNA expression and this seems to be species dependent. However, to what extent PUFA levels could result in regulation of enzyme activities and ultimately stimulate the LCPUFA metabolism has not been systemically examined. Animal models are an invaluable tool to identify consequences of tissue lipid status and potential mechanism that mediate the effect of tissue fatty acid accumulation and capacity for the cell membrane to incorporate particular fatty acids after dietary PUFA intervention. Interpretation of animal dietary intervention studies for evaluating enzyme activity, however, is often complicated by the presence of hormonal effects, β-oxidation, carbon recycling and various dietary fatty acids that compete for common enzymes in the desaturation and elongation pathway and affect fatty acid conversion. The use of *in vitro* cell systems such as human HepG2 cells to examine the accumulation of fatty acids are advantageous from their flexibility of a single fatty acid substrate supplement. For example HepG2 study revealed that in the absence of competing fatty acid substrates, a limitation in the accumulation of DHA from ALA in HepG2 cell phospholipids was still observed, and it has been suggested that competition between ALA and 24:5 n-3 for the use of Δ6 desaturase in the conversion of ALA to DHA may be the main factor limiting the accumulation of DHA in cell phospholipids (53, 312). Though the HepG2 cell seems a suitable model to mimic the *in vivo* environments to study fatty acid conversion and accumulation, the combined effect of other fatty acid enzymes in pathways including fatty acid conversion and phospholipid biosynthesis may also affect substrate concentrations and cannot specifically address the enzymatic reactions involved in the desaturation and elongation.

Biochemical characterization of membrane-bound proteins such as desaturases and elongases remains challenging due to the difficulties in isolating the proteins in a functional form which hinder detailed studies of these fatty acid pathway enzymes. Using a system such as isolated yeast microsomes that contain only one of the target genes of interest, without the complication of these being other cell types and enzymes present, provides advantages in studying the properties of the enzymes. However, it is evident that membrane proteins require specific lipids, be it as cofactors for their functions or as co-structures for their correct folding

and stability thus a possible bottleneck in the yeast expression could be due to a shortage of cholesterol of mammalian membrane proteins, that does not result in fully functional heterologous proteins (311). Recently a human stearyl-CoA desaturase has been successfully isolated and purified in a cell-free system (313). The cell-free approaches mimic the natural cytoplasmic environments, yet remain independent from the requirements and sensitivity of living cells and thus overcome the general limitations of conventional cellular expression systems. Therefore, using new concepts and techniques to elucidate the currently unknown enzyme kinetics of the key enzymes in the fatty acid pathway *in vitro* in a pure and functional state is essential to unlock roles/mechanisms of the desaturases and elongases in LCPUFA synthesis. Furthermore, systematic evaluation of dietary effects on gene expression and protein abundance of the key genes (FADS1, FADS2, ELOVL2 and ELOVL5) and their encoded proteins (Δ5, Δ6 desaturase, elongases 2 and 5) in the fatty acid pathway in tissues (primarily liver) under a full range of PUFA and LCPUFA is needed to define dietary approaches on the impacts of LCPUFA conversions and tissue fatty acid incorporation and, ultimately, to identify more sustainable sources of n-3 LCPUFA in the diet.

One of the strategies to improve n-3 status and also maintain the sustainability of n-3 source is to have animal diets enriched with plant derived n-3 PUFA. Since dietary ALA is consumed with LA at the same time; a balance between the n-6 and n-3 PUFA plays a vital role in maintaining health and this is not just because they are EFA but also precursors for n-3 LCPUFA. Applying n-3 rich vegetable oil or other non-fish ingredients through dietary manipulation in animal feeds such as feeds for poultry and livestock, for animals to convert dietary ALA into EPA and DHA endogenously, enable the beneficial effect of n-3 LCPUFA that are primarily found in fish to be conferred to other non-fish foods. This strategy for increasing the n-3 LCPUFA status of food animals such as chicken and pigs, is likely to be beneficial in increasing the n-3 status of the majority of Australians, who rarely consume fish (159).

Manipulation of n-3 fatty acid status through dietary interventions could also be directly applied to human diets. Excessive amounts of n-6 PUFA and high LA:ALA ratios, as found in current Australian diets, has been reported to negatively affect the n-3 status of the population, and may have negative effects on health (62). Mediterranean diets have been well-recognized for its protective effects on death from heart disease and other chronic diseases. The lipid composition of Mediterranean diets are characterized as being low in saturates, high in monoenes, low in PUFA with a low LA:ALA ratio (314). The efficacy of the Mediterranean

diet has been tested in the Lyon Diet Heart Study on the rate of coronary events in people who have had a first heart attack. The results suggest that a Mediterranean-style diet may help reduce recurrent events in patients with heart disease (315). Results from this thesis highlight that in order to increase n-3 LCPUFA levels in tissues, there are two essential features of the lipid profile of Mediterranean diets that need to be applied: a sufficient supply of ALA (with an optimum ratio of LA:ALA) and a low total PUFA level in the diet (lower than current Australian diets). Thus, alternatives to fish consumption to maximise the intake of n-3 fatty acids in human are feeding food animals with plant oil which contains ALA during animal rearing or through manipulating human diets directly.

It goes without saying that the studies in this thesis represent a few details in the vast field of fatty acid research. There is much to be done, if only improving the accuracy and robustness of current methodologies. Below is a short list of some of the most immediate work that could be done to improve the understanding of DHA conversion pathway:

1. The thesis investigated the LCPUFA conversion under a narrow range of dietary PUFA at specific concentrations. There is room for research into the response for different PUFA amounts and fat content to determine gene/enzyme regulation and n-3 LCPUFA conversion efficiency.
2. Only male rats were used to investigate the LCPUFA synthesis, whether there is a gender specific effect needs to be clarified by investigating the response in female rats.
3. Protein quantity could be measured in assays to determine how protein availability may affect n-3 LCPUFA conversion efficiencies.
4. While the condition for culturing/expressing the yeast cells was consistent with standard methods, there may be more ideal conditions for specifically studying the fatty acid pathway, such as manipulating the yeast expression system to maintain a steady supply of the fatty acid substrates of interest.
5. This thesis only experimented with one fish species. There are many fish and other farmable animal species that may be better at converting dietary PUFA to n-3 LCPUFA.

Despite the long history of fatty acid research, the enzyme kinetics for ALA and LA with isolated fatty acid desaturases and elongases are still missing. Studies of pure enzymes in the fatty acid pathway are urgently needed in order to address the direct effects of individual fatty acids and evaluate possible competitive and or inhibitory effects.

References

1. Brenna, J. T., N. Salem, Jr., A. J. Sinclair, and S. C. Cunnane. 2009. Alpha-linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. *Prostaglandins Leukot Essent Fatty Acids* **80**: 85-91.
2. Burdge, G. C., and P. C. Calder. 2005. Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod Nutr Dev* **45**: 581-597.
3. Sinclair, A., N. Attar-Bashi, and D. Li. 2002. What is the role of alpha-linolenic acid for mammals? *Lipids* **37**: 1113-1123.
4. Blank, C., M. A. Neumann, M. Makrides, and R. A. Gibson. 2002. Optimizing DHA levels in piglets by lowering the linoleic acid to alpha-linolenic acid ratio. *J Lipid Res* **43**: 1537-1543.
5. Park, W. J., K. S. Kothapalli, P. Lawrence, C. Tyburczy, and J. T. Brenna. 2009. An alternate pathway to long chain polyunsaturates: The FADS2 gene product Δ8-desaturates 20:2n-6 and 20:3n-3. *J Lipid Res*: **50**: 1195-1202.
6. Tremblay, A., G. Plourde, J. P. Despres, and C. Bouchard. 1989. Impact of dietary fat content and fat oxidation on energy intake in humans. *Am J Clin Nutr* **49**: 799-805.
7. Brown, D. A., and E. London. 1998. Structure and origin of ordered lipid domains in biological membranes. *J Membr Biol* **164**: 103-114.
8. Spector, A. A., and M. A. Yorek. 1985. Membrane lipid composition and cellular function. *J Lipid Res* **26**: 1015-1035.
9. Hu, F. B., M. J. Stampfer, J. E. Manson, A. Ascherio, G. A. Colditz, F. E. Speizer, C. H. Hennekens, and W. C. Willett. 1999. Dietary saturated fats and their food sources in relation to the risk of coronary heart disease in women. *Am J Clin Nutr* **70**: 1001-1008.
10. Bloch, K., and D. Vance. 1977. Control mechanisms in the synthesis of saturated fatty acids. *Annu Rev Biochem* **46**: 263-298.
11. Green, C. D., C. G. Ozguden-Akkoc, Y. Wang, D. B. Jump, and L. K. Olson. 2010. Role of fatty acid elongases in determination of de novo synthesized monounsaturated fatty acid species. *J Lipid Res* **51**: 1871-1877.
12. Lunn, J. 2007. Monounsaturates in the diet. *Nutr Bull* **32**: 378-391.
13. Arterburn, L. M., E. B. Hall, and H. Oken. 2006. Distribution, interconversion, and dose response of n-3 fatty acids in humans. *Am J Clin Nutr* **83**: 1467S-1476S.
14. Taber, L., C.-H. Chiu, and J. Whelan. 1998. Assessment of the arachidonic acid content in foods commonly consumed in the American diet. *Lipids* **33**: 1151-1157.
15. Gibson, R. 1983. Australian fish—An excellent source of both arachidonic acid and ω-3 polyunsaturated fatty acids. *Lipids* **18**: 743-752.
16. Kromann, N., and A. Green. 1980. Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. *Acta Med Scand* **208**: 401-406.
17. Makhoul, Z., A. R. Kristal, R. Gulati, B. Luick, A. Bersamin, B. Boyer, and G. V. Mohatt. 2010. Associations of very high intakes of eicosapentaenoic and docosahexaenoic acids with biomarkers of chronic disease risk among Yup'ik Eskimos. *Am J Clin Nutr* **91**: 777-785.
18. Fritsche, K. 2006. Fatty acids as modulators of the immune response. *Annu Rev Nutr* **26**: 45-73.
19. Bouwens, M., O. van de Rest, N. Dellschaft, M. G. Bromhaar, L. C. de Groot, J. M. Geleijnse, M. Muller, and L. A. Afman. 2009. Fish-oil supplementation induces antiinflammatory gene expression profiles in human blood mononuclear cells. *Am J Clin Nutr* **90**: 415-424.
20. Sampath, H., and J. M. Ntambi. 2005. Polyunsaturated fatty acid regulation of genes of lipid metabolism. *Annu Rev Nutr* **25**: 317-340.
21. Stoffel, W., B. Holz, B. Jenke, E. Binczek, R. H. Gunter, C. Kiss, I. Karakesisoglou, M. Thevis, A.-A. Weber, S. Arnhold, and K. Addicks. 2008. Δ6-Desaturase (FADS2) deficiency unveils the role of omega-3- and omega-6-polyunsaturated fatty acids. *EMBO J* **27**: 2281-2292.
22. Tinoco, J. 1982. Dietary requirements and functions of alpha-linolenic acid in animals. *Prog Lipid Res* **21**: 1-45.
23. Martin, R. E., E. B. Rodriguez de Turco, and N. G. Bazan. 1994. Developmental maturation of hepatic n-3 polyunsaturated fatty acid metabolism: Supply of docosahexaenoic acid to retina and brain. *J Nutr Biochem* **5**: 151-160.

24. Bazan, N. G. 2003. Synaptic lipid signaling: significance of polyunsaturated fatty acids and platelet-activating factor. *J Lipid Res* **44**: 2221-2233.
25. Simopoulos, A. P. 1999. Essential fatty acids in health and chronic disease. *Am J Clin Nutr* **70**: 560S-569S.
26. Goyens, P. L., M. E. Spilker, P. L. Zock, M. B. Katan, and R. P. Mensink. 2006. Conversion of alpha-linolenic acid in humans is influenced by the absolute amounts of alpha-linolenic acid and linoleic acid in the diet and not by their ratio. *Am J Clin Nutr* **84**: 44-53.
27. Kaminskas, A., B. Zieden, B. Elving, M. Kristenson, A. Abaravicius, B. Bergdahl, A. G. Olsson, and Z. Kucinskiene. 1999. Adipose tissue fatty acids in men from two populations with different cardiovascular risk: the LiVicordia study. *Scand J Clin Lab Invest* **59**: 227-232.
28. Tang, A. B., K. Y. Nishimura, and S. D. Phinney. 1993. Preferential reduction in adipose tissue alpha-linolenic acid (18:3 omega 3) during very low calorie dieting despite supplementation with 18:3 omega-3. *Lipids* **28**: 987-993.
29. Flachs, P., M. Rossmeisl, M. Bryhn, and J. Kopecky. 2009. Cellular and molecular effects of n-3 polyunsaturated fatty acids on adipose tissue biology and metabolism. *Clin Sci (Lond)* **116**: 1-16.
30. Cunnane, S. C., R. Ross, J. L. Bannister, and D. J. Jenkins. 2001. Beta-oxidation of linoleate in obese men undergoing weight loss. *Am J Clin Nutr* **73**: 709-714.
31. Cunnane, S. C., M. A. Ryan, Y. H. Lin, S.-Y. Lim, and N. J. Salem. 2006. Suckling rats actively recycle carbon from alpha-linolenate into newly synthesized lipids even during extreme dietary deficiency of n-3 polyunsaturates. *Pediatric Research* **59**: 107-110.
32. Sprecher, H., D. L. Luthria, B. S. Mohammed, and S. P. Baykousheva. 1995. Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids. *J Lipid Res* **36**: 2471-2477.
33. Polinati, P. P., P. Eskelin, and T. Tyni. 2001. Fatty Acid Oxidation Disorders John Wiley & Sons, Ltd.
34. Bjorkhem, I. 1978. On the quantitative importance of omega-oxidation of fatty acids. *J Lipid Res* **19**: 585-590.
35. Tserng, K. Y., and S. J. Jin. 1991. Metabolic conversion of dicarboxylic acids to succinate in rat liver homogenates. A stable isotope tracer study. *J Biol Chem* **266**: 2924-2929.
36. Sanders, R. J., R. Ofman, F. Valianpour, S. Kemp, and R. J. Wanders. 2005. Evidence for two enzymatic pathways for omega-oxidation of docosanoic acid in rat liver microsomes. *J Lipid Res* **46**: 1001-1008.
37. Eaton, S., K. Bartlett, and M. Pourfarzam. 1996. Mammalian mitochondrial beta-oxidation. *Biochem. J.* **320**: 345-357.
38. Wanders, R. J., and H. R. Waterham. 2006. Biochemistry of mammalian peroxisomes revisited. *Annu Rev Biochem* **75**: 295-332.
39. Verhoeven, N. M., D. S. Roe, R. M. Kok, R. J. Wanders, C. Jakobs, and C. R. Roe. 1998. Phytanic acid and pristanic acid are oxidized by sequential peroxisomal and mitochondrial reactions in cultured fibroblasts. *J Lipid Res* **39**: 66-74.
40. Senadheera, S. D., G. M. Turchini, T. Thanuthong, and D. S. Francis. 2011. Effects of dietary alpha-linolenic acid (18:3n-3)/linoleic acid (18:2n-6) ratio on fatty acid metabolism in Murray cod (Maccullochella peelii peelii). *J Agric Food Chem* **59**: 1020-1030.
41. Gavino, V., S. Cordeau, and G. Gavino. 2003. Kinetic analysis of the selectivity of acylcarnitine synthesis in rat mitochondria. *Lipids* **38**: 485-490.
42. Irving, C. S., W. W. Wong, R. J. Shulman, E. O. Smith, and P. D. Klein. 1983. [13C]bicarbonate kinetics in humans: intra- vs. interindividual variations. *Am J Physiol* **245**: R190-202.
43. DeLany, J. P., M. M. Windhauser, C. M. Champagne, and G. A. Bray. 2000. Differential oxidation of individual dietary fatty acids in humans. *Am J Clin Nutr* **72**: 905-911.
44. Brenna, J. T. 2002. Efficiency of conversion of alpha-linolenic acid to long chain n-3 fatty acids in man. *Curr Opin Clin Nutr Metab Care* **5**: 127-132.
45. Vermunt, S. H., R. P. Mensink, M. M. Simonis, and G. Hornstra. 2000. Effects of dietary alpha-linolenic acid on the conversion and oxidation of 13C-alpha-linolenic acid. *Lipids* **35**: 137-142.
46. Burdge, G. C., A. E. Jones, and S. A. Wootton. 2002. Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men. *Br J Nutr* **88**: 355-363.
47. Burdge, G. C., and S. A. Wootton. 2002. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. *Br J Nutr* **88**: 411-420.

48. Burdge, G. C., and S. A. Wootton. 2003. Conversion of alpha-linolenic acid to palmitic, palmitoleic, stearic and oleic acids in men and women. *Prostaglandins Leukot Essent Fatty Acids* **69**: 283-290.
49. Cunnane, S. C., and M. J. Anderson. 1997. The majority of dietary linoleate in growing rats is beta-oxidized or stored in visceral fat. *J Nutr* **127**: 146-152.
50. Lin, Y. H., and N. Salem, Jr. 2007. Whole body distribution of deuterated linoleic and alpha-linolenic acids and their metabolites in the rat. *J Lipid Res* **48**: 2709-2724.
51. Brenner, R. 1971. The desaturation step in the animal biosynthesis of polyunsaturated fatty acids. *Lipids* **6**: 567-575.
52. Ves-Losada, A., and R. O. Peluffo. 1993. Effect of L-triiodothyronine on liver microsomal Δ6 and Δ5 desaturase activity of male rats. *Mol Cell Biochem* **121**: 149-153.
53. Portolesi, R., B. C. Powell, and R. A. Gibson. 2007. Competition between 24:5n-3 and ALA for Δ6 desaturase may limit the accumulation of DHA in HepG2 cell membranes. *J Lipid Res* **48**: 1592-1598.
54. Langelier, B., J. M. Alessandri, M. H. Perruchot, P. Guesnet, and M. Lavialle. 2005. Changes of the transcriptional and fatty acid profiles in response to n-3 fatty acids in SH-SY5Y neuroblastoma cells. *Lipids* **40**: 719-728.
55. Su, H. M., M. C. Huang, N. M. Saad, P. W. Nathanielsz, and J. T. Brenna. 2001. Fetal baboons convert 18:3n-3 to 22:6n-3 in vivo. A stable isotope tracer study. *J Lipid Res* **42**: 581-586.
56. Neuringer, M., W. E. Connor, D. S. Lin, L. Barstad, and S. Luck. 1986. Biochemical and functional effects of prenatal and postnatal omega 3 fatty acid deficiency on retina and brain in rhesus monkeys. *Proc Natl Acad Sci U S A* **83**: 4021-4025.
57. Igarashi, M., J. C. DeMar, Jr., K. Ma, L. Chang, J. M. Bell, and S. I. Rapoport. 2007. Upregulated liver conversion of alpha-linolenic acid to docosahexaenoic acid in rats on a 15 week n-3 PUFA-deficient diet. *J Lipid Res* **48**: 152-164.
58. Igarashi, M., J. C. DeMar, Jr., K. Ma, L. Chang, J. M. Bell, and S. I. Rapoport. 2007. Docosahexaenoic acid synthesis from alpha-linolenic acid by rat brain is unaffected by dietary n-3 PUFA deprivation. *J Lipid Res* **48**: 1150-1158.
59. Louheranta, A. M., E. K. Porkkala-Sarataho, M. K. Nyyssonen, R. M. Salonen, and J. T. Salonen. 1996. Linoleic acid intake and susceptibility of very-low-density and low density lipoproteins to oxidation in men. *Am J Clin Nutr* **63**: 698-703.
60. Regnstrom, J., J. Nilsson, P. Tornvall, C. Landou, and A. Hamsten. 1992. Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* **339**: 1183-1186.
61. de Lorgeril, M., S. Renaud, P. Salen, I. Monjaud, N. Mamelle, J. L. Martin, J. Guidollet, P. Touboul, and J. Delaye. 1994. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. *Lancet* **343**: 1454-1459.
62. Simopoulos, A. P. 2006. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. *Biomed Pharmacother* **60**: 502-507.
63. Lin, Y. H., and N. Salem, Jr. 2005. In vivo conversion of 18- and 20-C essential fatty acids in rats using the multiple simultaneous stable isotope method. *J Lipid Res* **46**: 1962-1973.
64. Mohrhauer, H., and R. T. Holman. 1963. The Effect of dose level of essential fatty acids upon fatty acid composition of the rat liver. *J Lipid Res* **4**: 151-159.
65. Mohrhauer, H., and R. T. Holman. 1963. Effect of linolenic acid upon the metabolism of linoleic acid. *J Nutr* **81**: 67-74.
66. Arbuckle, L. D., M. J. MacKinnon, and S. M. Innis. 1994. Formula 18:2(n-6) and 18:3(n-3) content and ratio influence long-chain polyunsaturated fatty acids in the developing piglet liver and central nervous system. *J Nutr* **124**: 289-298.
67. Woods, J., G. Ward, and N. Salem. 1996. Is docosahexaenoic acid necessary in infant formula? Evaluation of high linolenate diets in the neonatal rat. *Pediatric Research* **40**: 687-694.
68. Bowen, R. A. R., A. A. Wierzbicki, and M. T. Clandinin. 1999. Does increasing dietary linolenic acid content increase the docosahexaenoic acid content of phospholipids in neuronal cells of neonatal rats? *Pediatric Research* **45**: 815-819.
69. Araya, J., R. Rodrigo, L. A. Videla, L. Thielemann, M. Orellana, P. Pettinelli, and J. Poniachik. 2004. Increase in long-chain polyunsaturated fatty acid n-6/n-3 ratio in relation to hepatic steatosis in patients with non-alcoholic fatty liver disease. *Clin. Sci.* **106**: 635-643.

70. Muskiet, F. A. J., S. A. van Goor, R. S. Kuipers, F. V. Velzing-Aarts, E. N. Smit, H. Bouwstra, D. A. Janneke Dijck-Brouwer, E. Rudy Boersma, and M. Hadders-Algra. 2006. Long-chain polyunsaturated fatty acids in maternal and infant nutrition. *Prostaglandins Leukot Essent Fatty Acids* **75**: 135-144.
71. Burdge, G. 2004. alpha-Linolenic acid metabolism in men and women: nutritional and biological implications. *Curr Opin Clin Nutr Metab Care* **7**: 137-144.
72. Harper, C. R., M. J. Edwards, A. P. DeFilipis, and T. A. Jacobson. 2006. Flaxseed oil increases the plasma concentrations of cardioprotective (n-3) fatty acids in humans. *J Nutr* **136**: 83-87.
73. Francois, C. A., S. L. Connor, L. C. Bolewicz, and W. E. Connor. 2003. Supplementing lactating women with flaxseed oil does not increase docosahexaenoic acid in their milk. *Am J Clin Nutr* **77**: 226-233.
74. Mantzioris, E., M. J. James, R. A. Gibson, and L. G. Cleland. 1994. Dietary substitution with an alpha-linolenic acid-rich vegetable oil increases eicosapentaenoic acid concentrations in tissues. *Am J Clin Nutr* **59**: 1304-1309.
75. Galli, C., and F. Marangoni. 1997. Recent advances in the biology of n-6 fatty acids. *Nutrition* **13**: 978-985.
76. Voss, A., M. Reinhart, S. Sankarappa, and H. Sprecher. 1991. The metabolism of 7,10,13,16,19-docosapentaenoic acid to 4,7,10,13,16,19-docosahexaenoic acid in rat liver is independent of a 4-desaturase. *J Biol Chem* **266**: 19995-20000.
77. Burdge, G. C., Y. E. Finnegan, A. M. Minihane, C. M. Williams, and S. A. Wootton. 2003. Effect of altered dietary n-3 fatty acid intake upon plasma lipid fatty acid composition, conversion of [13C]alpha-linolenic acid to longer-chain fatty acids and partitioning towards beta-oxidation in older men. *Br J Nutr* **90**: 311-321.
78. Jensen, C., H. Chen, J. Kennard Fraley, R. Anderson, and W. Heird. 1996. Biochemical effects of dietary linoleic/α-linolenic acid ratio in term infants. *Lipids* **31**: 107-113.
79. Jensen, C. L., T. C. Prager, J. K. Fraley, H. Chen, R. E. Anderson, and W. C. Heird. 1997. Effect of dietary linoleic/alpha-linolenic acid ratio on growth and visual function of term infants. *J Pediatr* **131**: 200-209.
80. Makrides, M., M. A. Neumann, B. Jeffrey, E. L. Lien, and R. A. Gibson. 2000. A randomized trial of different ratios of linoleic to alpha-linolenic acid in the diet of term infants: effects on visual function and growth. *Am J Clin Nutr* **71**: 120-129.
81. Emken, E. A., R. O. Adlof, and R. M. Gulley. 1994. Dietary linoleic acid influences desaturation and acylation of deuterium-labeled linoleic and linolenic acids in young adult males. *Biochim Biophys Acta - Lipids and Lipid Metabolism* **1213**: 277-288.
82. Emken, E. A., R. O. Adlof, S. M. Duval, and G. J. Nelson. 1999. Effect of dietary docosahexaenoic acid on desaturation and uptake in vivo of isotope-labeled oleic, linoleic, and linolenic acids by male subjects. *Lipids* **34**: 785-791.
83. Sprecher, H. 2000. Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochim Biophys Acta - Molecular and Cell Biology of Lipids* **1486**: 219-231.
84. Sayanova, O., F. Beaudoin, B. Libisch, A. Castel, P. R. Shewry, and J. A. Napier. 2001. Mutagenesis and heterologous expression in yeast of a plant Δ6-fatty acid desaturase. *J Exp Bot* **52**: 1581-1585.
85. Howton, D. R., R. H. Davis, and J. C. Nevenzel. 1954. Unsaturated fatty acids. III. Preparation of 1-C14-linoleic acid. *J Am Chem Soc* **76**: 4970-4974.
86. Howton, D. R., and J. F. Mead. 1960. Metabolism of essential fatty acids. X. Conversion ox 8, 11, 14-eicosatrienoic acid to arachidonic acid in the rat. *J Biol Chem* **235**: 3385-3386.
87. Mead, J. F., and D. R. Howton. 1957. Metabolism of essential fatty acids. VII. Conversion of gamma-linolenic acid to arachidonic acid. *J Biol Chem* **229**: 575-582.
88. Steinberg, G., W. H. Slaton, Jr., D. R. Howton, and J. F. Mead. 1957. Metabolism of essential fatty acids. V. Metabolic pathway of linolenic acid. *J Biol Chem* **224**: 841-849.
89. Sprecher, H. 1996. New advances in fatty-acid biosynthesis. *Nutrition* **12**: S5-S7.
90. Sprecher, H., Q. Chen, and F. Yin. 1999. Regulation of the biosynthesis of 22:5n-6 and 22:6n-3: A complex intracellular process. *Lipids* **34**: S153-S156.
91. Nakamura, M. T., and T. Y. Nara. 2004. Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. *Annu Rev Nutr* **24**: 345-376.

92. Aki, T., Y. Shimada, K. Inagaki, H. Higashimoto, S. Kawamoto, S. Shigeta, K. Ono, and O. Suzuki. 1999. Molecular cloning and functional characterization of rat Δ6 fatty acid desaturase. *Biochem Biophys Res Commun* **255**: 575-579.
93. Cho, H. P., M. Nakamura, and S. D. Clarke. 1999. Cloning, expression, and fatty acid regulation of the human Δ5 desaturase. *J Biol Chem* **274**: 37335-37339.
94. Cho, H. P., M. T. Nakamura, and S. D. Clarke. 1999. Cloning, expression, and nutritional regulation of the mammalian Δ6 desaturase. *J Biol Chem* **274**: 471-477.
95. Leonard, A. E., B. Kelder, E. G. Bobik, L. T. Chuang, J. M. Parker-Barnes, J. M. Thurmond, P. E. Kroeger, J. J. Kopchick, Y. S. Huang, and P. Mukerji. 2000. cDNA cloning and characterization of human Δ5-desaturase involved in the biosynthesis of arachidonic acid. *Biochem. J.* **347**: 719-724.
96. Matsuzaka, T., H. Shimano, N. Yahagi, M. Amemiya-Kudo, T. Yoshikawa, A. H. Hasty, Y. Tamura, J. Osuga, H. Okazaki, Y. Iizuka, A. Takahashi, H. Sone, T. Gotoda, S. Ishibashi, and N. Yamada. 2002. Dual regulation of mouse Δ5- and Δ6-desaturase gene expression by SREBP-1 and PPARalpha. *J Lipid Res* **43**: 107-114.
97. Zheng, X., I. Seiliez, N. Hastings, D. R. Tocher, S. Panserat, C. A. Dickson, P. Bergot, and A. J. Teale. 2004. Characterization and comparison of fatty acyl Δ6 desaturase cDNAs from freshwater and marine teleost fish species. *Comp Biochem Physiol B* **139**: 269-279.
98. Zolfaghari, R., C. J. Cifelli, M. D. Banta, and A. C. Ross. 2001. Fatty acid Δ5 desaturase mRNA is regulated by dietary vitamin A and exogenous retinoic acid in liver of adult rats. *Arch Biochem Biophys* **391**: 8-15.
99. Li, Y., O. Monroig, L. Zhang, S. Wang, X. Zheng, J. R. Dick, C. You, and D. R. Tocher. 2010. Vertebrate fatty acyl desaturase with Δ4 activity. *Proc Natl Acad Sci U S A* **107**: 16840-16845.
100. Shanklin, J., E. Whittle, and B. G. Fox. 1994. Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. *Biochemistry* **33**: 12787-12794.
101. Paton, C. M., and J. M. Ntambi. 2009. Biochemical and physiological function of stearoyl-CoA desaturase. *Am J Physiol Endocrinol Metab* **297**: E28-37.
102. Tripodi, K. E., L. V. Buttigliero, S. G. Altabe, and A. D. Uttaro. 2006. Functional characterization of front-end desaturases from trypanosomatids depicts the first polyunsaturated fatty acid biosynthetic pathway from a parasitic protozoan. *FEBS J* **273**: 271-280.
103. Meyer, A., P. Cirpus, C. Ott, R. Schlecker, U. Zahringer, and E. Heinz. 2003. Biosynthesis of docosahexaenoic acid in Euglena gracilis: biochemical and molecular evidence for the involvement of a Delta4-fatty acyl group desaturase. *Biochemistry* **42**: 9779-9788.
104. Tonon, T., D. Harvey, T. R. Larson, and I. A. Graham. 2003. Identification of a very long chain polyunsaturated fatty acid Δ4-desaturase from the microalga Pavlova lutheri. *FEBS Lett* **553**: 440-444.
105. Qiu, X., H. Hong, and S. L. MacKenzie. 2001. Identification of a Δ4 fatty acid desaturase from Thraustochytrium sp. involved in the biosynthesis of docosahexanoic acid by heterologous expression in Saccharomyces cerevisiae and Brassica juncea. *J Biol Chem* **276**: 31561-31566.
106. Cook, H. W., D. M. Byers, F. B. Palmer, M. W. Spence, H. Rakoff, S. M. Duval, and E. A. Emken. 1991. Alternate pathways in the desaturation and chain elongation of linolenic acid, 18:3(n-3), in cultured glioma cells. *J Lipid Res* **32**: 1265-1273.
107. Schenck, P. A., H. Rakoff, and E. A. Emken. 1996. Δ8 desaturation in vivo of deuterated eicosatrienoic acid by mouse liver. *Lipids* **31**: 593-600.
108. Brenner, R. R., and R. O. Peluffo. 1966. Effect of saturated and unsaturated fatty acids on the desaturation in vitro of palmitic, stearic, oleic, linoleic, and linolenic acids. *J Biol Chem* **241**: 5213-5219.
109. Mathers, L., and M. J. Bailey. 1975. Enzyme deletions and essential fatty acid metabolism in cultured cells. *J Biol Chem* **250**: 1152-1153.
110. Goyens, P. L., and R. P. Mensink. 2005. The dietary alpha-linolenic acid to linoleic acid ratio does not affect the serum lipoprotein profile in humans. *J Nutr* **135**: 2799-2804.
111. Reddy, A. S., M. L. Nuccio, L. M. Gross, and T. L. Thomas. 1993. Isolation of a Δ6-desaturase gene from the cyanobacterium Synechocystis sp. strain PCC 6803 by gain-of-function expression in Anabaena sp.strain PCC 7120. *Plant Mol Biol* **22**: 293-300.

112. Sayanova, O., M. A. Smith, P. Lapinskas, A. K. Stobart, G. Dobson, W. W. Christie, P. R. Shewry, and J. A. Napier. 1997. Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of Δ6-desaturated fatty acids in transgenic tobacco. *Proc Natl Acad Sci U S A* **94**: 4211-4216.
113. Napier, J. A., S. J. Hey, D. J. Lacey, and P. R. Shewry. 1998. Identification of a Caenorhabditis elegans Δ6-fatty-acid-desaturase by heterologous expression in Saccharomyces cerevisiae. *Biochem J* **330**: 611-614.
114. Michaelson, L. V., J. A. Napier, M. Lewis, G. Griffiths, C. M. Lazarus, and A. K. Stobart. 1998. Functional identification of a fatty acid Δ5 desaturase gene from Caenorhabditis elegans. *FEBS Lett* **439**: 215-218.
115. Michaelson, L. V., C. M. Lazarus, G. Griffiths, J. A. Napier, and A. K. Stobart. 1998. Isolation of a Δ5-fatty acid desaturase gene from Mortierella alpina. *J Biol Chem* **273**: 19055-19059.
116. Knutzon, D. S., J. M. Thurmond, Y. S. Huang, S. Chaudhary, E. G. Bobik, Jr., G. M. Chan, S. J. Kirchner, and P. Mukerji. 1998. Identification of Δ5-desaturase from Mortierella alpina by heterologous expression in Bakers' yeast and canola. *J Biol Chem* **273**: 29360-29366.
117. Leonard, A. E., E. G. Bobik, J. Dorado, P. E. Kroeger, L. T. Chuang, J. M. Thurmond, J. M. Parker-Barnes, T. Das, Y. S. Huang, and P. Mukerji. 2000. Cloning of a human cDNA encoding a novel enzyme involved in the elongation of long-chain polyunsaturated fatty acids. *Biochem J* **350 Pt 3**: 765-770.
118. Zheng, X., D. Tocher, C. Dickson, J. Bell, and A. Teale. 2005. Highly unsaturated fatty acid synthesis in vertebrates: New insights with the cloning and characterization of a Δ6 desaturase of Atlantic salmon. *Lipids* **40**: 13-24.
119. Hastings, N., M. Agaba, D. R. Tocher, M. J. Leaver, J. R. Dick, J. R. Sargent, and A. J. Teale. 2001. A vertebrate fatty acid desaturase with Δ5 and Δ6 activities. *Proc Natl Acad Sci U S A* **98**: 14304-14309.
120. Seiliez, I., S. Panserat, S. Kaushik, and P. Bergot. 2001. Cloning, tissue distribution and nutritional regulation of a Δ6-desaturase-like enzyme in rainbow trout. *Comp Biochem Physiol B* **130**: 83-93.
121. Seiliez, I., S. Panserat, G. Corraze, S. Kaushik, and P. Bergot. 2003. Cloning and nutritional regulation of a Δ6-desaturase-like enzyme in the marine teleost gilthead seabream (Sparus aurata). *Comp Biochem Physiol B* **135**: 449-460.
122. Zheng, X., Z. Ding, Y. Xu, O. Monroig, S. Morais, and D. R. Tocher. 2009. Physiological roles of fatty acyl desaturases and elongases in marine fish: Characterisation of cDNAs of fatty acyl Δ6 desaturase and elovl5 elongase of cobia (Rachycentron canadum). *Aquaculture* **290**: 122-131.
123. Tocher, D. R., X. Zheng, C. Schlechtriem, N. Hastings, J. R. Dick, and A. J. Teale. 2006. Highly unsaturated fatty acid synthesis in marine fish: cloning, functional characterization, and nutritional regulation of fatty acyl Δ6 desaturase of Atlantic cod (Gadus morhua L.). *Lipids* **41**: 1003-1016.
124. Mohd-Yusof, N. Y., O. Monroig, A. Mohd-Adnan, K. L. Wan, and D. R. Tocher. 2010. Investigation of highly unsaturated fatty acid metabolism in the Asian sea bass, Lates calcarifer. *Fish Physiol Biochem* **36**: 827-843.
125. Hastings, N., M. K. Agaba, D. R. Tocher, X. Zheng, C. A. Dickson, J. R. Dick, and A. J. Teale. 2004. Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs involved in the production of eicosapentaenoic and docosahexaenoic acids from alpha-linolenic acid in Atlantic salmon (Salmo salar). *Mar Biotechnol (NY)* **6**: 463-474.
126. Stroud, C. K., T. Y. Nara, M. Roqueta-Rivera, E. C. Radlowski, P. Lawrence, Y. Zhang, B. H. Cho, M. Segre, R. A. Hess, J. T. Brenna, W. M. Haschek, and M. T. Nakamura. 2009. Disruption of FADS2 gene in mice impairs male reproduction and causes dermal and intestinal ulceration. *J Lipid Res* **50**: 1870-1880.
127. D'Andrea, S., H. Guillou, S. Jan, D. Catheline, J. N. Thibault, M. Bouriel, V. Rioux, and P. Legrand. 2002. The same rat Δ6-desaturase not only acts on 18- but also on 24-carbon fatty acids in very-long-chain polyunsaturated fatty acid biosynthesis. *Biochem J* **364**: 49-55.
128. Geiger, M., B. S. Mohammed, S. Sankarappa, and H. Sprecher. 1993. Studies to determine if rat liver contains chain-length-specific acyl-CoA 6-desaturases. *Biochim Biophys Acta* **1170**: 137-142.

129. Cinti, D. L., L. Cook, M. N. Nagi, and S. K. Suneja. 1992. The fatty acid chain elongation system of mammalian endoplasmic reticulum. *Prog Lipid Res* **31**: 1-51.
130. Jakobsson, A., R. Westerberg, and A. Jacobsson. 2006. Fatty acid elongases in mammals: Their regulation and roles in metabolism. *Prog Lipid Res* **45**: 237-249.
131. Leonard, A. E., S. L. Pereira, H. Sprecher, and Y. S. Huang. 2004. Elongation of long-chain fatty acids. *Prog Lipid Res* **43**: 36-54.
132. Nagi, M. N., L. Cook, D. Ghesquier, and D. L. Cinti. 1986. Site of inhibition of rat liver microsomal fatty acid chain elongation system by dec-2-ynoyl coenzyme A. Possible mechanism of inhibition. *J Biol Chem* **261**: 13598-13605.
133. Moon, Y. A., N. A. Shah, S. Mohapatra, J. A. Warrington, and J. D. Horton. 2001. Identification of a mammalian long chain fatty acyl elongase regulated by sterol regulatory element-binding proteins. *J Biol Chem* **276**: 45358-45366.
134. Tvrdik, P., A. Asadi, L. P. Kozak, J. Nedergaard, B. Cannon, and A. Jacobsson. 1997. Cig30, a mouse member of a novel membrane protein gene family, is involved in the recruitment of brown adipose tissue. *J Biol Chem* **272**: 31738-31746.
135. Tvrdik, P., R. Westerberg, S. Silve, A. Asadi, A. Jakobsson, B. Cannon, G. Loison, and A. Jacobsson. 2000. Role of a new mammalian gene family in the biosynthesis of very long chain fatty acids and sphingolipids. *J Cell Biol* **149**: 707-718.
136. Inagaki, K., T. Aki, Y. Fukuda, S. Kawamoto, S. Shigeta, K. Ono, and O. Suzuki. 2002. Identification and expression of a rat fatty acid elongase involved in the biosynthesis of C18 fatty acids. *Biosci Biotechnol Biochem* **66**: 613-621.
137. Leonard, A., B. Kelder, E. Bobik, L.-T. Chuang, C. Lewis, J. Kopchick, P. Mukerji, and Y.-S. Huang. 2002. Identification and expression of mammalian long-chain PUFA elongation enzymes. *Lipids* **37**: 733-740.
138. Tamura, K., A. Makino, F. Hullin-Matsuda, T. Kobayashi, M. Furihata, S. Chung, S. Ashida, T. Miki, T. Fujioka, T. Shuin, Y. Nakamura, and H. Nakagawa. 2009. Novel lipogenic enzyme ELOVL7 is involved in prostate cancer growth through saturated long-chain fatty acid metabolism. *Cancer Res* **69**: 8133-8140.
139. Agbaga, M. P., R. S. Brush, M. N. Mandal, K. Henry, M. H. Elliott, and R. E. Anderson. 2008. Role of Stargardt-3 macular dystrophy protein (ELOVL4) in the biosynthesis of very long chain fatty acids. *Proc Natl Acad Sci U S A* **105**: 12843-12848.
140. Agbaga, M. P., M. N. Mandal, and R. E. Anderson. 2010. Retinal very long-chain PUFAs: new insights from studies on ELOVL4 protein. *J Lipid Res* **51**: 1624-1642.
141. Agaba, M., D. R. Tocher, C. A. Dickson, J. R. Dick, and A. J. Teale. 2004. Zebrafish cDNA encoding multifunctional fatty acid elongase involved in production of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. *Mar Biotechnol (NY)* **6**: 251-261.
142. Morais, S., O. Monroig, X. Zheng, M. J. Leaver, and D. R. Tocher. 2009. Highly unsaturated fatty acid synthesis in Atlantic salmon: characterization of ELOVL5- and ELOVL2-like elongases. *Mar Biotechnol (NY)* **11**: 627-639.
143. Meyer, A., H. Kirsch, F. Domergue, A. Abbadi, P. Sperling, J. Bauer, P. Cirpus, T. K. Zank, H. Moreau, T. J. Roscoe, U. Zahringer, and E. Heinz. 2004. Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. *J Lipid Res* **45**: 1899-1909.
144. Monroig, Ó., J. Rotllant, E. Sánchez, J. M. Cerdá-Reverter, and D. R. Tocher. 2009. Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish Danio rerio early embryogenesis. *Biochim Biophys Acta - Molecular and Cell Biology of Lipids* **1791**: 1093-1101.
145. Moon, Y. A., R. E. Hammer, and J. D. Horton. 2009. Deletion of ELOVL5 leads to fatty liver through activation of SREBP-1c in mice. *J Lipid Res* **50**: 412-423.
146. Wang, Y., D. Botolin, B. Christian, J. Busik, J. Xu, and D. B. Jump. 2005. Tissue-specific, nutritional, and developmental regulation of rat fatty acid elongases. *J Lipid Res* **46**: 706-715.
147. Xiang, M., M. A. Rahman, H. Ai, X. Li, and L. S. Harbige. 2006. Diet and gene expression: Δ5 and Δ6 desaturases in healthy Chinese and European subjects. *Ann Nutr Metab* **50**: 492-498.
148. Izquierdo, M., L. Robaina, E. Juárez-Carrillo, V. Oliva, C. Hernández-Cruz, and J. Afonso. 2007. Regulation of growth, fatty acid composition and delta 6 desaturase expression by dietary lipids in gilthead seabream larvae (Sparus aurata). *Fish Physiol Biochem* **34**: 117-127.

149. Tocher, D. R., M. Agaba, N. Hastings, J. G. Bell, J. R. Dick, and A. J. Teale. 2001. Nutritional regulation of hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition in zebrafish (Danio rerio) and tilapia (Oreochromis niloticus). *Fish Physiol Biochem* **24**: 309-320.
150. Extier, A., B. Langelier, M. H. Perruchot, P. Guesnet, P. P. Van Veldhoven, M. Lavialle, and J. M. Alessandri. 2010. Gender affects liver desaturase expression in a rat model of n-3 fatty acid repletion. *J Nutr Biochem* **21**: 180-187.
151. Giltay, E. J., L. J. G. Gooren, A. W. F. T. Toorians, M. B. Katan, and P. L. Zock. 2004. Docosahexaenoic acid concentrations are higher in women than in men because of estrogenic effects. *Am J Clin Nutr* **80**: 1167-1174.
152. Giltay, E. J., E. J. J. Duschek, M. B. Katan, P. L. Zock, S. J. Neele, and J. C. Netelenbos. 2004. Raloxifene and hormone replacement therapy increase arachidonic acid and docosahexaenoic acid levels in postmenopausal women. *J Endocrinol* **182**: 399-408.
153. Mater, M. K., A. P. Thelen, D. A. Pan, and D. B. Jump. 1999. Sterol response element-binding protein 1c (SREBP1c) is involved in the polyunsaturated fatty acid suppression of hepatic S14 gene transcription. *J Biol Chem* **274**: 32725-32732.
154. Miller, M. R., A. R. Bridle, P. D. Nichols, and C. G. Carter. 2008. Increased elongase and desaturase gene expression with stearidonic acid enriched diet does not enhance long-chain (n-3) content of seawater Atlantic salmon (Salmo salar L.). *J Nutr* **138**: 2179-2185.
155. Woods, V. B., and A. M. Fearon. 2009. Dietary sources of unsaturated fatty acids for animals and their transfer into meat, milk and eggs: A review. *Livest Sci* **126**: 1-20.
156. Bourre, J. M. 2005. Effect of increasing the omega-3 fatty acid in the diets of animals on the animal products consumed by humans. *Med Sci (Paris)* **21**: 773-779.
157. Davis, B. C., and P. M. Kris-Etherton. 2003. Achieving optimal essential fatty acid status in vegetarians: current knowledge and practical implications. *Am J Clin Nutr* **78**: 640S-646S.
158. Lunn, J., and H. E. Theobald. 2006. The health effects of dietary unsaturated fatty acids. *Nutr Bull* **31**: 178-224.
159. Howe, P., B. Meyer, S. Record, and K. Baghurst. 2006. Dietary intake of long-chain omega-3 polyunsaturated fatty acids: contribution of meat sources. *Nutrition* **22**: 47-53.
160. Borowitzka, M. 1997. Microalgae for aquaculture: Opportunities and constraints. *J Appl Phycol* **9**: 393-401.
161. Spolaore, P., C. Joannis-Cassan, E. Duran, and A. Isambert. 2006. Commercial applications of microalgae. *J Biosci Bioeng* **101**: 87-96.
162. Naylor, R. L., R. J. Goldburg, J. H. Primavera, N. Kautsky, M. C. M. Beveridge, J. Clay, C. Folke, J. Lubchenco, H. Mooney, and M. Troell. 2000. Effect of aquaculture on world fish supplies. *Nature* **405**: 1017-1024.
163. Tacon, A. G. J., and M. Metian. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture* **285**: 146-158.
164. Ursin, V. M. 2003. Modification of plant lipids for human health: development of functional land-based omega-3 fatty acids. *J Nutr* **133**: 4271-4274.
165. Chong, E. W. T., A. J. Sinclair, and R. H. Guymer. 2006. Facts on fats. *Clin Exp Ophthalmol* **34**: 464-471.
166. Lee, D.-S., B.-S. Noh, S.-Y. Bae, and K. Kim. 1998. Characterization of fatty acids composition in vegetable oils by gas chromatography and chemometrics. *Anal Chim Acta* **358**: 163-175.
167. Moser, B. R., and S. F. Vaughn. 2010. Evaluation of alkyl esters from Camelina sativa oil as biodiesel and as blend components in ultra low-sulfur diesel fuel. *Bioresource Technol* **101**: 646-653.
168. Rao, S., M. Abdel-Reheem, R. Bhella, C. McCracken, and D. Hildebrand. 2008. Characteristics of high α-linolenic acid accumulation in seed oils. *Lipids* **43**: 749-755.
169. Giada Mde, L. 2010. Food applications for flaxseed and its components: products and processing. *Recent Pat Food Nutr Agric* **2**: 181-186.
170. Coates, W., and R. Ayerza. 1996. Production potential of chia in northwestern. *Argentina Ind Crop Prod* **5**: 229-233.
171. Dubois, V., S. Breton, M. Linder, J. Fanni, and M. Parmentier. 2007. Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. *Eur J Lipid Sci Technol* **109**: 710-732.

172. Karvonen, H. M., A. Aro, N. S. Tapola, I. Salminen, M. I. Uusitupa, and E. S. Sarkkinen. 2002. Effect of alpha-linolenic acid-rich Camelina sativa oil on serum fatty acid composition and serum lipids in hypercholesterolemic subjects. *Metabolism* **51**: 1253-1260.
173. Simopoulos, A. P. 2010. Nutrigenetics/Nutrigenomics. *Annu Rev Public Health* **31**: 53-68.
174. Rivers, J. P., A. G. Hassam, and C. Alderson. 1976. The absence of Δ6-desaturase activity in the cat. *Proc Nutr Soc* **35**: 67A-68A.
175. Rivers, J. P., A. J. Sinclair, and M. A. Craqford. 1975. Inability of the cat to desaturate essential fatty acids. *Nature* **258**: 171-173.
176. de Souza, N. E., M. Matsushita, C. C. de Oliveira, M. R. B. Franco, and J. V. Visentainer. 2007. Manipulation of fatty acid composition of Nile tilapia (Oreochromis niloticus) fillets with flaxseed oil. *J Sci Food Agric* **87**: 1677-1681.
177. Bessa, R. J. B., S. P. Alves, E. Jerónimo, C. M. Alfaia, J. A. M. Prates, and J. Santos-Silva. 2007. Effect of lipid supplements on ruminal biohydrogenation intermediates and muscle fatty acids in lambs. *Eur J Lipid Sci Technol* **109**: 868-878.
178. González-Esquerra, R., and S. Leeson. 2001. Alternatives for enrichment of eggs and chicken meat with omega-3 fatty acids. *Can J Anim Sci* **81**: 12.
179. Rey, A. I., C. J. Lopez-Bote, J. P. Kerry, P. B. Lynch, D. J. Buckley, and P. A. Morrissey. 2004. Modification of lipid composition and oxidation in porcine muscle and muscle microsomes as affected by dietary supplementation of n-3 with either n-9 or n-6 fatty acids and alpha-tocopheryl acetate. *Anim Feed Sci Technol* **113**: 223-238.
180. Lopez-Ferrer, S., M. D. Baucells, A. C. Barroeta, J. Galobart, and M. A. Grashorn. 2001. n-3 enrichment of chicken meat. 2. Use of precursors of long-chain polyunsaturated fatty acids: linseed oil. *Poult Sci* **80**: 753-761.
181. Rymer, C., G. F. Hartnell, and D. I. Givens. 2010. The effect of feeding modified soyabean oil enriched with C18 : 4 n-3 to broilers on the deposition of n-3 fatty acids in chicken meat. *Br J Nutr*: 1-13.
182. Specht-Overholt, S., J. R. Romans, M. J. Marchello, R. S. Izard, M. G. Crews, D. M. Simon, W. J. Costello, and P. D. Evenson. 1997. Fatty acid composition of commercially manufactured omega-3 enriched pork products, haddock, and mackerel. *J Anim Sci* **75**: 2335-2343.
183. Guillevic, M., M. Kouba, and J. Mourot. 2009. Effect of a linseed diet on lipid composition, lipid peroxidation and consumer evaluation of French fresh and cooked pork meats. *Meat Sci* **81**: 612-618.
184. Nuernberg, K., K. Fischer, G. Nuernberg, U. Kuechenmeister, D. Klosowska, G. Eliminowska-Wenda, I. Fiedler, and K. Ender. 2005. Effects of dietary olive and linseed oil on lipid composition, meat quality, sensory characteristics and muscle structure in pigs. *Meat Sci* **70**: 63-74.
185. Raes, K., S. De Smet, and D. Demeyer. 2004. Effect of dietary fatty acids on incorporation of long chain polyunsaturated fatty acids and conjugated linoleic acid in lamb, beef and pork meat: a review. *Anim Feed Sci Technol* **113**: 199-221.
186. Saadiah, I., A. Abol-Munafi, and C. Che Utama. 2010. Replacement of fishmeal in cobia (Rachycentron canadum) diets using poultry by-product meal. *Aquacult Int*: 1-12.
187. Craig, S. R., M. H. Schwarz, and E. McLean. 2006. Juvenile cobia (Rachycentron canadum) can utilize a wide range of protein and lipid levels without impacts on production characteristics. *Aquaculture* **261**: 384-391.
188. Almaida-Pagán, P. F., M. D. Hernández, B. García García, J. A. Madrid, J. De Costa, and P. Mendiola. 2007. Effects of total replacement of fish oil by vegetable oils on n-3 and n-6 polyunsaturated fatty acid desaturation and elongation in sharpsnout seabream (Diplodus puntazzo) hepatocytes and enterocytes. *Aquaculture* **272**: 589-598.
189. Jobling, M., and E. Å. Bendiksen. 2003. Dietary lipids and temperature interact to influence tissue fatty acid compositions of Atlantic salmon, Salmo salar L., parr. *Aquacult Res* **34**: 1423-1441.
190. Williams, K. C., B. D. Paterson, C. G. Barlow, A. Ford, and R. Roberts. 2003. Potential of meat meal to replace fish meal in extruded dry diets for barramundi, Lates calcarifer (Bloch). II. Organoleptic characteristics and fatty acid composition. *Aquacult Res* **34**: 33-42.
191. Tocher, D., and C. Ghioni. 1999. Fatty acid metabolism in marine fish: Low activity of fatty acyl Δ5 desaturation in gilthead sea bream (Sparus aurata) cells. *Lipids* **34**: 433-440.

192. Izquierdo, M. S., A. Obach, L. Arantzamendi, D. Montero, L. Robaina, and G. Rosenlund. 2003. Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. *Aquacult Nutr* **9**: 397-407.
193. Bell, J. G., D. R. Tocher, R. J. Henderson, J. R. Dick, and V. O. Crampton. 2003. Altered fatty acid compositions in atlantic salmon (Salmo salar) fed diets containing linseed and rapeseed oils can be partially restored by a subsequent fish oil finishing diet. *J Nutr* **133**: 2793-2801.
194. Lattka, E., T. Illig, B. Koletzko, and J. Heinrich. 2010. Genetic variants of the FADS1 FADS2 gene cluster as related to essential fatty acid metabolism. *Curr Opin Lipidol* **21**: 64-69.
195. Simopoulos, A. P. 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother* **56**: 365-379.
196. Laaksonen, D. E., K. Nyyssonen, L. Niskanen, T. H. Rissanen, and J. T. Salonen. 2005. Prediction of cardiovascular mortality in middle-aged men by dietary and serum linoleic and polyunsaturated fatty acids. *Arch Intern Med* **165**: 193-199.
197. Harris, W. S., D. Mozaffarian, E. Rimm, P. Kris-Etherton, L. L. Rudel, L. J. Appel, M. M. Engler, M. B. Engler, and F. Sacks. 2009. Omega-6 fatty acids and risk for cardiovascular disease: a science advisory from the American Heart Association Nutrition Subcommittee of the Council on Nutrition, Physical Activity, and Metabolism; Council on Cardiovascular Nursing; and Council on Epidemiology and Prevention. *Circulation* **119**: 902-907.
198. Bourre, J. M., M. Francois, A. Youyou, O. Dumont, M. Piciotti, G. Pascal, and G. Durand. 1989. The effects of dietary alpha-linolenic acid on the composition of nerve membranes, enzymatic activity, amplitude of electrophysiological parameters, resistance to poisons and performance of learning tasks in rats. *J Nutr* **119**: 1880-1892.
199. Akabas, S. R., and R. J. Deckelbaum. 2006. Summary of a workshop on n-3 fatty acids: current status of recommendations and future directions. *Am J Clin Nutr* **83**: 1536S-1538S.
200. Harris, W. S., and C. von Schacky. 2004. The Omega-3 Index: a new risk factor for death from coronary heart disease? *Prev Med* **39**: 212-220.
201. International Society for the Study of Fatty Acids and Lipids (ISSFAL). 2004. Report of the sub-committee on recommendations for intake of polyunsaturated fatty acids in healthy adults. http://www.issfal.org.uk/pufa-recommendations.html.
202. Krauss, R. M., R. H. Eckel, B. Howard, L. J. Appel, S. R. Daniels, R. J. Deckelbaum, J. W. Erdman, Jr., P. Kris-Etherton, I. J. Goldberg, T. A. Kotchen, A. H. Lichtenstein, W. E. Mitch, R. Mullis, K. Robinson, J. Wylie-Rosett, S. St Jeor, J. Suttie, D. L. Tribble, and T. L. Bazzarre. 2000. AHA Dietary Guidelines: revision 2000: A statement for healthcare professionals from the Nutrition Committee of the American Heart Association. *Circulation* **102**: 2284-2299.
203. Institute of Medicine (IOM). 2005. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (macronutrients) (Sep 2002). The National Academies Press (Washington).
204. Kris-Etherton, P. M., W. S. Harris, and L. J. Appel. 2003. Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association. *Arterioscler Thromb Vasc Biol* **23**: 151-152.
205. International Society for the Study of Fatty Acids and Lipids (ISSFAL). 2004. Recommendations for intake of polyunsaturated fatty acids in healthy adults. www.issfal.org.uk.
206. Australian National Health and Medical Research Council (NHMRC) and the New Zealand Ministry of Health (MoH). 2006. Nutrient reference values for Australia and New Zealand including recommended dietary intakes. Australian Government Publishing Service.
207. FAO/WHO. 2008. Expert consultation on fats and fatty acids in human: Interim summary of conclusions and dietary recommendations on total fat and fatty acids. Nutrition, WHO HQ (Switzerland).
208. Iacono, J. M., and R. M. Dougherty. 1993. Effects of polyunsaturated fats on blood pressure. *Annu Rev Nutr* **13**: 243-260.
209. Bell, J. G., and J. R. Sargent. 2003. Arachidonic acid in aquaculture feeds: current status and future opportunities. *Aquaculture* **218**: 491-499.
210. Cowey, C. B., J. M. Owen, J. W. Adron, and C. Middleton. 1976. Studies on the nutrition of marine flatfish. The effect of different dietary fatty acids on the growth and fatty acid composition of turbot (Scophthalmus maximus). *Br J Nutr* **36**: 479-486.

211. Sargent, J., G. Bell, L. McEvoy, D. Tocher, and A. Estevez. 1999. Recent developments in the essential fatty acid nutrition of fish. *Aquaculture* **177**: 191-199.
212. Sargent, J. R., L. A. McEvoy, and J. G. Bell. 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture* **155**: 117-127.
213. Bell, J. G., J. McEvoy, D. R. Tocher, F. McGhee, P. J. Campbell, and J. R. Sargent. 2001. Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (Salmo salar) affects tissue lipid compositions and hepatocyte fatty acid metabolism. *J Nutr* **131**: 1535-1543.
214. Menoyo, D., C. J. Lopez-Bote, A. Obach, and J. M. Bautista. 2005. Effect of dietary fish oil substitution with linseed oil on the performance, tissue fatty acid profile, metabolism, and oxidative stability of Atlantic salmon. *J Anim Sci* **83**: 2853-2862.
215. Tocher, D., J. Fonseca-Madrigal, J. Bell, J. Dick, R. Henderson, and J. Sargent. 2002. Effects of diets containing linseed oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes in Atlantic salmon (Salmo salar). *Fish Physiol Biochem* **26**: 157-170.
216. de Souza, N. E., F. B. Stevanato, E. E. Garcia, J. E. L. Visentainer, R. F. Zara, and J. V. Visentainer. 2008. Supplemental dietary flaxseed oil affects both neutral and phospholipid fatty acids in cultured tilapia. *Eur J Lipid Sci Technol* **110**: 707-713.
217. Tocher, D. R., J. G. Bell, P. MacGlaughlin, F. McGhee, and J. R. Dick. 2001. Hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition of liver in salmonids: effects of dietary vegetable oil. *Comp Biochem Physiol B* **130**: 257-270.
218. Bligh, E. G., and W. J. Dyer. 1959. A rapid method for total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911-917.
219. Broekhuyse, R. M. 1974. Improved lipid extraction of erythrocytes. *Clin Chim Acta* **51**: 341-343.
220. Muller, P. Y., H. Janovjak, A. R. Miserez, and Z. Dobbie. 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* **32**: 1372-1374, 1376, 1378-1379.
221. Simon, P. 2003. Q-Gene: processing quantitative real-time RT-PCR data. *Bioinformatics* **19**: 1439-1440.
222. Wong, M. L., and J. F. Medrano. 2005. Real-time PCR for mRNA quantitation. *Biotechniques* **39**: 75-85.
223. Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J. A. Leunissen. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res* **35**: W71-74.
224. Giniger, E., S. M. Varnum, and M. Ptashne. 1985. Specific DNA binding of GAL4, a positive regulatory protein of yeast. *Cell* **40**: 767-774.
225. Sherman, F. 1991. [1] Getting started with yeast. *In* Methods in Enzymology. G. Christine and R. F. Gerald, editors. Academic Press. 3-21.
226. Jump, D. B., and S. D. Clarke. 1999. Regulation of gene expression by dietary fat. *Annu Rev Nutr* **19**: 63-90.
227. Kersten, S. 2001. Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep* **2**: 282-286.
228. Sul, H. S., and D. Wang. 1998. Nutritional and hormonal regulation of enzymes in fat synthesis: studies of fatty acid synthase and mitochondrial glycerol-3-phosphate acyltransferase gene transcription. *Annu Rev Nutr* **18**: 331-351.
229. Raz, A., N. Kamin-Belsky, F. Przedecki, and M. Obukowicz. 1998. Dietary fish oil inhibits Δ6-desaturase activity in vivo. *J Am Oil Chem Soc* **75**: 241-245.
230. Beck, F., S. Plummer, P. V. Senior, S. Byrne, S. Green, and W. J. Brammar. 1992. The ontogeny of peroxisome-proliferator-activated receptor gene expression in the mouse and rat. *Proc Biol Sci* **247**: 83-87.
231. Keller, H., C. Dreyer, J. Medin, A. Mahfoudi, K. Ozato, and W. Wahli. 1993. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc Natl Acad Sci U S A* **90**: 2160-2164.
232. Brown, M. S., and J. L. Goldstein. 1997. The SREBP Pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* **89**: 331-340.
233. Tang, C., H. P. Cho, M. T. Nakamura, and S. D. Clarke. 2003. Regulation of human Δ-6 desaturase gene transcription: identification of a functional direct repeat-1 element. *J Lipid Res* **44**: 686-695.

234. Xu, J., M. T. Nakamura, H. P. Cho, and S. D. Clarke. 1999. Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. *J Biol Chem* **274**: 23577-23583.
235. Punchard, N. A., A. T. Green, J. G. Ll. Mullins, and R. P. H. Thompson. 2000. Analysis of the intestinal absorption of essential fatty acids in vivo in the rat. *Prostaglandins Leukot Essent Fatty Acids* **62**: 27-33.
236. Li, Y., T. Y. Nara, and M. T. Nakamura. 2005. Peroxisome proliferator-activated receptor alpha is required for feedback regulation of highly unsaturated fatty acid synthesis. *J Lipid Res* **46**: 2432-2440.
237. Hansen, A. E., M. E. Haggard, A. N. Boelsche, D. J. Adam, and H. F. Wiese. 1958. Essential fatty acids in infant nutrition. III. Clinical manifestations of linoleic acid deficiency. *J Nutr* **66**: 565-576.
238. Holman, R. T. 1998. The slow discovery of the importance of omega 3 essential fatty acids in human health. *J Nutr* **128**: 427S-433S.
239. Rahm, J. J., and R. T. Holman. 1964. Effect of linoleic acid upon the metabolism of linolenic acid. *J Nutr* **84**: 15-19.
240. Johnson, R. R., P. Bouchard, J. Tinoco, and R. L. Lyman. 1967. Fatty acid changes in liver and plasma lipid fractions after safflower oil was fed to rats deficient in essential fatty acids. *Biochem J* **105**: 343-350.
241. Eberlé, D., B. Hegarty, P. Bossard, P. Ferré, and F. Foufelle. 2004. SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie* **86**: 839-848.
242. Kersten, S., J. Seydoux, J. M. Peters, F. J. Gonzalez, B. Desvergne, and W. Wahli. 1999. Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* **103**: 1489-1498.
243. Kim, H. J., M. Takahashi, and O. Ezaki. 1999. Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. *J Biol Chem* **274**: 25892-25898.
244. Patsouris, D., J. K. Reddy, M. Muller, and S. Kersten. 2006. Peroxisome proliferator-activated receptor alpha mediates the effects of high-fat diet on hepatic gene expression. *Endocrinology* **147**: 1508-1516.
245. Vega, R. B., J. M. Huss, and D. P. Kelly. 2000. The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* **20**: 1868-1876.
246. Nakatani, T., H. J. Kim, Y. Kaburagi, K. Yasuda, and O. Ezaki. 2003. A low fish oil inhibits SREBP-1 proteolytic cascade, while a high-fish-oil feeding decreases SREBP-1 mRNA in mice liver: relationship to anti-obesity. *J Lipid Res* **44**: 369-379.
247. Knight, B. L., A. Hebbachi, D. Hauton, A. M. Brown, D. Wiggins, D. D. Patel, and G. F. Gibbons. 2005. A role for PPARalpha in the control of SREBP activity and lipid synthesis in the liver. *Biochem J* **389**: 413-421.
248. Igarashi, M., K. Ma, L. Chang, J. M. Bell, and S. I. Rapoport. 2007. Dietary n-3 PUFA deprivation for 15 weeks upregulates elongase and desaturase expression in rat liver but not brain. *J Lipid Res* **48**: 2463-2470.
249. Howe, P. R., J. A. Downing, B. F. Grenyer, E. M. Grigonis-Deane, and W. L. Bryden. 2002. Tuna fishmeal as a source of DHA for n-3 PUFA enrichment of pork, chicken, and eggs. *Lipids* **37**: 1067-1076.
250. Sioutis, S., A. M. Coates, J. D. Buckley, T. W. Murphy, H. A. Channon, and P. R. C. Howe. 2008. N-3 enrichment of pork with fishmeal: Effects on production and consumer acceptability. *Eur J Lipid Sci Technol* **110**: 701-706.
251. FAO. 2008. State of world fisheries and aquaculture *In*. Food and agriculture organization of the United Nations, Rome 196.
252. Tidwell, J. H., and G. L. Allan. 2001. Fish as food: aquaculture's contribution. Ecological and economic impacts and contributions of fish farming and capture fisheries. *EMBO Rep* **2**: 958-963.

253. Bostock, J., B. McAndrew, R. Richards, K. Jauncey, T. Telfer, K. Lorenzen, D. Little, L. Ross, N. Handisyde, I. Gatward, and R. Corner. 2010. Aquaculture: global status and trends. *Philos Trans R Soc Lond B Biol Sci* **365**: 2897-2912.
254. Francis, D. S., G. M. Turchini, P. L. Jones, and S. S. De Silva. 2007. Effects of fish oil substitution with a mix blend vegetable oil on nutrient digestibility in Murray cod, Maccullochella peelii peelii. *Aquaculture* **269**: 447-455.
255. Ghioni, C., D. R. Tocher, M. V. Bell, J. R. Dick, and J. R. Sargent. 1999. Low C18 to C20 fatty acid elongase activity and limited conversion of stearidonic acid, 18:4 (n-3), to eicosapentaenoic acid, 20:5(n-3), in a cell line from the turbot, Scophthalmus maximus. *Biochim Biophys Acta - Molecular and Cell Biology of Lipids* **1437**: 170-181.
256. Mackinnon, M. R. 1989. Status and potential of Australian Lates calcarifer culture., Tahiti.
257. Catacutan, M. R., and R. M. Coloso. 1995. Effect of dietary protein to energy ratios on growth, survival, and body composition of juvenile Asian seabass, Lates calcarifer. *Aquaculture* **131**: 125-133.
258. Williams, K. C., C. G. Barlow, L. J. Rodgers, and I. Ruscoe. 2003. Potential of meat meal to replace fish meal in extruded dry diets for barramundi, Lates calcarifer (Bloch). I. Growth performance. *Aquacult Res* **34**: 23-32.
259. Glencross, B. 2006. The nutritional management of barramundi, Lates calcarifer - a review. *Aquacult Nutr* **12**: 291-309.
260. Raso, S., and T. A. Anderson. 2003. Effects of dietary fish oil replacement on growth and carcass proximate composition of juvenile barramundi (Lates calcarifer). *Aquacult Res* **34**: 813-819.
261. Smith, D. M., B. J. Hunter, G. L. Allan, D. C. K. Roberts, M. A. Booth, and B. D. Glencross. 2004. Essential fatty acids in the diet of silver perch (Bidyanus bidyanus): effect of linolenic and linoleic acid on growth and survival. *Aquaculture* **236**: 377-390.
262. Mariotti, F., D. Tome, and P. P. Mirand. 2008. Converting nitrogen into protein--beyond 6.25 and Jones' factors. *Crit Rev Food Sci Nutr* **48**: 177-184.
263. Filby, A. L., and C. R. Tyler. 2007. Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish. *BMC Mol Biol* **8**: 10.
264. He, J. Q., A. J. Sandford, I. M. Wang, S. Stepaniants, D. A. Knight, A. Kicic, S. M. Stick, and P. D. Pare. 2008. Selection of housekeeping genes for real-time PCR in atopic human bronchial epithelial cells. *Eur Respir J* **32**: 755-762.
265. Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**: Research0034 (epub).
266. Tucker, J. W., M. R. Mackinnon, D. J. Russell, J. J. O'Brien, and E. Cazzola. 1988. Growth of juvenile barramundi (Lates calcarifer) on dry feeds. *Prog Fish-Cult* **50**: 81-85.
267. Catacutan, M. R., and R. M. Coloso. 1997. Growth of juvenile Asian seabass, Lates calcarifer, fed varying carbohydrate and lipid levels. *Aquaculture* **149**: 137-144.
268. Glencross, B., and N. Rutherford. 2011. A determination of the quantitative requirements for docosahexaenoic acid for juvenile barramundi (Lates calcarifer). *Aquacult Nutr* **17**: e536-e548.
269. Castell, J. D., R. O. Sinnhuber, D. J. Lee, and J. H. Wales. 1972. Essential fatty acids in the diet of rainbow trout (Salmo gairdneri): physiological symptoms of EFA deficiency. *J Nutr* **102**: 87-92.
270. Bell, M. V., R. J. Henderson, and J. R. Sargent. 1986. The role of polyunsaturated fatty acids in fish. *Comp Biochem Physiol B* **83**: 711-719.
271. Tocher, D. R., J. Carr, and J. R. Sargent. 1989. Polyunsaturated fatty acid metabolism in fish cells: differential metabolism of (n-3) and (n-6) series acids by cultured cells originating from a freshwater teleost fish and from a marine teleost fish. *Comp Biochem Physiol B* **94**: 367-374.
272. Buranapanidgit, J., M. Boonyaratpalin, T. Watanabe, T. Pechmanee, and R. Yashiro. 1988. Essential fatty acid requirement of juvenile seabass *Lates calcarifer*. *In* Technical paper National Institute of Coastal Aquaculture. Department of Fisheries, Thailand. 21 pp.
273. Buranapanidgit, J., M. Boonyaratpalin, T. Watanabe, T. Pechmanee, and R. Yashiro. 1989. Optimum level of ω3HUFA on juvenile seabass Lates calcarifer. *In* IDRC Fish Nutrition Project Annual Report. Department of Fisheries, Thailand. 23 pp.

274. Dhert, P., P. Lavens, M. Duray, and P. Sorgeloos. 1990. Improved larval survival at metamorphosis of Asian seabass (Lates calcarifer) using omega3-HUFA-enriched live food. *Aquaculture* **90**: 63-74.
275. Williams, K. C., C. G. Barlow, L. Rodgers, and C. Agcopra. 2006. Dietary composition manipulation to enhance the performance of juvenile barramundi (Lates calcarifer, Bloch) reared in cool water. *Aquacult Res* **37**: 914-927.
276. Tacon, A. G. 1996. Lipid nutritional pathology in farmed fish. *Arch Tierernahr* **49**: 33-39.
277. Ji, H., J. Li, and P. Liu. 2011. Regulation of growth performance and lipid metabolism by dietary n-3 highly unsaturated fatty acids in juvenile grass carp, Ctenopharyngodon idellus. *Comp Biochem Physiol B* **159**: 49-56.
278. Mourente, G., and J. G. Bell. 2006. Partial replacement of dietary fish oil with blends of vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass (Dicentrarchus labrax L.) over a long term growth study: effects on muscle and liver fatty acid composition and effectiveness of a fish oil finishing diet. *Comp Biochem Physiol B* **145**: 389-399.
279. Alhazzaa, R., A. R. Bridle, P. D. Nichols, and C. G. Carter. 2011. Replacing dietary fish oil with Echium oil enriched barramundi with C18 PUFA rather than long-chain PUFA. *Aquaculture* **312**: 162-171.
280. Geay, F., E. Santigosa I Culi, C. Corporeau, P. Boudry, Y. Dreano, L. Corcos, N. Bodin, M. Vandeputte, J. L. Zambonino-Infante, D. Mazurais, and C. L. Cahu. 2010. Regulation of FADS2 expression and activity in European sea bass (Dicentrarchus labrax, L.) fed a vegetable diet. *Comp Biochem Physiol B* **156**: 237-243.
281. González-Rovira, A., G. Mourente, X. Zheng, D. R. Tocher, and C. Pendón. 2009. Molecular and functional characterization and expression analysis of a Δ6 fatty acyl desaturase cDNA of European Sea Bass (Dicentrarchus labrax L.). *Aquaculture* **298**: 90-100.
282. Izquierdo, M., L. Robaina, E. Juárez-Carrillo, V. Oliva, C. Hernández-Cruz, and J. Afonso. 2008. Regulation of growth, fatty acid composition and Δ6 desaturase expression by dietary lipids in gilthead seabream larvae (Sparus aurata). *Fish Physiol Biochem* **34**: 117-127.
283. Schlechtriem, C., J. E. Bron, and D. R. Tocher. 2007. Inter-individual variation in total fatty acid compositions of flesh of Atlantic salmon smolts-fed diets containing fish oil or vegetable oil. *Aquacult Res* **38**: 1045-1055.
284. Innis, S. M. 2003. Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. *J Pediatr* **143**: 1-8.
285. McCann, J. C., and B. N. Ames. 2005. Is docosahexaenoic acid, an n-3 long-chain polyunsaturated fatty acid, required for development of normal brain function? An overview of evidence from cognitive and behavioral tests in humans and animals. *Am J Clin Nutr* **82**: 281-295.
286. Bell, M., R. Batty, J. Dick, K. Fretwell, J. Navarro, and J. Sargent. 1995. Dietary deficiency of docosahexaenoic acid impairs vision at low light intensities in juvenile herring (Clupea harengus L.). *Lipids* **30**: 443-449.
287. Rainuzzo, J. R., K. I. Reitan, and Y. Olsen. 1997. The significance of lipids at early stages of marine fish: a review. *Aquaculture* **155**: 103-115.
288. Domergue, F., A. Abbadi, and E. Heinz. 2005. Relief for fish stocks: oceanic fatty acids in transgenic oilseeds. *Trends Plant Sci* **10**: 112-116.
289. Glencross, B. D., M. Booth, and G. L. Allan. 2007. A feed is only as good as its ingredients - a review of ingredient evaluation strategies for aquaculture feeds. *Aquacult Nutr* **13**: 17-34.
290. Glencross, B. D. 2009. Exploring the nutritional demand for essential fatty acids by aquaculture species. *Rev Aquaculture* **1**: 71-124.
291. Beaudoin, F., L. V. Michaelson, S. J. Hey, M. J. Lewis, P. R. Shewry, O. Sayanova, and J. A. Napier. 2000. Heterologous reconstitution in yeast of the polyunsaturated fatty acid biosynthetic pathway. *Proc Natl Acad Sci U S A* **97**: 6421-6426.
292. Gibson, R. A., M. J. James, M. A. Neumann, J. S. Hawkes, and L. G. Cleland. 1992. Incorporation of dietary oleate, linoleate, alpha-linolenate and eicosapentaenoate into the plasma lipid fractions of four strains of rat. *Biochim Biophys Acta - Lipids and Lipid Metabolism* **1126**: 49-52.
293. Hussein, N., E. Ah-Sing, P. Wilkinson, C. Leach, B. A. Griffin, and D. J. Millward. 2005. Long-chain conversion of [13C]linoleic acid and alpha-linolenic acid in response to marked changes in their dietary intake in men. *J Lipid Res* **46**: 269-280.

294. de Antueno, R. J., S. J. Allen, A. Ponton, and M. D. Winther. 2001. Activity and mRNA abundance of Δ-5 and Δ-6 fatty acid desaturases in two human cell lines. *FEBS Lett* **491**: 247-251.
295. Buzzi, M., R. J. Henderson, and J. R. Sargent. 1997. Biosynthesis of docosahexaenoic acid in trout hepatocytes proceeds via 24-carbon intermediates. *Comp Biochem Physiol B* **116**: 263-267.
296. Frohman, M. A. 1994. On beyond classic RACE (rapid amplification of cDNA ends). *PCR Methods Appl* **4**: S40-58.
297. Smith, S. J., and L. W. Parks. 1997. Requirement of heme to replace the sparking sterol function in the yeast Saccharomyces cerevisiae. *Biochim Biophys Acta - Lipids and Lipid Metabolism* **1345**: 71-76.
298. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**: 1596-1599.
299. Hirokawa, T., S. Boon-Chieng, and S. Mitaku. 1998. SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* **14**: 378-379.
300. Lewis, T. E., P. D. Nichols, and T. A. McMeekin. 1999. The biotechnological potential of Thraustochytrids. *Mar Biotechnol (NY)* **1**: 580-587.
301. Mourente, G., and D. R. Tocher. 1994. In vivo metabolism of [1-14C]linolenic acid (18:3(n-3)) and [1-14C]eicosapentaenoic acid (20:5(n-3)) in a marine fish: Time-course of the desaturation/elongation pathway. *Biochim Biophys Acta - Lipids and Lipid Metabolism* **1212**: 109-118.
302. Tu, W. C., R. J. Cook-Johnson, M. J. James, B. S. Mühlhäusler, and R. A. Gibson. 2010. Omega-3 long chain fatty acid synthesis is regulated more by substrate levels than gene expression. *Prostaglandins Leukot Essent Fatty Acids* **83**: 61-68.
303. Albert, D., R. Rhamy, and J. Coniglio. 1979. Desaturation of eicosa-11,14-dienoic acid in human testes. *Lipids* **14**: 498-500.
304. Albert, D. H., and J. G. Coniglio. 1977. Metabolism of eicosa-11,14-dienoic acid in rat testes evidence for Δ8-desaturase activity. *Biochim Biophys Acta - Lipids and Lipid Metabolism* **489**: 390-396.
305. Ward, R. D., B. H. Holmes, and G. K. Yearsley. 2008. DNA barcoding reveals a likely second species of Asian sea bass (barramundi) (Lates calcarifer). *J Fish Biol* **72**: 458-463.
306. van Roermund, C. W. T., H. R. Waterham, L. Ijlst, and R. J. A. Wanders. 2003. Fatty acid metabolism Saccharomyces cerevisiae. *Cell Mol Life Sci* **60**: 1838-1851.
307. Gregory, M. K., V. H. See, R. A. Gibson, and K. A. Schuller. 2010. Cloning and functional characterisation of a fatty acyl elongase from southern bluefin tuna (Thunnus maccoyii). *Comp Biochem Physiol B* **155**: 178-185.
308. Agaba, M. K., D. R. Tocher, X. Zheng, C. A. Dickson, J. R. Dick, and A. J. Teale. 2005. Cloning and functional characterisation of polyunsaturated fatty acid elongases of marine and freshwater teleost fish. *Comp Biochem Physiol B* **142**: 342-352.
309. Abedin, L., E. Lien, A. Vingrys, and A. Sinclair. 1999. The effects of dietary α-linolenic acid compared with docosahexaenoic acid on brain, retina, liver, and heart in the guinea pig. *Lipids* **34**: 475-482.
310. Aziz, A. A., C. Cruz-Hernandez, L. J. Plouffe, V. Casey, C. Xiao, and W. M. Nimal Ratnayake. 2010. Increasing dietary alpha-linolenic acid enhances tissue levels of long-chain n-3 PUFA when linoleic acid intake is low in hamsters. *Ann Nutr Metab* **57**: 50-58.
311. Opekarová, M., and W. Tanner. 2003. Specific lipid requirements of membrane proteins--a putative bottleneck in heterologous expression. *Biochim Biophys Acta - Biomembranes* **1610**: 11-22.
312. Portolesi, R., B. C. Powell, and R. A. Gibson. 2005. Accumulation of DHA in HepG2 cell membranes is elevated following supplementation with fatty acids found in dairy fat. *Aust J Dairy Technol* **60**: 143-145.
313. Goren, M. A., and B. G. Fox. 2008. Wheat germ cell-free translation, purification, and assembly of a functional human stearoyl-CoA desaturase complex. *Protein Expr Purif* **62**: 171-178.
314. Giugliano, D., and K. Esposito. 2005. Mediterranean diet and cardiovascular health. *Ann NY Acad Sci* **1056**: 253-260.
315. de Lorgeril, M., P. Salen, J. L. Martin, I. Monjaud, J. Delaye, and N. Mamelle. 1999. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. *Circulation* **99**: 779-785.