The Effect of Omega-3 Polyunsaturated Fatty Acid Supplementation on Lipid Composition, Oxidative Stress, and Aldehyde Concentrations in Rat Liver and Brain

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IMRAN MALIK

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Abstract

Oxidative stress is caused by an imbalance between the production and removal of reactive oxygen species (ROS). Polyunsaturated fatty acids (PUFA), due to their multiple carbon-carbon double bonds, react readily with ROS in a process termed lipid peroxidation. Lipid peroxidation generates a number of potentially harmful secondary products, including the aldehydes ethanal, propanal, and hexanal. Indeed, raised aldehyde levels have been associated with various diseases, including cancer, leading researchers to consider them as potential diagnostic markers. However the abundance of their fatty acid precursors is dependent on dietary intake. As such, tissue aldehyde content may be diet-dependent, reducing their desirability as markers. To investigate this I fed 32 male Wistar rats diets containing 90% fat-free rat chow, 9% palm oil (mostly saturated fat), and 1% omega-3 fatty acid (EPA, DHA, ALA) for 8 weeks. The different diets resulted in changed fatty acid lipid composition in rat brain and liver. Compared to controls, DHA and EPA diets decreased liver arachidonic acid levels by 10%, while increasing levels of EPA and DHA by 7-11%. A similar effect was seen in brain lipid composition, although the changes, while statistically significant, weren't as pronounced. In brain lipids, feeding omega-3 PUFA did not lead to great changes in their concentrations, while DHA was found to be maintained at high levels even in the absence of any omega-3 PUFA in the diet. However, selected ion flow mass-spectrometry (SIFT-MS) analysis of the rat livers and brains suggests that diet does not significantly affect the concentrations of various aldehydes ($P>0.05$), or overall levels of oxidative stress (as measured by TBARS assays) in liver or brain ($P>0.05$). My results suggest that volatile aldehydes may represent a useful marker for oxidative stress with potential applications in a variety of common disorders.

Lay Summary

Oxidative stress has been found to be a primary cause of a number of disease conditions. This can result in lipid peroxidation that leads to the generation of free radicals, which in turn, can cause a chain reaction that leads to the destruction of DNA and proteins. The degradation of lipid peroxides produces aldehydes, which are more stable than reactive oxygen species. This may make them a more stable and reliable biomarker of oxidative stress. However, past studies have found that an increase in the omega-3 fatty acid content of the diet can lead to increased propanal concentrations in tissue, and conversely that an increase in the omega-6 polyunsaturated fatty acid (PUFA) content of the diet can lead to increased tissue hexanal concentrations. My research examined the effect of feeding different types of omega-3 PUFA on aldehyde concentrations, oxidative stress, and lipid compositions in rat brain and liver. I found that although omega-3 PUFA supplementation significantly affected lipid compositions, it did not affect lipid peroxidation or tissue aldehyde concentrations. These results may signify that tissue aldehyde concentrations are unaffected by diet, and may represent a reliable, more stable biomarker for oxidative stress.

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Table of Contents

List of Tables

List of Abbreviations

1) Fatty Acids: SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids

2) Omega-6 Polyunsaturated Fatty Acids: LA – linoleic acid, AA- arachidonic acid

3) Omega-3 Polyunsaturated Fatty Acids: ALA – alpha-linolenic acid, EPA - eicosapentaenoic acid, DHA – doccosahexaenoic acid

4) Control: CTRL – palm oil control group

5) Aldehydes: MDA – malondialdehyde, 4-HNE – 4 hydroxynonenal

List of Figures

A) Introduction

Oxidative stress is caused by the imbalance between the production and removal of reactive oxygen species (ROS), and has been associated with numerous disease conditions (Valko *et al.,* 2007) The increased amount of double bonds in polyunsaturated fatty acids (PUFA) makes them increasingly susceptible to ROS, and can lead to a chain reaction of free radical production that causes lipid peroxidation (Halliwell, 2006). The degradation of lipid peroxides can in turn produce secondary metabolites, including aldehydes that can have toxic effects to our cells through the generation of DNA and protein-adducts. It has been suggested that an increase in total PUFA consumption could lead to an increase in lipid peroxidation, with a subsequent increase in tissue aldehyde concentrations (Halliwell, 2006).

i) Structure and Classification of Fats and Fatty Acids

Fatty acids are one of the main building blocks of fats. Fatty acids themselves are composed of a carbon chain usually from 4-28 carbons long, bonded to hydrogen atoms, with a methyl group at one end, and a carboxylic acid group at the other end. However, there are many different types of fatty acids.

Fatty acids are monocarboxylic acids derived from animal or vegetable fat, oils, or waxes (Altar, 2006). Fatty acids can be classified based on their chain length, or by the presence of double bonds in their carbon chains. Those with no double bonds are said to be fully saturated (saturated fatty acids or SFA). Those with one double bond are monounsaturated (MUFA), and those with more than one double bond are known as

polyunsaturated fatty acids (PUFA). In this experiment I will be analyzing the effect of dietary omega-3 fatty acid supplementation on the fatty acid composition of the livers and brains of male Wistar rats. This will include several SFA, MUFA, and PUFA (Table 1).

ii) Polyunsaturated Fatty Acids

Fatty acids are important structural components of phospholipid cell membrane tissues. They form protective, semi-permeable barriers around cells and our sub-cellular structures that allow the cell can to regulate ion movement into and out of the cell with the use of proteins called ion pumps (Figure 1). Most naturally produced fatty acid double bonds are in *cis*-configuration, which creates the kinks in the fatty acid chains (Gunstone *et al.,* 1994). The kinks in the chain cause them to pack more loosely together than SFA, which form straight chains. This allows PUFA to create more fluid and biologically active structures, although their increased number of double bonds may lead to an increased risk of oxidative injury. While SFA and MUFA can be synthesized by the body *de novo*, certain PUFA cannot as we lack the necessary desaturase enzymes to create double bonds closer to the methyl end than the omega-9 carbon (Gunstone *et al.,* 1994).

For the purpose of my experiment PUFA can be split into two main groups: omega-3 PUFA and omega-6 PUFA. The number following the omega indicates the first carbon containing a double bond, from the terminal, or omega, methyl end of the fatty acid. Both omega-6 and omega-3 fatty acids have an essential 18 carbon type, linoleic acid (LA, omega-6) and alpha-linolenic acid (ALA, omega-3). Due to the fact that these polyunsaturated fatty acids are necessary for body and brain health, and because they

cannot be synthesized by the body de novo and must be obtained through the diet, they are classified as 'essential fatty acids'. Once ingested, the 18-carbon LA and ALA can be converted by the body into longer chained PUFA, as I describe below. The fatty acid content of various fat sources is given in Table 2.

iii) Omega-6 Polyunsaturated Fatty Acids

Omega-6 PUFA (also known as n-6 PUFA, with 'n' referring to the number of carbon atoms in the molecule) play important roles throughout the body, particularly in the skin. Omega-6 PUFA in the form of gamma-linolenic acid (GLA) and linoleic acid (LA) are found in the plant seed oils of evening primrose, black currant, borage, and fungal oils, while arachidonic acid (AA) of the omega-6 series is found in egg yolk, meats in general, particularly organ meats, and other animal-based foods (Institute of Medicine, 2005). The shortest chained omega-6 PUFA, the 18-carbon linoleic acid, is incorporated into skin ceramides, and is essential for maintaining the water permeability barrier of the skin (European Food Safety Authority, 2005). A prolonged lack of dietary omega-6 PUFA has been shown to increase trans-epidermal water loss, causing rough and scaly skin, and may contribute to the development of dermatitis (Institute of Medicine, 2005). Linoleic acid has also been shown to lower LDL and total cholesterol levels when taken in place of SFA. However, high levels of omega-6 PUFA have also been linked with an increased risk of myocardial infarction and coronary thrombosis (Simopoulos *et al.,* 2000).

In 1950, Widmer and Holman found that linoleic acid (18-carbon) was the precursor for arachidonic acid, a 20-carbon omega-6 PUFA (Holman, 1998). In 1963, it was discovered that arachidonic acid was converted into pro-inflammatory prostaglandins by the body. By the start of the 1980's, more pro-inflammatory agents; thromboxanes, prostacyclins, leukotrienes, and hydroxyeicosatetraenoic acids (HETE) were discovered. All of these pro-inflammatory agents were classified into a group known as eicosanoids, given their name for the fact that they are derived from the 20-carbon arachidonic acid, with eicosa- meaning twenty in Greek.

Upon stimulation of a tissue, the formation of the omega-6 eicosanoids from arachidonic acid is often associated with an explosive, short-lasting, burst of synthesis (Rose and Connolly, 1999). This burst provides active eicosanoids that can activate specific receptors briefly before being converted to inactive metabolites by selective catabolic enzymes (Lands, 1992). If the rate of synthesis is too slow, there will be insufficient amounts of active eicosanoids to occupy receptors, whereas if the rate of synthesis is too fast, excess active eicosanoids can have deleterious effects, such as heart attacks, strokes, arthritis, lupus, and asthma. Because of these potential harmful effects, it appears important to limit the amount of arachidonic acid substrate available for transformation into eicosanoids. One way of doing this is to simply limit the intake of omega-6 PUFA. However, recently another potential solution has started to receive significant attention, the use of omega-3 PUFA.

iv) Omega-3 Polyunsaturated Fatty Acids

Omega-3 PUFA (also known as n-3 PUFA) originate in the green leaves of plants, as well as plankton and algae. Flaxseed is very high in ALA, while fish, who consume the plankton or plants, have relatively high concentrations of the 20 carbon eicosapentaenoic acid (EPA) and the 22 carbon docossahexaenoic acid (DHA). Some of these fatty acids are then passed on to humans as we consume these foods.

It was believed that a small amount of omega−3 PUFA in the diet (~1% of total calories) enabled normal growth, and increasing the amount had little to no additional effect on growth (Burr and Burr, 1929). Small amounts of omega-3 PUFA prevented impaired growth, and so these fatty acids were designated as essential fatty acids. Although omega-3 PUFA have been known as essential to normal growth and health since the 1930s, awareness of their health benefits has dramatically increased in the past few decades.

Recognition of the potential benefits of omega-3 PUFA occurred in the 1970s with the study of Greenland Inuits. From these studies (Dyerberg *et al.,* 1975), it was observed that while the Greenland Inuits consumed a diet high in fat, they displayed virtually no cardiovascular disease or coronary atherosclerosis. This was believed to be because the meat consumed originated from the sea, and that their dietary fat compositions were characterized by their maritime origin. The researchers noticed that although their diets were high in fat, the composition of the fats had led to reduced triglyceride levels, heart rate, blood pressure and atherosclerosis, as well as a 2/3 decrease in the amount of

linoleic acid, and a decrease in the amount of arachidonic acid in the phospholipids (Dyerberg *et al.,* 1975). These findings are reinforced by similar findings in Japanese populations, in which we see lower rates of atherosclerosis and mortality from heart failure than Caucasian populations (Yano *et al.,* 1988). These observations led to more studies, which have supported a protective effect of omega-3 PUFA, although it is important to distinguish between omega-3 PUFA to determine which fatty acid provides us with what health benefit.

v) Omega-3 Polyunsaturated Fatty Acid Conversions

Omega-3 PUFA have shown a variety of potential health benefits, but it is important to note that there are different variations of omega-3 fatty acids PUFA, and that they do not all play the same role, and do not all have the same effect on the body and brain. There are three main types of omega-3 PUFA; ALA (18:3, n-3), EPA (20:5, n-3), and DHA (22:6, n-3) [the numbers in parentheses refer to – the number of carbon atoms in the fatty acid: the number of double bonds, and followed, in the case of PUFA and MUFA by the family of the fatty acid). As mentioned earlier, these fatty acids cannot be synthesized *de novo* by the body, and must be taken up through the diet. However, once ALA is consumed it can then be converted into EPA and later DHA by the body. Because of this it was often thought that it was sufficient enough to consume ALA alone, and that it would be converted into sufficient amounts of EPA and DHA by the body. It has since been shown that this is not the case.

ALA is converted by the body through a series of desaturation and elongation events into EPA, which is then converted into DHA, again through desaturation and elongation (Figure 2). However, ALA's conversion rate into EPA and DHA is low. A study (Gerster, 1998) using radioisotope labeled ALA showed that with a diet high in SFA, the conversion efficiency of ALA was about 6% for EPA, and only 3.8% for DHA. He also found that a diet high in omega-6 PUFA reduced the conversion rate by about 40-50% (Gerster, 1998). Goyens *et al*. 2006, found that the amount of linoleic acid (omega-6) in the diet affected the conversion rate of ALA to EPA and DHA, and estimated the conversion rate of ALA to EPA to be in the area of 0.2-8%. Because of this data, and that of further studies, it can be said that ALA, EPA and DHA are not all present in the same amounts when simply ALA is consumed. Further research has shown that these fatty acids do not all have the same effect on disease pathology, and need to be studied separately.

vi) Omega-3 Polyunsaturated Fatty Acids and Cardiovascular Disease

In 2000, the U.S. Food and Drug Administration (FDA) announced a qualified health claim for dietary supplements containing EPA and DHA omega-3 fatty acids and the reduced risk of coronary heart disease (U.S. Food and Drug Administration, 2004). In 2004, the FDA gave qualified health claim status to EPA and DHA, stating that "supportive but not conclusive research shows that consumption of EPA and DHA omega-3 PUFA may reduce the risk of coronary heart disease" (U.S. Food and Drug Administration, 2004). Omega-3 PUFA have been known to be essential to normal

growth and development of the body and the brain, but as the breadth of research and information on omega-3 PUFA continues to grow, new and exciting potential health benefits are being demonstrated.

The primary health benefit of omega-3 PUFA seems to be in reducing cholesterol and triglycerides in the blood stream that lead to atherosclerosis and eventually heart disease. While ALA has shown beneficial effects in some studies, it has also shown opposing effects in other studies (Anderson and Ma, 2009). Meanwhile EPA and DHA are now known as potent hypotriacylglycerolaemic agents, due to what is thought to be their ability to decrease triglyceride synthesis, and also increase triglyceride clearance (Anderson and Ma, 2009) through the regulation of arachidonic acid produced eicosanoids, lowering of plasma triglycerides and blood pressure, regulation of ion flux in cardiac cells, and regulation of gene expression via the peroxisomal proliferation system (PPARs) (Lands, 1992). More importantly, their ability to lower triglyceride levels has been demonstrated at intake levels that are achievable through our diet (Harris, 1997). This gives us a possible method by which North American populations can lower their risk of cardiovascular disease simply by making modifications to their diets.

Gester in Switzerland (Gerster, 1998) found that a diet that included 2-3 portions of fatty fish per week, or approximately 1.25 g of EPA + DHA per day, showed a beneficial role in the prevention of cardiovascular and inflammatory diseases. By modulating inflammation within the arterial wall, omega-3 PUFA alter the cellular and the structural composition of advanced atherosclerotic plaques in a manner that could reduce the incidence of plaque rupture or ulceration (Harris, 1997). Fish oil has also been

shown to decrease blood pressure, and improve blood flow in hyperlipidaemic, overweight men (Davidson *et al.,* 2007). While it is now fairly well accepted that small amounts of omega-3 PUFA can aid with cardiovascular function, there are also many other potential health benefits that are being researched, particularly in brain.

vii) Omega-3 Polyunsaturated Fatty Acids and Brain Health

In 1984 it was found that omega-3 PUFA were required for proper differentiation and function of the brain (Holman, 1998). Today we now know that omega-3 PUFA, particularly DHA, are required for proper development of not only the body, but also of the brain. The late David Horrobin, a pioneer in fatty acid research, says that omega-3 PUFA comprise approximately eight percent of the average human brain (Horrobin, 1987). Another leading essential fatty acid researcher, and the man who gave omega-3 PUFA their name, Ralph Holman, says that "DHA is structure, EPA is function" in the human brain (Holman, 1998). The Canadian Government has also recognized the importance of omega-3 PUFA, stating that: "DHA, on omega-3 PUFA, supports the normal development of the brain, eyes and nerves" (Fitzpatrick, 2002).

In 2007, a published article studied the effect of omega-3 PUFA on the accumulation of beta amyloid and tau proteins in mice brain, proteins that are abnormal, and may contribute to the development of memory loss in later years. The study found that all the groups that were supplemented with DHA experienced lower accumulation of these proteins (National Institute on Aging, 2007). Improvements in poor developmental

health with essential fatty acid supplementation have been found, and have linked omega-3 fatty acid deficiency with a variety of mental disorders (Perica and Delas, 2011).

In 1998, a study done at Harvard University (Stoll *et al.,* 1999) found that subjects with bipolar disorder were less likely to show a relapse of symptoms when given omega-3 PUFA and also showed significantly greater recovery when fed omega-3 PUFA than found in the placebo group. Biological marker studies have also shown that omega-3 fatty acid deficiencies are often found in people with depressive disorders, and that supplementation with omega-3 PUFA have led to therapeutic benefits (U.S. Food and Drug Administration, 2006). Another study found that out of 100 patients that had attempted suicide, on average most had significantly lower levels of EPA in their blood when compared to control subjects (Hibbeln *et al.,* 2009). In 2006, the Omega-3 Fatty Acids Subcommittee stated that: "The preponderance of epidemiologic and tissue compositional studies supports a protective effect of omega-3 essential fatty acid intake, particularly EPA and DHA, in mood disorders (Hibbeln *et al.,* 2009)," after studies showed that EPA and DHA had potential benefits in treating and preventing bipolar disorder and major depressive disorder. Recent works have also shown an omega-3 PUFA deficiency in patients with anxiety disorders (Ross, 2009), which could contribute to depression. Although we cannot be certain of these benefits until the mechanisms by which they act are fully established, we can see that there is strong evidence linking omega-3 PUFA supplementation with the treatment of depressive disorders. It is important to note that the health benefits of omega-3 PUFA are not solely affected by their dietary intake, but can also be significantly affected by competition with other fatty

acids. It is this competition however, that is the proposed mechanism by which omega-3 PUFA exert many of their beneficial effects.

viii) Competition between Polyunsaturated Fatty Acids

One possible explanation for the beneficial effects of omega-3 PUFA consumption, particularly in cardiovascular health, may be their ability to compete with omega-6 PUFA for transformation into secondary metabolites. In 1963, Holman and Mohrhauer (Holman, 1998) found that omega-6 and omega-3 PUFA competed for the same enzymatic sites involved in the metabolism of the essential fatty acids, and that they could strongly suppress omega-6 metabolism with less than 2% caloric intake coming from ALA. The reason for the beneficial effects of this competition appears to be due to the fact that both omega-6 and omega-3 PUFA are converted into eicosanoids by the body, and that these eicosanoids have opposing effects. While those from omega-6 PUFA are known to be pro-inflammatory, those from omega-3 PUFA are said to be anti-inflammatory.

This competition between omega-6 and omega-3 PUFA was first noticed when it was found that the 2-series thromboxanes produced by omega-6 PUFA were an important part of the clumping of platelets during thrombosis (Lands, 2005). This discovery presented a potential mechanism for slowing thrombosis by simply interfering with omega-6 production of thromboxane A_2. It was later found that omega-3 PUFA supplementation, as well as the use of Asprin, decreased omega-6 PUFA conversion into thromboxanes (Lands, 2005). It was also found that omega-3 PUFA supplementation

could interfere or slow the production of other omega-6 eicosanoids such as leukotrienes and prostaglandins (Lands, 2005). In 1989, a study concluded from their results that dietary fish oil may have greater triglyceride lowering effects in individuals eating diets high in saturated fats compared with those consuming mainly omega-6 PUFA (Garg *et al.,* 1990), showing that not only can omega-3 supplementation decrease omega-6 PUFA produced effects, but that the relationship is reciprocal, and that omega-6 PUFA supplementation can also decrease the effects produced by omega-3 PUFA. It has become increasingly clearer that these two PUFA compete with each other by means of an increasingly well understood mechanism.

Recognition that the rate of biosynthesis of prostaglandins was much slower with omega-3 derived eicosanoids than with their omega-6 counterparts led immediately to the recognition that omega-3 PUFA competitively attenuate the rate of omega-6 eicosanoid formation (Lands, 1992). The 20-carbon EPA (omega-3) and AA (omega-6) have been found to be converted into different types of prostaglandins (Figure 3). Arachidonic acid is converted into 2-series prostaglandins, while EPA is converted into 3-series prostaglandins (Lands, 1992). Omega-6 PUFA are converted into prostaglandins and thromboxanes by cyclooxygenase (COX)-derived metabolites, while lipoxygenase (LOX)-derived metabolites convert them into leukotrienes and HETE eicosanoids (Lands, 1992; Anderson and Ma, 2009). Not only does the omega-6 PUFA, arachidonic acid, use these pathways, but the omega-3 PUFA, EPA, also uses the same enzymes to be transformed (Bell *et al.,* 1997), and the rate of eicosanoid production for each is directly affected by the amounts of each precursor fatty acid present in the diet. It is now commonly felt that the ratio of omega-3 to omega-6 PUFA is directly responsible for the

amount of each type of eicosanoids produced through these COX and LOX pathways (Lands, 1992). In the past 100 years we have seen a great shift in dietary omega-6 to omega-3 fatty acid ratios in Western populations, a shift that can lead to harmful effects.

ix) Omega-6/Omega-3 Polyunsaturated Fatty Acid Ratio

A result of the nonselective metabolism of AA and EPA is that the relative availability of each in the diet strongly influences the proportions of the types of PUFA that are accumulated in tissue lipids. The resultant proportions influence, in turn, the proportions of eicosanoid precursor that can be mobilized by tissues upon stimulation, and thereby the intensity of the omega-6 eicosanoid response (Lands, 1992). The typical Western diet has omega-6: omega-3 PUFA ratios in the area of 10:1 to 40:1. However, these are not the ideal ratios one should strive for. Studies suggest that the evolutionary human diet, which focused around game animals, seafood, and other marine sources that were rich in omega-3 PUFA has led to a need for a balanced ratio between omega-6 and omega-3 PUFA (Simopoulos *et al.,* 2000). Typically one should try and achieve a 1:1 to 4:1 ratio of omega-6 to omega-3 PUFA in order to maximize the health benefits that both can provide (Simopoulos *et al.,* 2000).

PUFA appear to provide numerous health benefits when taken in place of SFA, however as with most potential treatments, we must take into consideration that there may be some negative side effects. As mentioned, the increased number of double bonds in these PUFA may present a potential health risk in high doses, through lipid peroxidation and free radical damage.

x) Omega-3 Polyunsaturated Fatty Acids and Lipid Peroxidation

An increased degree of desaturation will lead to an increase in lipid peroxidation (Sevanian and Hochstein, 1985), which should lead to an increase in lipid peroxidation in rats fed EPA over ALA, with an even further increase in those fed DHA over EPA as their omega-3 PUFA source.

Lipid peroxidation is a degradative process which is the consequence of the production and propagation of free radicals, primarily involving membrane PUFA, and has been associated with numerous diseases (Mutia and Uchida, 1999). Lipid peroxidation occurs when reactive oxygen species (ROS) attack fatty acids and initiate the production of a fatty acid radical, which combines with water to make a lipid radical. This radical is not very stable, and reacts readily with molecular oxygen, creating a peroxyl-fatty acid radical. This too is unstable, and reacts with another free fatty acid, producing a different fatty acid radical and a lipid peroxide. This cycle continues, as the new fatty acid radical reacts in the same way. When a radical reacts with a non-radical, it always produces another radical, which is why the process is called a "chain reaction mechanism." The radical reaction stops when two radicals react and produce a non-radical species. This happens only when the concentration of radical species is high enough for there to be a high probability of collision of two radicals (Mylonas and Kouretas, 1999). But it all stems from the production of reactive oxygen species by the body.

Reactive oxygen species are generated during oxidative metabolism, where biochemical energy is converted into ATP. In about 0.1–2% of electrons passing through the chain, oxygen is prematurely and incompletely reduced to give the superoxide radical ($\cdot O_2^-$). Superoxide is in itself highly reactive, but nevertheless, can inactivate specific enzymes or can initiate the process of lipid peroxidation (Hill and Batnagar, 1999). Superoxide can also leak out of the mitochondria where it is converted into hydrogen peroxide (H_2O_2), which is a major contributor to oxidative damage (Muller, 2000). ROS are also generated in response to pathogens, as a defense mechanism (Hill and Batnagar, 2009). Due to the damaging effects of ROS, the body has evolved a variety of ways to protect itself against these ROS.

This protection is done through enzymatic and non-enzymatic antioxidants (Hill and Batnagar, 2009). These antioxidants include vitamins C and E in sufficient amounts in the diet, among other things, and are the most attractive targets for ROS. If ROS are not removed they can react with proteins, DNA, and lipids, as mentioned above. These reactions, leading to lipid peroxidation, have long been considered to be involved in the initiation, progression, and metastasis of cancer (Pizzimenti *et al.,* 2010), and appear to be increased in situations of high dietary omega-3 PUFA consumption.

In 2011, a study (Tsuduki *et al.,* 2011) showed that plasma thiobarbaturic acid reactive substances (TBARS) and liver TBARS were approximately doubled when fish oil was given to rats. TBARS assays provide a simple, reproducible and standardized way of measuring lipid peroxidation in tissue homogenates, in that thiobarbaturic acid reacts with a secondary product of lipid peroxidation, malondialdehyde, to form a

coloured product (Cayman Chemical Company 2011). They (Tsuduki *et al.,* 2011) also found that mice given long-term fish oil developed severe oxidative stress and had a shortened lifespan, and found that the mitochondrial and peroxisomal fatty acid oxidation rate was more than doubled with perilla oil (ALA) supplementation, while fish oil more than tripled the oxidation rates. This study confirmed an earlier study that also found increased fatty acid oxidation in rats fed diets high in omega-3 fatty acids (Wong *et al.,* 1984). It remains to be determined at just exactly what level of omega-3 PUFA consumption do these increased oxidation rates lead to harmful effects on the body, and my experiment will try to determine if ALA, EPA, and DHA have different effects. Although we are not yet sure if it is harmful, it does appear that omega-3 PUFA supplementation increases oxidation rates in various parts of the body, possibly through the increased generation of lipid peroxidation- derived aldehydes, and that this needs to be taken into consideration when performing tests that use oxidation rates as a biomarker of a particular disease.

Although ROS have been shown to have damaging effects in the body, they are short-lived due to their high reactivity, which should limit their harmful effects to the site of origin (Thannickal and Fanburg, 2000). The fact that ROS are short-lived in the body, but that they are still able to cause significant harm throughout the body, may indicate that the ROS generate secondary products that can spread injury and amplify damage (Thannickal and Fanburg, 2000). PUFA are the most common target of ROS, and when oxidized, they generate a variety of bioactive molecules, of which aldehydes are amongst the most abundant and reactive (Hill and Batnagar, 2009).

xi) Lipid Peroxidation and Aldehydes

There is increasing evidence that endogenous aldehydes generated from lipid peroxidation are causally involved in most of the pathophysiological states associated with oxidative stress in cells and tissues (Srivastava *et al.,* 2002). When compared with free radicals themselves, lipid-derived aldehydes are much more stable and can diffuse within, or even escape from the cell and attack targets far from the original free radical-initiated site (Mutia and Uchida, 1999). Not only are the aldehydes able to diffuse to distant sites, but they also possess a rich variety of structural features (either naturally, or acquired by conjugation with receptive nucleophiles) that allow them to be recognized by cell constituents as signalling molecules (Hill and Batnagar, 2009). These features may make them an important secondary metabolite of ROS by which oxidative injury can be spread throughout the body.

Two aldehydes of great interest relating to lipid peroxidation are malondialdehyde (as measured in the TBARS assay) (MDA) and 4-hydroxy-trans-2-nonenal (4-HNE). MDA [$CH_2(CHO)_2$] occurs naturally, produced from the degradation of PUFA by reactive oxygen species (Figure 4), and is very reactive (Pryor and Stanley, 1975). This reactive ability allows MDA to form covalent protein and DNA adducts through reactions with deoxyadenosine and deoxyguanosine in DNA (Marnett, 1999). These DNA-adducts are toxic to cells, and commonly referred to as advanced lipoxidation end-products (Farmer and Davoine, 2007). Because of this, the production of MDA is often used as a biomarker to measure the level of oxidative stress in an organism (Del Rio *et al.,* 2005). 4-HNE is an unsaturated aldehyde that is also produced by lipid peroxidation in cells

(Figure 4). 4-HNE, like MDA, has also been found to be increased in oils high in PUFA composition, and has been linked with numerous diseases, such as chronic inflammation, diabetes, and cancer (Zarkovic, 2003). The increased consumption of PUFA has been proposed to increase the production of these aldehydes, which may lead to disease and cell death (Surh *et al.,* 2007).

There is increasing evidence that endogenous aldehydes generated from lipid peroxidation are causally involved in most of the pathophysiological states associated with oxidative stress in cells and tissues (Hill *et al.,* 2008). As early as 1985 studies in Chile had found that rats fed a diet high in fish meal and hence, PUFA, showed raised blood acetaldehyde levels (Quintanilla, 1993). A connection between high dietary omega-6 PUFA levels and increased hexanal levels has been proposed (Jarr *et al.,* 1999), while a similar correlation has been suggested between omega-3 PUFA and propanal levels (Shen, *et al.,* 2007). The reason for these connections are that a lipid hydroperoxide formed at the site of double bonds in omega-3 PUFA should lead to a cleavage between the third and fourth carbons (site of first double bond) which will generate water, and the 3-carbon aldehyde, propanal (Shen *et al.,* 2007). The same theory applies to omega-6 PUFA, with cleavage generating water and the 6-carbon aldehyde, hexanal (Jarr *et al.,* 1999). It is possible that a diet high in omega-3 PUFA could be linked to raised acetaldehyde and propanal concentrations, while decreasing hexanal concentrations in tissue.

Propanal and hexanal are volatile aldehydes (MDA and HNE are not) which may make them useful biomarkers of lipid peroxidation that can be detected in breath or by other non-invasive means. The ability to detect propanal and hexanal non-invasively may

have great applications in the battle against cancer, diabetes, and other diseases. However since these aldehydes are derived from specific fatty acid types, diet could potentially change their concentrations, making them unreliable biomarkers. I studied this potential link using a novel machine, the Selected Ion Flow Tube- Mass Spectrometer (SIFT-MS) to analyze the headspace aldehyde concentrations in rat brain and liver, as outlined in the methods section.

xii) Aldehyde Metabolism

There are enzymes capable of detoxifying these aldehydes, specifically glutathione-S-transferases, aldose reductase, and aldehyde dehydrogenase (Chen *et al.,* 2008). Aldehydes can have toxic effects in the body if not metabolized and removed in an orderly fashion. Part of this toxicity is due to their ability to bond nucleic acids and proteins and form various DNA and protein-adducts, due to their electrophilic nature (for example see O'Brien *et al.,* 2006). Past studies have shown that aldehydes are oxidized to carboxylic acids, reduced to alcohols, or conjugated with cellular nucleophiles such as proteins (O'Brien *et al.,* 2006). This is done metabolically, and in general aldehydes are oxidized by NAD+, and aldehyde dehydrogenase (ALDH) to form carboxylic acids and secondary alcohols (O'Brien *et al.,* 2006). In cardiovascular tissue, aldehyde reduction to primary alcohols is catalyzed by aldose reductase (AR) (Hill and Batnagar, 2009), but they can also be reduced a variety of ways including by alcohol dehydrogenase. Aldehydes generated from oxidized lipids have been found in tissues that have experienced oxidative injury throughout the body, including in ischemic, hypertrophic, and failing hearts; in artherosclerotic lesions, apoptotic cells, and even damaged

mitochondria (Hill and Bhatnagar, 2009), and may provide a volatile biomarker for cancer.

xiii) Aldehydes and Cancer

Recent research is now suggesting a possible link between raised aldehyde levels in the body and an increased risk of cancer. It is commonly known that increasing ethanol consumption may lead to deleterious effects on the body, one of which is an increased risk of developing cancer. The principle metabolite of ethanol is the small chain aldehyde, acetaldehyde. It has been suggested that acetaldehyde is responsible for the increased risk of cancer associated with high concentrations of ethanol consumption. However, the body does have ways of detoxifying and removing excess aldehydes, to try and protect itself from the danger these aldehydes appear to present.

xiv) Dietary Influence on Tissue Aldehyde Concentrations

This link between a raised aldehyde level and an increased risk of cancer has led to some suggestions that this can be used as a biomarker for cancer. However, although high acetaldehyde levels have been linked with an increased risk of cancer in the lab, there is still information pointing to the contrary. A study done on the levels of aldehyde dehydrogenase 1 (ALDH1) found that raised levels were correlated with a poor prognosis, and shorter disease free and survival times (Deng *et al.,* 2010). However, they also found that the pattern and level of ALDH1 varied depending on the area of tissue examined, and that different body parts had significantly different background levels.

This led them to conclude that, although ALDH1 may be promising as a cancer stem cell biomarker, that it may not be suitable for use in tissues with high background ALDH1 levels, such as the liver and pancreas (Deng *et al.,* 2010). If we can see variations in ALDH1 levels within the same individual, then we have to ask whether or not we see variations between individuals, and if so, what might be causing this variation?

I used a novel instrument known as a selected ion flow tube- mass spectrometer (SIFT-MS), which provides real-time quantification of several trace gases in breath or air, or for our purpose, in the headspace of a beaker (Smith & Spanel, 2005, 1999).

The SIFT-MS (Figure 5) generates positive precursor ions using an ion source. From the mixture of ion species generated, a current of ions of a given mass-to-charge ratio is obtained using a quadrupole mass filter (Smith & Spanel, 2005, 1999). One can then select one of three precursor ions (H_3O^+, NO^+, or O_2^+), which react with trace gas molecules from the air/breath/headspace introduced through a downstream sampler line. No sample work-up is required as the chemical ionization agents are unreactive with the major components of air. The sample is simply a small amount of air leaked directly into the flow tube. The ions, both product ions and unreacted reagent ions, are carried along the flow tube in a stream of helium gas and exit the reaction region through a small orifice at the far end. The flow tube pressure is maintained at approximately Torr and the reaction time between the ionization agents and the analytes is around 1ms. After traversing through the orifice, the ions are focused by a lens array into a second quadrupole mass filter located at the downstream end of the instrument where they are identified by mass and counted by an ion multiplier. The concentrations of the trace

species of interest are found by measuring the ratios of the product ion signals to the reagent ion signals providing the relevant kinetic parameters are known. Based on this mass-to-charge ratio, and the knowledge of the previously calculated reaction rate coefficient, one can deduce the type and concentration of gaseous compounds.

Detection limits are usually in the parts per billion range (ppb), although parts per trillion sensitivity (ppt) have been reported (Smith and Spanel, 1999). The SIFT-MS machine, unlike the gas chromatography-mass spectrometry does not require water to be removed by an equation after each test, in fact it can help in the determination of certain chemicals. This reduces the time of each test, and makes breath or headspace quantification of volatile chemicals much easier. However, one problem with this machine is that we run the risk of mistaking the product produced by the combination of a compound with a precursor ion for another one with the same mass-to-charge ratio. To date, no one has detected any products that mimic the product ion produced by the reaction of NO^+ with propanal or hexanal, apart from the ^{18}O isotopologue of hydrated hydronium, which has the same m/z as propanal, at m/z 57 (Smith and Spanel, 1999). This overlap should be limited in my experiment, which should ensure that this is not a problem.

The purpose of my study was to determine whether changes in dietary fatty acid intake, which lead to changes in membrane fatty acids concentrations, will alter the overall amounts of aldehydes present through changes in overall oxidative stress. I also wanted to observe how the relative amounts of propanal and hexanal concentrations vary

as the relative omega-3 and -6 PUFA concentrations change. If the volatile aldehydes are greatly affected by total omega-3/omega-6 PUFA concentrations, then these markers will have large dietary influences, which may make them unsuitable as diagnostic biomarkers. Past studies have shown that this may be the case, however these studies used crude oils (combination flax/fish oils, containing omega-6 PUFA, SFA, and MUFA as well). We now have access to cheaper, highly purified omega-3 PUFA, which allow us to investigate the individual effects of each omega-3 PUFA.

xv) New Purified Omega-3 Polyunsaturated Fatty Acids

Recently highly purified, vacuum-distilled versions of omega-3 PUFA have become available. These new, 95% pure versions of omega-3 PUFA (EPA, DHA, ALA), now allow us to isolate specific omega-3 PUFA and use them individually at high levels of purity, instead of simply using flax or fish oil. These fatty acids are prepared using a 2-step process known as cold fractionation. This process takes advantage of the higher freezing point of SFA and MUFA, to solidify them, leaving oil containing only PUFA. These PUFA are then separated using high performance-liquid chromatography based on their size and structural features.

Studies have already shown a benefit of the purified PUFA against cardiovascular disease and diabetes (Horrobin, 2001; Yokohama *et al.;* 2007, Westerveld *et al.,* 1993), and have also shown anti-psychotic and anti-depressive effects (Wood *et al.;* 2007, Maes *et al.,* 1996). With the aid of these purified omega-3 PUFA, I hope to fill in the gaps left by previous research, and hope to identify the relationships between omega-3 PUFA, lipid peroxidation, and tissue aldehyde concentrations, and to determine whether or not

volatile aldehydes present us with a potential biomarker for cancer which is unaffected by dietary composition.

xvi) Hypotheses

Using the highly purified omega-3 PUFA, I hope to clarify the differences between them. I hypothesize that diets supplemented with omega-3 PUFA will show increased concentrations of omega-3 PUFA in their tissue phospholipids, while showing a decrease in omega-6 PUFA concentrations. Specifically, I hypothesize that EPA and DHA supplementation will lead to a greater increase in longer-chained omega-3 PUFA, and decrease omega-6 AA concentrations, than ALA supplementation, when compared to the palm oil CTRL group. ALA supplementation should lead to increased ALA concentrations, with a smaller increase in EPA and DHA concentrations, while EPA supplementation should lead to an increase in EPA and DHA concentrations. DHA supplementation should only increase DHA concentrations, as the omega-3 PUFA conversion pathway (Figure 2) only creates longer-chained PUFA from smaller-chained ones.

I hypothesize that the different omega-3 PUFA will have differential effects on lipid peroxidation in rat brains and livers fed diets containing different omega-3 PUFA as their principle fat source, due mainly to their different degrees of unsaturation. I also hypothesize that I will see differences in the liver and brain headspace aldehyde concentrations of the same rats. Specifically, I hypothesize that rats fed diets containing EPA and DHA will show higher levels of lipid peroxidation in their livers and brains as compared to those fed on either a diet containing ALA as the principal fat source or palm

oil control groups, due to their increased number of double bonds. Here I propose that a rat with access to a diet high in omega-3 PUFA may show raised acetaldehyde and propanal concentrations (through a cleavage at the double bond between the 3rd and 4th carbons) in their livers and brains as compared to palm oil control groups. Conversely, I expect that diets showing high omega-6 lipid concentrations will give higher headspace hexanal concentrations, caused by a cleavage at the double bond between the 6th and 7th carbons. These dietary influences on lipid peroxidation, and subsequently tissue aldehyde concentrations, should render the analyzed volatile aldehydes unreliable biomarkers of oxidative stress and cancer.

B) Materials and Methods

i) Rat Storage and Handling

Male Wistar rats were randomly assignedinto pairs and placed into cages containing access to water and fat-free rat chow and kept in the the Thunder Bay Regional Research Institute, Thunder Bay, Ontario. During this period rats were handled periodically to familiarize them with human contact. Cages were cleaned once a weekand experiments were carried out ethically under the Lakehead University animal utlilization protocol (Lakehead University, 2012).

ii) Fatty Acid Administration

Rats were fed for 8 weeks on supplementation diets containing 90% rat chow (containing protein, nutrients, and everything else in a balanced diet apart from fat) and 10% fat. Control groups were also given palm oil (50% SFA [palmitic acid], 40% MUFA [oleic acid], 10% omega-6 PUFA [linoleic acid], and 0.3% ALA) as their fat source (to try and replicate the typical Western human diet), while other groups were given 90% palm oil and 10% of a specific omega-3 PUFA as their fat source. After food preparation, omega-3 PUFA containing canisters were fogged with nitrogen gas to prevent oxidation, and stored at -20ºC. Each serving of food contained 315g basal rat mix + 31.5g palm oil + 3.5g omega-3 fatty acid (315g basal rat mix + 35g palm oil for control group), and rats were given unlimited access to food.

iii) Euthanizing Procedure

After 8 weeks on the experimental diets, the rats were euthanized by placing them in a glass box under a fume hood, and adding a mixture of isofluorane gas and oxygen into the gas box to anaesthetize the animals. Rats were then removed from the box and decapitated using a small animal guillotine. The livers, brains, and plasma of the rats were harvested and stored in a -80°C freezer.

iv) Headspace Aldehyde Analysis

As mentioned, I used a SIFT-MS instrument to analyze tissue headspace aldehyde concentrations. This instrument uses the reactions of precursor ions with headspace concentrations to produce product ions that can be identified based on their mass-to-charge ratio (Smith and Spanel, 1999). Before tissue headspace concentrations could be analyzed, preliminary work had to be performed.

v) Tissue Preparation and Preliminary Work

The SIFT-MS was used in my experiment to measure the headspace concentrations of the saturated, one to eight carbon (C1-C8) aldehydes in the brains and livers of our test rats to investigate the effect of dietary omega-3 PUFA supplementation. Each aldehyde in the headspace over the tissue will react with the selected precursor ions (either H_3O^+ or NO^+) to produce characteristic product ions that can be identified from their mass to charge ratio. The H_3O^+ precursor ion was used for formaldehyde and

acetaldehyde, as only the protonated parent ion is produced in this reaction, whereas when reacted with NO^+, we get no reaction occurring, due to the lack of exothermic channels, and charge transfer (Smith and Spanel, 1999). All the other aldehydes were analyzed using the NO^+ precursor ion, which proceeds via hydride (H^-) transfer to produce a protonated product and HNO in saturated aldehydes. (Smith and Spanel, 1999). Typical ion counts varied for each particular aldehyde, but in general I was looking for concentrations in the parts per billion range. However, before I could start using experimental organs, I first had to run preliminary tests to set the experimental parameters.

Firstly, the effect of beaker size on aldehyde measurements was determined. At first we were using 250 ml beakers, but after comparison with 125 ml beakers, it was determined that the smaller beakers gave more consistent results, and required less tissue to produce results in the range we wanted (ideal range was different for each aldehyde, but always in ppb).

Before determining how much brain and liver were required to give aldehyde concentrations in the optimal range, I first had to determine that range using propanal and hexanal standard curves. Hexanal or propanal were mixed with deionized water at various dilutions to give readings in the 100-1000 ppb range. Propanal and hexanal quality controls (QCs) were used as indicators of the consistency and performance of the SIFT-MS, and were prepared daily, as their headspace concentrations decrease over time due to the volatile nature of the aldehydes (Yasuhara and Shibamoto, 1995). A range of headspace aldehyde concentrations was created by creating 4 separate QCs (in triplicates)

for each propanal and hexanal, and taking headspace readings of each. From this I determined a suitable range for the QCs, with each required to fall within 2 standard deviations of the calculated mean (range of 400-800 ppb for propanal, and 100-300 ppb for hexanal).

The amount of brain required to give a reliable aldehyde measurement was determined. Livers and brains were ground individually and separately in a mortar and pestle with the use of liquid nitrogen and separated 125 ml beakers. Using weights of 50 mg, 100 mg, 150 mg, 300 mg, 500 mg, and 750 mg, headspace aldehyde concentrations were recorded at room temperature (22°C), 40°C, and 60°C to check for any effect of increasing oxidation at higher temperatures on headspace aldehyde concentrations, and also to account for the varying volatility of the aldehydes under consideration. Each experiment was repeated twice, with triplicate samples, arriving at an optimal weight of 200 mg.

The optimal duration of each reading was set by running various spectra of different time lengths, checking for dramatic increases or decreases in aldehyde concentrations with respect to time. It was determined that 60 seconds was an appropriate scan time to allow for accurate and stable results, while keeping the time of each test to a minimum.

vi) Experimental Procedure

After allowing the machine to warm up for 45-60 minutes, the water level of my breath was analyzed by removing the sampler from the sampling line, and breathing into the sampling line. Using the H_3O^+ precursor ion, the percent of water in breath was recorded, and used as a quality control for the SIFT-MS machine, with correct breath water vapour concentrations being approximately 6%. Precursor ion purities were recorded by turning off the sampling line and using the 'cycle precursors' function in scan mode. Machine blank concentrations were recorded for each aldehyde by using the scan mode on the SIFT-MS and the 'select compounds' function to select for aldehydes of choice, then running spectra with the sampling line off. These were then subtracted from aldehyde concentrations taken from the headspace of the experimental brains and livers, recorded with the use of the sampling line. Aldehyde concentrations were recorded for a minute for each aldehyde and an average concentration calculated. Quantifiable aldehyde concentrations were considered approximately three times higher than machine blank concentrations (the recorded concentration of aldehydes with no gas sampling occurring due to spectrometer noise and/or carry over contaminants).

Aldehyde concentrations, as mentioned, were first recorded at machine blank concentrations, with a source pressure of 0.60 Torr, a tube pressure of 1.012 Torr, a tube length of 3 cm and a gas temperature of 25.1˚C, and a microwave power of 92%. The direct sample flow rate was 0.43 TorrL/s. After that they were recorded at ambient concentrations by placing the sampling line in an empty 125 ml beaker. Finally, experimental aldehyde concentrations were recorded by placing the sampling line in the

headspace of a 125 ml beaker containing an experimental rat brain or liver. All experimental livers and brains ground in liquid nitrogen, weighed, and then immediately placed in a 125 ml beaker on dry ice, to prevent any oxidation from taking place. Each liver and brain was analyzed in triplicate to reduce experimental error. Once all beakers were prepared the first beaker (ambient) was removed from the dry ice and placed at room temperature for 5 minutes. Aldehyde concentrations were recorded after 5 minutes using the SIFT-MS. After sampling the beaker was resealed using aluminum foil and tape, and left at room temperature. While spectra were being run for the first beaker, the second beaker was placed at room temperature for 5 minutes. The second beaker was then sampled in the same manner as the first beaker, while the third beaker was placed at room temperature for 5 minutes. This procedure was followed until all the beakers had been sampled at room temperature.

After sampling all beakers at room temperature, machine blank levels were re-recorded in the same manner as before. While that was being done the first beaker (ambient) was placed in a 40°C water bath for 5 minutes to induce oxidation. After 5 minutes aldehyde levels were recorded using the SIFT-MS, and the beaker was re-sealed and left at room temperature as done earlier. The same procedure was the followed for all other beakers, using the 40˚C water bath and the SIFT-MS. Machine blanks were then re-recorded and the same process was repeated at 60˚C.

vii) Lipid Peroxidation Analysis

I measured oxidative stress using a thiobarbaturic acid reactive substances (TBARS) assay kit from Cayman Chemical Company. This assay uses thiobarbituric acid (TBA), along with diluted acetic acid and diluted sodium hydroxide to prepare a colour reagent that is then mixed with sodium dodecyl sulfate (SDS) and either the malondialdehyde (MDA) standard or with tissue homogenates. As mentioned above, MDA [$CH_2(CHO)_2$] is a highly reactive compound that is formed from the lipid peroxidation of PUFA, making it a reactive oxygen species, and a common marker of oxidative stress.

Tissue homogenates were prepared by first grinding tissue (brain or liver) into a powder using a mortar and pestle and liquid nitrogen. The powder was then weighed out into 100 mg samples and placed in 2 ml microcentrifuge tubes. I then added 350 ul of degassed water and the tissue was homogenized using a Polytron machine to make a 1/3.5 x dilution. 100 ul of each dilution was then added to separate 10 ml test tubes and mixed with 100 ul of SDS solution (equilibrated to room temperature) and 4mls of colour reagent [a mixture of TBA, dilute acetic acid (20%), and dilute NaCl (10%)]. The tubes were capped and boiled for an hour in a 100ºC water bath, then cooled on ice for 10 minutes to stop the reaction, transferred to plastic centrifuge tubes and centrifuged for 10 minutes at 1,600 * g. Supernatant (150 ul) from each tube was transferred into wells on the 96-well assay plate and absorbance readings were taken at 530 nm, using the plate spectrophotometer. Absorbances were compared with an MDA standard curve (Figure 6),

which was prepared using the MDA standard provided in the assay kit, mixed with water to various dilutions.

viii) Calculation of TBARS Concentration

Data reduction was performed according to manufacturer's instructions, against a standard curve of MDA (Cayman Chemical Company, 2010). Blank absorbances were subtracted from each tissue absorbance to remove any absorbance associated with anything other than tissue composition. Corrected absorbances were used to calculate MDA concentration using the formula: MDA (uM) = [Corrected absorbance – y-intercept/ slope]. The assay kit has a range of 0-50 uM, and typically has an intra-assay coefficient of variance between 5.5-7.6%.

ix) Lipid Analysis

Rat brains were warmed on a metal tray over an ice slurry (mix of ice and water) to prevent them from warming to quickly. Brains were then cut in half using a scalpel, and half a brain was placed in the -80°C freezer for tissue aldehyde and TBARS analysis. The other half was ground into a powder using a mortar, pestle, and liquid nitrogen, weighed (300 mg +/- 10 mg) and placed in labeled scintillation vials. The vials were then sent to Guelph Lipid Analytical Laboratories in Guelph, Ontario for analysis of the brain lipid compositions. Lipids were analyzed by first homogenizing the tissue using a Polytron homogenizer. Lipids were then extracted from the samples according to the method of Bligh & Dyer (Bligh and Dyer, 1959) in the presence of the internal standard,

tritridecanoin. Fatty acid methyl esters were prepared using boron trichloride in methanol, and heating the methylation tubes in a boiling water bath. The resulting fatty acid methyl esters were analyzed on a Varian 3400 gas-liquid chromatograph, with a 60-m DB-23 capillary column (0.32 mm internal diameter).

Experimental livers were prepared in a similar fashion, however due to their larger size and homogenous nature, only a small piece had to be cut off each liver (not cut in half). Final weights were 300 mg +/- 50 mg.

x) Statistical Analysis

Concentrations of fatty acids, aldehydes or TBARS were compared using ANOVA and Tukey Tests to test for any significant differences between dietary groups. Correlational analysis was conducted using linear Pearson regression using a software program (Statistica).

C) Results

i) Lipid Composition

Liver

Lipid analysis showed that dietary omega-3 PUFA supplementation did significantly affect the lipid composition of rat livers, when compared to the palm oil CTRL group. Results were reported in percent composition of total liver lipids. The percent abundance of 11 out of the 15 PUFA was significantly affected by dietary composition (Table 3). Of particular importance was that the concentrations of arachidonic acid (C20:4 n-6), EPA (C20:5 n-3), and DHA (C22:6 n-3) were all significantly affected (Table 3). Liver AA concentrations were highest in palm oil CTRL rats (15.17%), while being significantly reduced ($p<0.01$) in DHA (5.74%) and EPA-fed (6.1%) rats, although ALA supplementation (10.13%) significantly reduced ($p<0.05$) AA concentrations from those of CTRL rats as well (Table 4). In contrast, linoleic acid concentrations were unaffected by dietary composition ($p>0.05$).

ALA concentrations were significantly increased ($p<0.05$) in the ALA-supplemented group (1.34%) when compared to all other groups [DHA (0.24%), EPA (0.18%), CTRL (0.1%)] (Table 4). EPA concentrations were significantly increased ($p<0.005$) in the EPA-supplemented group (7.81%) when compared to the palm oil CTRL group (0.11%, Figure 7). DHA-supplementation also significantly increased ($p<0.01$) EPA concentrations to 4.23% of total liver lipids (Figure 8), while ALA-supplementation significantly increased ($p<0.05$) EPA concentrations from those of the palm oil CTRL group to 2.14% (Figure 9). DHA concentrations were significantly

increased ($p<0.005$) in the DHA-supplemented group (15.23%), while EPA and ALA-supplementation also produced significant increases ($p<0.05$) from the palm oil CTRL group (7.14% and 7.07% respectively, Table 4). It is also important to note that the concentration of docosapentaenoic acid (DPA, C22:5 n-3) was significantly higher ($p<0.05$) in EPA-fed groups (2.96%) when compared to other dietary groups (ALA - 1.2%, DHA - 1%, CTRL - 0.29%, Table 4), as this PUFA is the one that links EPA to DHA in the omega-3 fatty acid transition pathway.

Only 2 of the 6 MUFA were significantly affected ($p<0.05$) by dietary composition [physeteric acid (C14:1 n-9) and oleic acid (C18:1 n-9)] (Table 3). Both of these MUFA had higher lipid percent concentrations in control groups (0.07% and 28.5% respectively), and both had lowest concentrations in the DHA-supplemented group (0.026% and 19.7% respectively, Table 4). Oleic acid was found to be significantly decreased ($p<0.05$) in the EPA-supplemented group (22.75%) relative to the palm oil CTRL group, but not in the ALA-supplemented group (24.9%, Table 4). The SFA were relatively unaffected by changes in the omega-3 content of their diet (Table 3). Only 2 of the 7 SFA measured were found to be significantly affected (14:0 and 15:0). C14:0 (myristic acid) was highest in control groups (0.86%) and was found to be significantly different ($p<0.05$) from all omega-3 groups, with DHA (0.53%) again causing the largest decrease, followed by EPA (0.6%) and ALA (0.67%, Table 4). C15:0 was actually lowest in the palm oil control group (0.09%), while DHA-supplementation (0.13%) caused the most significant increase ($p<0.05$), while ALA-supplementation (0.11%) also caused a significant increase ($p<0.05$, Table 4).

Brain

The brain lipid compositions also had some significant differences between omega-3 PUFA supplemented groups and the palm oil CTRL group, as analyzed by ANOVA analysis, although not as pronounced as in liver (Table 5). DHA concentrations were significantly affected only by supplementation with DHA in the diet (Table 6). The DHA-supplemented group had brain DHA concentrations (13.71%) significantly higher ($p<0.05$) than the palm oil CTRL group (11.86%, Figure 10). EPA (C20:5 n-3) concentration was significantly increased ($p<0.01$) in the EPA-supplemented group (0.21%) when compared to the palm oil CTRL group (0%, Figure 11). The DHA-supplemented group (0.09%) also had significantly higher ($p<0.05$) EPA concentrations than ALA-supplemented (0.01%) and palm oil CTRL groups while ALA-fed and control groups were not significantly different ($p>0.05$) from each other (Table 6). DPA (C22:5 n-3) concentrations were also highest in the EPA-supplemented group (0.71%), again significantly higher ($p<0.05$) than all other groups (Table 6). However, this time DHA (0.35%) and ALA-fed (0.31%) groups both showed significantly higher ($p<0.05$) percentages of DPA in their brain lipids than the palm oil CTRL group (0.12%, Table 6). The remaining 4 omega-3 PUFA analyzed, including ALA (Figure 12) were not found to be significantly affected by diet ($p>0.05$, Table 5).

Three of the omega-6 PUFA, including linoleic acid, were not significantly affected ($p>0.05$) by dietary composition (Table 5). Arachidonic acid (C20:4, n-6) concentration was highest in the palm oil CTRL group (9.61%, Table 6). It was not found to be significantly affected ($p>0.05$) by ALA supplementation (9.34%), however, both palm oil

CTRL and ALA-supplemented groups were found to have significantly higher ($p<0.05$) AA concentrations than both EPA (8.22%) and DHA (8.19%) supplemented groups (Table 6). The omega-6 PUFA, C22:4 and C22:5 followed a similar pattern to AA, with palm oil control groups having significantly higher ($p<0.05$) concentrations (2.64% and 0.48% respectively), followed by ALA (2.3%, 0.11%), EPA (1.98%, 0.12%), and DHA-supplemented groups (1.88%, 0.02%, Table 6). All omega-3 PUFA supplemented groups showed significantly higher ($p<0.05$) concentrations of the omega-6 PUFA C20:3 than the palm oil CTRL group (0.39%, Table 6). Again DHA supplementation (0.62%) showed the greatest difference ($p<0.05$), while EPA supplementation (0.54%) and ALA supplementation (0.52%) also had significant ($p<0.05$), but less pronouced effects (Table 6). The concentrations of SFA and MUFA were not significantly affected ($p>0.05$) by supplementation with omega-3 fatty acids based on ANOVA analysis (Table 5).

ii) Liver Lipid Composition by Fatty Acid Class

Comparing total omega-6 and omega-3 fatty acid concentrations, I found that total omega-6 PUFA content was significantly decreased ($p<0.05$) by EPA (15.61%), and DHA (15.44%) supplementation, while ALA supplementation (20.32%) did not have a significant effect ($p>0.05$) when compared to the palm oil CTRL group (23.94%, Figure 13). Total omega-3 PUFA concentrations were significantly increased ($p<0.05$) by omega-3 PUFA dietary supplementation (Figure 14). ALA-supplemented rats did have significantly higher ($p<0.05$) total omega-3 concentrations (11.94%) when compared to the palm oil CTRL group (4.83%), however, EPA-supplemented (18.17%) and DHA-supplemented (20.8%) groups showed a significantly higher increase ($p<0.01$, Figure 14).

I found that EPA supplementation and DHA supplementation significantly increased ($p<0.01$) the ratio of omega-3/omega-6 PUFA (1.18 and 1.35 respectively), while ALA supplementation (0.59) increased the ratio to a smaller degree ($p<0.05$) when all groups were compared the palm oil CTRL group (Figure 15). Rats fed control diets had an average omega-3/omega-6 PUFA ratio of only 0.2 (Figure 15).

iii) Brain Lipid Composition by Fatty Acid Class

Total brain omega-3 PUFA concentrations were significantly increased ($p<0.05$) by DHA supplementation (14.15%) and EPA supplementation (13.64%), whereas ALA supplementation (13.1%) did not significantly differ ($p>0.05$) from the palm oil CTRL group (11.99%, Figure 16).

EPA and DHA supplementation significantly decreased ($p<0.05$) total omega-6 PUFA concentrations (12.42% and 12.09%) when compared to the palm oil CTRL group (14.35%), and again ALA supplementation (13.59%) failed to significantly differ ($p>0.05$) from the palm oil CTRL group (Figure 17). ALA supplementation did significantly increase ($p<0.05$) the ratio of total omega-3/omega-6 fatty acid concentration (0.96) from the palm oil CTRL group (0.84), although not as significantly ($p<0.01$) as EPA (1.1) or DHA (1.7) supplementation (Figure 18).

iv) TBARS Analysis

TBARS analysis showed no significant effect ($p>0.05$) of dietary composition on malondialdehyde concentrations in rat livers (Figure 19). Brain TBARS analysis (Figure 20) also showed no significant difference ($p>0.05$) in MDA concentrations between dietary groups.

v) Headspace Aldehyde Analysis

At room temperature (22°C, Table 7) brain pentanal concentrations were found to be significantly higher ($p<0.05$) in the DHA-supplemented group (44 ppb) when compared to the palm oil CTRL group (24 ppb, Figure 21). That was the only aldehyde found to be significantly affected ($p<0.05$) by dietary composition at room temperature based on ANOVA and Tukey HSD analysis (Table 7). At 40°C no significant differences ($p>0.05$) were found between any of the dietary groups for any of the aldehydes analyzed (Table 8). Again at 60°C a significant difference ($p<0.05$) was found in brain pentanal concentrations (Table 9), although Tukey HSD analysis failed to show a significant difference ($p>0.05$) between any of the 4 groups. Analysis with a less conservative LSD test showed a significant difference ($p<0.05$) between the ALA-supplemented (49 ppb) and DHA-supplemented (48 ppb) groups, when compared to the EPA-supplemented (33.66 ppb) group (Figure 22). This was the only significant difference found at 60°C (Table 9).

SIFT-MS analysis of liver aldehyde levels at room temperature showed no significant differences ($p>0.05$) in any of the 4 dietary groups (Table 10). At 40°C ANOVA analysis found a significant difference ($p<0.05$) in butanal concentrations (Table 11) between EPA-supplemented (11 ppb) and DHA-supplemented (5 ppb) groups (Figure 23). A significant difference ($p<0.05$) was also found at 60°C for butanal concentrations (Table 12), however this time it was found between the ALA-supplemented (10 ppb) and palm oil CTRL (3 ppb) groups (Figure 24). None of the other aldehydes were found to be significantly affected ($p>0.05$) by dietary composition at 40°C or 60°C (Tables 11, 12).

Total omega-3 PUFA content did not significantly correlate ($p>0.05$) with propanal concentrations in either liver (Figure 25) or brain, while total omega-6 PUFA content did not significantly correlate ($p>0.05$) with tissue hexanal concentrations (Figure 26).

D) Discussion

To the best of my knowledge, this is the first time that the effects of highly purified omega-3 PUFA have been tested in relation to tissue aldehyde concentrations. The increased availability of these purified fatty acids allowed me to cost-effectively measure the individual effects of each omega-3 fatty acid, and to differentiate between them, as opposed to using crude oils. It is important to be able to differentiate between the three main types of omega-3 PUFA (ALA, EPA, and DHA), and to be able to discern the individual effects of each. Numerous experiments have now suggested differences between the actions and therapeutic value of each omega-3 PUFA (see Introduction). It may be important for the consumer to look beyond the label, and to read the ingredients to determine which omega-3 PUFA are in fact present in the product.

i) Omega-3 Polyunsaturated Fatty Acids in Liver Phospholipids

My results are in accordance with previous work (von Schacky and Weber, 1985), which also found that ALA supplementation, and not EPA or DHA supplementation affected ALA concentrations (Table 3). This is because although metabolism can generate longer chained omega-3 PUFA from ALA in mammals, it appears unable to synthesize the shorter ALA from EPA and DHA.

Dietary supplementation with EPA led to an increase in the incorporation of EPA into liver phospholipids (Figure 7). The observation that ALA-supplementation did not significantly increase ($p>0.05$) EPA lipid concentrations, while DHA supplementation

did ($p<0.05$) when compared to the palm oil CTRL group (Table 4), may seem surprising at first, but it has been proposed that DHA may be retro-converted into EPA (von Schacky and Weber, 1985). This retro-conversion indicates that DHA may serve as a reservoir for EPA in times of low EPA concentrations, possibly through partial β-oxidation and saturation of the resulting trans-double bond (von Schacky and Weber, 1985). To date however, researchers have not identified the enzymes needed to perform this retro-conversion, and although it appears to occur in von Schacky and Weber's experiment, these results have not been replicated. Perhaps more likely is that the increased EPA concentrations found in DHA-supplemented rats could be due to impurities in the DHA source used. The source used was 95% pure, and it is likely that the remaining 5% could have included EPA.

DHA concentrations showed a statistically significant increase in lipid DHA composition with dietary DHA supplementation, as hypothesized (Figure 8). The observation that ALA-supplemented rats did not differ significantly in their DHA concentrations from EPA-supplemented rats (Table 4) may indicate that conversion of EPA into DHA is just as inefficient as the conversion of ALA into DHA, possibly showing that the conversion of EPA to DHA is a rate-limiting step in the overall conversion process, which is in accordance with previous works (Harper *et al.,* 2005; Stark *et al.,* 2008)

EPA dietary supplementation also had a significant effect on DPA concentrations (Table 4), which is in accordance with previous work in human plasma using omega-3 ethyl esters, where it was also found that EPA, and not DHA, supplementation led to

increased concentrations of DPA in platelet phospholipids (von Schacky and Weber, 1985). This makes sense, as we have seen above (Figure 2), EPA can be converted to DHA in a 4-step process, the first of which is the 2 carbon elongation of the omega-3 EPA (C20:5) into DPA (C22:5). This may be because EPA supplementation leads to significantly higher concentrations of DPA. Interestingly, while DHA supplementation significantly increased the levels of EPA, it did not significantly affect DPA levels when analyzed by an ANOVA test ($p>0.05$). This supports the conclusion that DHA is not retro-converted into EPA.

ii) Arachidonic Acid in Liver

Arachidonic acid acts as the substrate for a variety of eicosanoids. Cyclooxygenase (COX) activity converts AA into PGG_2, which is then reduced to PGH_2 by peroxidase activity (Oates et al. 1988). PGH_2 then becomes the precursor for various prostaglandins (PGD_2, PGE_2, PGF_2, PGI_2) and for thromboxane A_2 (Figure 27). As mentioned earlier, the formation of AA-derived eicosanoids is usually associated with a brief, explosive burst of synthesis of eicosanoids, that if too great, has been associated with many inflammatory diseases (Lands, 1992). It has become clear that managing AA-derived eicosanoid concentrations is of great importance.

My results show that AA concentrations can be managed by dietary omega-3 PUFA supplementation in liver (Table 4). Rats fed the palm oil CTRL diet had on average 15.2% of their lipids made up of arachidonic acid (Table 4). This value is at the upper range of what past studies have found (Neuringer *et al.,* 1988). Arachidonic acid is

present in all biological membranes, and represents approximately 5-15% of the total fatty acid composition of most tissue phospholipids (Neuringer *et al.,* 1988). The high percentage found is probably indicative of the average Western diet, containing high amounts of SFA and oleic acid (n-9 MUFA), along with about 10% of their fat source coming from omega-6 PUFA, with little to no omega-3 PUFA in the diet.

In contrast to the palm oil control group, ALA-supplemented rats were found to significantly decrease AA concentrations (Figure 9), indicating that flax seed, and consequently ALA, may be sufficient alone to decrease AA concentrations in liver. Whether this action is through the limited conversion of ALA to EPA and DHA, or if ALA is exerting some other protective effect remains to be determined. It has been shown that not only do ALA and LA compete for the same Δ4 and Δ6 desaturases, but that the omega-3 PUFA has a greater affinity for these enzymes (Rose and Connolly, 1999). This leads to a reduction in AA production from LA in the presence of enough ALA (Rose and Connolly, 1999). The significance found in rat livers may also be in part due to the fact that both the omega-3 PUFA, ALA, and the omega-6 PUFA, LA, are metabolized into their respective longer chain fatty acids, for the most part in the liver (Rose and Connolly, 1999), leading to an increase in the concentrations of DHA, and in particular EPA, in the liver, which is consistent with a study (Goyens *et al.,* 2006) which found 6% of ALA from the plasma phospholipid pool used was converted into EPA. It has become apparent that EPA can displace AA from membrane phospholipids.

Supplementation with ALA significantly reduced ($p<0.05$) the amount of arachidonic acid concentrations, however EPA and DHA supplementation produced

greater and statistically significant decreases ($p<0.01$) than ALA (Table 4). It has been established that competition exists between AA and EPA to be converted by COX and LOX enzymes into their respective eicosanoids (Rose and Connolly, 1999; Lands, 1992). As with AA, EPA is converted into PGG, and then reduced into PGH, which is then further metabolized into PGE, PGI, and thromboxane A (Figure 28), except EPA is converted into 3-series prostanoids, while AA is converted into 2-series prostanoids with much more inflammatory effects (Lands, 1992).

My findings (Figure 7) support earlier work (Calder, 2002) which found that EPA supplementation decreased AA concentrations in liver by displacing them from membranes, as well as decreasing concentrations of AA-derived eicosanoids. My results are also in agreement with a study done in 2009 (Shearer *et al.,* 2010), using purified omega-3 PUFA ethyl esters, which found that EPA supplementation decreased AA concentrations by an average of 20%. They also found that these purified omega-3 PUFA lowered the LOX-catalyzed, AA-derived HODE isomers by 18%, again indicative of an increase in the omega-3/omega-6 PUFA ratio of substrates (Shearer *et al.,* 2010).

There is evidence that DHA may decrease AA phospholipid levels by decreasing Δ6 and Δ5 desaturation, thereby decreasing the formation of AA-derived eicosanoids (Grimsgaard *et al.,* 1997), however unlike EPA, DHA is not of the same chain length as AA, and does not produce eicosanoids that compete with those of AA, although it does produce its own oxylipins. Not only did I see a significant reduction ($p>0.05$) in AA concentrations with DHA supplementation (Figure 8), but I actually saw a slight (though not statistically significant) decrease AA concentrations in DHA-supplemented rats from

EPA-supplemented rats (Table 4). Although DHA is 2 carbons longer than AA, it has been found to have a similar effect to EPA in displacing AA from membrane phospholipids (Calder, 2002). DHA supplementation has also been found to decrease omega-6 eicosanoids and HODE isomers, while having no effect on LOX-catalyzed, EPA-derived HETE isomers (Shearer *et al.,* 2010). Whether this reduction is through competition for enzymes or phospholipases remains to be determined, but it is becoming clear that DHA supplementation is as capable as EPA at reducing AA concentrations in liver membrane phospholipids, and that both marine-derived omega-3 PUFA, DHA and EPA, are more likely to have AA lowering capabilities than the flaxseed-derived ALA.

iii) Other Omega-6 Polyunsaturated Fatty Acids in Liver Phospholipids

Two of the omega-6 PUFA were not significantly affected by diet ($p>0.05$), while other did not follow any specific pattern (Table 3), indicating that omega-3 PUFA supplementation does not appear to alter the concentrations of very long chain omega-6 PUFA. Of note was that the 18-carbon precursor for arachidonic acid, linoleic acid, did not significantly differ in its overall percentage with any omega-3 PUFA supplementation (Table 3). This is consistent with the findings in plasma and platelet phospholipids (von Schacky and Weber, 1985; Shearer *et al.,* 2010).

iv) Saturated and Monounsaturated Fatty Acids in Liver Phospholipids

The observation that the percentages of only 2 of the 7 SFA were significantly affected by omega-3 PUFA dietary supplementation (Table 3) is not a surprise, as we

know that omega-3 PUFA compete with omega-6 PUFA to be transformed into secondary products, and not with SFA. We know that omega-3 PUFA accumulate in phosphatidylethanolamine, phosphatidylcholine, and in triglycerides (de Bravo *et al.,* 1991). Unsaturated fatty acids in *de novo* synthesis of triglycerides are usually placed originally at the *sn*-2 position, but are then replaced by PUFA through the actions of phospholipid retailoring acyltransferases (Lands, 1992). Because of this, omega-3 PUFA usually occupy the sn-2 position of triglycerides, while SFA occupy the *sn*-1 and *sn*-3 positions (Zock *et al.,* 1995). This could explain why we do not see significant decreases in liver phospholipid SFA content.

Only 2 of the 6 MUFA were significantly affected ($p<0.05$) by dietary supplementation (Table 3). However, both C14:1 and C18:1 are of much smaller chain length then DHA, and it is uncertain if there is any physiological significance to this finding, and more work on the interactions between MUFA and PUFA are required.

v) Liver Lipid Composition by Fatty Acid Class

My finding that total omega-6 PUFA content was significantly decreased ($p<0.05$) by EPA and DHA supplementation, while ALA supplementation did not have a significant effect ($p>0.05$) when compared to the palm oil control groups (Figure 13) is important , and is in agreement with what I hypothesized. The omega-6 PUFA lowering advantage of fish oil derived omega-3 PUFA, EPA and DHA, over the flax seed derived ALA, has been shown in numerous studies (Shearer *et al.,* 2010; Lands, 1992; Anderson

and Ma, 2009), and may contribute to the various disease prognosis improvements we see with omega-3 PUFA dietary supplementation.

ALA-supplemented rats did have significantly higher total liver omega-3 PUFA lipid concentrations when compared to palm oil control groups ($p<0.05$), however, EPA and DHA-supplemented groups showed further decreases ($p<0.01$, Figure 14). This finding again suggests a benefit of marine-derived omega-3 PUFA sources over flax-seed sources.

Several studies have suggested that the ratio of omega-3 to omega-6 PUFA is of greater importance than the individual concentrations of each fatty acid class (Lands, 2008; Okuyama, 2001; Griffin 2008; Tribole *et al.,* 2006). As mentioned above, both PUFA compete for the same enzymes to be transformed into their respective eicosanoids, and the amount of substrate of each PUFA available appears to directly correlate with the amount of each type of eicosanoid produced (Rose and Connolly, 1999; von Schacky *et al.,* 1985; Neuringer *et al.,* 1988; Lands 1992). Again, it was found that EPA and DHA significantly increased ($p<0.01$) this ratio to the same degree, while ALA significantly increased ($p<0.05$) the ratio to a smaller degree compared to ratios of palm oil CTRL rats (Figure 15). Rats fed the palm oil CTRL diet showed an average omega-3/omega-6 in their liver lipids still higher than that of the average Western diet, usually between 0.1 and 0.025, while ALA supplementation produced liver lipids closer to the 1:1 ratio that has been suggested (Lands, 2005) to be optimal based on the evolutionary human diet. However, EPA and DHA supplementation produced liver lipids with a greater than a 1:1

ratio in favour of omega-3 PUFA (Figure 15). Again an added benefit of marine omega-3 PUFA over ALA, in modifying omega-6 eicosanoid production, was found.

vi) Omega-3 Polyunsaturated Fatty Acids in Brain Phospholipids

Although DHA supplementation significantly increased ($p<0.05$) DHA concentrations (Figure 10), EPA and ALA-supplementation did not ($p>0.05$) when compared to control groups (Table 6). Unlike in liver, I did not see a decrease in lipid DHA concentrations with the absence of DHA from the diet, but only a small decrease in DHA concentrations, although significant (Figure 10). In fact, it appears as though the brain has a homeostatic mechanism for maintaining high DHA concentrations even in the absence of DHA in the diet (Table 6). DHA makes up about one third of the total fatty acid content of ethanolamine and serine phospholipids in cerebral gray matter (Neuringer *et al.,* 1988), and its concentrations appear to be maintained regardless of dietary omega-3 PUFA intake (Neuringer *et al.,* 1988). Whether this is due to a much slower fatty acid turnover rate in the brain, due to the importance of DHA in maintaining proper brain function, or for another reason remains to be determined, but this observation leads us to an interesting avenue for future research to follow.

Brain EPA concentrations were significantly increased ($p<0.05$) by dietary supplementation with EPA and DHA (Table 6), however actual EPA abundance and increase was much lower than in liver (Figure 11). This finding may help explain PUFA composition in brain. It appears as though the brain has a mechanism for keeping EPA out of its phospholipids, even when high concentrations are provided through the diet.

Previous research (Chen *et al.,* 2011) has shown that EPA appears to be limited in brain phospholipids, and that this is probably due to extensive metabolism of EPA in brain, in part by β-oxidation, upon entry into the brain and upon de-esterification from phospholipids. However, the statistically significant increase in EPA concentrations with EPA supplementation (Figure 11), although small, appears to be sufficient enough to cause a significant (although much smaller than in liver) decrease in AA concentrations. Perhaps only a small increase in EPA, or decrease in AA, percent lipid composition is required to produce drastic alterations in brain function. This may be why the brain appears to maintain very low EPA concentrations, even when high amounts are fed in the diet (Figure 11).

ALA supplementation failed to significantly increase ($p>0.05$) EPA or DHA concentrations compared to those of palm oil control groups (Figure 12), again showing the importance of consuming EPA and DHA directly, and not relying solely on the conversion of ALA to obtain the longer chained omega-3 PUFA. A similar effect is also found when looking at brain ALA concentrations. A diet high in ALA concentrations failed to increase ALA concentrations (Figure 12). This suggests that ALA is may be rapidly converted into longer-chained PUFA in brain, or that it may have a potentially harmful effect on the brain, as discussed about with EPA, although future study is needed to justify these theories.

vii) Omega-6 Polyunsaturated Fatty Acids in Brain Phospholipids

As in liver , brains of DHA and EPA-supplemented rats had significantly ($p<0.05$) lower arachidonic acid concentrations than the ALA-supplemented and palm oil CTRL groups (Table 6). EPA's ability to significantly decrease AA concentrations (Figure 11) may show that only a small amount of EPA in the brain can have significant effects on AA metabolism (Philbrick *et al.,* 1987).These alterations in lipid composition may alter membrane fluidity, cellular responses, ion transport, as well as the synthesis of AA- and EPA-derived eicosanoids (Philbrick *et al.,* 1987), which may help to explain why the brain appears to be limiting EPA concentrations.

The increased incorporation of DHA into brain phospholipids may account for the decrease in AA concentrations with DHA supplementation (Figure 10), which has been suggested to have anti-bipolar effects (Bazinet *et al.,* 2006). It appears as though DHA supplementation may have the same ability as EPA supplementation to decrease AA uptake (Table 6), and potentially metabolism and eicosanoid production. Again, I found a significant difference ($p<0.05$) between marine and flaxseed generated omega-3 PUFA (Table 6), which may be due to the fact that ALA is converted to EPA, and consequently DHA, predominantly in the liver (Rose and Connolly, 1990). As suggested above, this may be the reason that I found a significant effect of ALA supplementation on AA concentrations in rat livers (through increased EPA and DHA competing with AA), and would be supported by the fact that I did not see a significant increase ($p>0.05$) in EPA concentrations with ALA supplementation in brain (Figure 12).

viii) Saturated and Monounsaturated Fatty Acids in Brain Phospholipids

The observation that dietary omega-3 PUFA supplementation failed to significantly affect ($p>0.05$) any of the seven SFA (Table 5) may not be surprising, given that, as I mentioned above, omega-3 PUFA and SFA tend to occupy different positions when incorporated into triglycerides, possibly preventing omega-3 PUFA from replacing SFA in the lipid bilayers of mammalian membranes (Lands, 1992).

The finding that omega-3 PUFA dietary supplementation failed to significantly affect ($p>0.05$) the levels of any of the six MUFA (Table 5) is more surprising, as unsaturated fatty acids are usually replaced by highly unsaturated fatty acids at the *sn*-2 position in triglycerides (Lands, 1992). It appears as though this phospholipid acyltransferase retailoring is either not significant enough to affect overall concentrations of MUFA, or that perhaps the *in vivo* production of these MUFA by the body increases to ensure that their concentrations do not decrease dramatically in the presence increased omega-3 PUFA concentrations, indicating a possible increased importance of MUFA in brain as opposed to liver, possibly to protect against age-related cognitive decline (Solfrizzi *et al.,* 1999), although the relatively small change in omega-3 PUFA concentrations observed may have been insufficient to impact MUFA concentrations.

ix) Brain Phospholipid Composition by Fatty Acid Class

Much like in liver, EPA and DHA supplementation had the greatest ability to increase ($p<0.05$) total brain omega-3 PUFA concentrations (Figure 16), however unlike

in liver, ALA supplementation did not significantly result in differences ($p>0.05$) in total omega-3 PUFA concentrations from those of palm oil control rats (Figure 16). The increases in total omega-3 PUFA concentrations with EPA and DHA supplementation again were balanced by significant decreases ($p<0.05$) in total omega-6 PUFA concentration compared to ALA-supplemented and palm oil CTRL groups (Figure 17). This may be explained by the fact that most of the conversion of ALA into the longer-chained omega-3 PUFA occurs in the liver (Rose and Connolly, 1990). Again, it is worth mentioning that the changes, while statistically significant, were much lower in brain than in liver. Although it has been shown that both AA and DHA have a rapid turnover rate in rodent brains (Rapoport, 2008), it appears from my results as though the brain more strictly regulates its fatty acid content than liver, even when dietary conditions are changed.

x) TBARS Analysis

Changes in MDA concentrations have been associated with various diseases, including schizophrenia (Mahadik *et al.,* 2006). My results provide some potentially interesting insight into the relationship of omega-3 PUFA and oxidative stress.

Contrary to my hypothesis, my results failed to show any significant difference ($p<0.05$) in liver or brain TBARS concentrations with dietary omega-3 supplementation of any kind (Figures 19, 20). I anticipated to see increases in oxidative stress in rats fed diets high in omega-3 PUFA, in comparison to the palm oil CTRL group, as omega-3

PUFA have more double bonds than SFA and MUFA, which should make them more susceptible to lipid peroxidation by ROS (Tsuduki *et al.,* 2011).

Previous works on omega-3 PUFA and oxidative stress have lead to similar findings to mine, with further reductions in oxidative stress being found with omega-3 PUFA treatment (Barbosa *et al,.* 2003; Wu *et al.,* 2004). Although PUFA contain more double bonds (a preferred site of attack for ROS) and show an increased propensity for lipid peroxidation *in vitro*, they do not show increased lipid peroxidation and oxidative stress *in vivo*. My results are consistent with such conclusions and I will review some of the mechanisms explaining how this may occur.

When we think of oxidative stress, we think of an antioxidant defense system incapable of combating the level of ROS in the tissue. This leads us to the possibility that omega-3 PUFA have the capability to reduce the production of free radicals. We know that EPA competes with AA to be transformed into their respective metabolites, and it has been proposed that this competition, and subsequent reduction in AA-derived metabolites, specifically leukotriene B4 through the LOX pathway, may be one of the most important ways that omega-3 PUFA can reduce oxidative stress (Barbosa *et al.,* 2003). It has been widely documented that the reduction of AA-derived metabolites by omega-3 PUFA competition has shown beneficial effects in a wide range of situations (Rose and Connolly, 1990; Lands, 1992), and this could be another benefit of this competition.

It is possible that omega-3 PUFA may be exerting a positive effect by boosting the antioxidant defense system. It has been proposed that omega-3 PUFA themselves may act as free radical scavengers, protecting the body against the overall effect of oxidative stress (Barbosa *et al.,* 2003). Not only did Barbosa and colleagues find a reduction in plasma lipid peroxide formation, but they also found that omega-3 PUFA treatment lead to a significant increase in plasma antioxidant capacity (Barbosa *et al.,* 2003). This hypothesis has been supported by another study (Palozza *et al.,* 2002), who studied the effects of omega-3 PUFA on oxidative stress and antioxidant levels in human erythrocytes (Palozza *et al.,* 2002). During their research they found that omega-3 PUFA supplementation not only changed lipid composition, but was also found to modulate α-tocopherol (a form of vitamin E, preferentially absorbed in humans) concentration. It was found that concentrations of this antioxidant increased with omega-3 PUFA supplementation after 30 days in red blood cell membranes (Palozza *et al.,* 2002). This presents a very interesting solution to the questions raised by their apparent ability to reduce oxidative stress. These results suggest the existence of a membrane mechanism of homeostasis that functions to maintain adequate tissue concentrations of vitamin E (α-tocopherol, a potent antioxidant) to avoid the increased lipid peroxidation that results from a higher membrane omega-3 PUFA content (Palozza *et al.,* 2002). This would help to explain the lack of dietary influence found in my results after 8 weeks (Figures 19, 20), through the increased concentration of α-tocopherol, although future work would be required to test this hypothesis.

However, Palozza *et al.* 2002 also found that while no changes in lipid peroxidation were noticed after 30 days of omega-3 PUFA supplementation, after 180

days of omega-3 PUFA supplementation lipid peroxidation was significantly increased in red blood cell membranes, combined with a return to baseline α-tocopherol concentrations (Palozza *et al.,* 2002). This decrease in α-tocopherol concentrations after 180 days led to increased lipid peroxidation in their study (Palozza *et al.,* 2002), and may help to explain why no significant increases were found in my experiment (Figures 19, 20), where the feeding period was only 8 weeks.

Although these are both hypotheses that would make sense, the mechanisms by which they accomplish either or both of these processes need to be explained. This leads me to suggest that a combination of omega-3 PUFA and antioxidants in the diet may be the best way to obtain the cardiovascular benefits of omega-3 PUFA, while limiting their potential negative effects. This combination has been suggested by other researchers as well (Mahadik *et al.,* 2006).

xi) Tissue Aldehyde Analysis

SIFT-MS was used to analyze the headspace aldehyde concentrations of my tissue. As with my TBARS analysis, I hypothesized that the extra double bonds in the marine-type omega-3 dietary supplemented groups would lead to increased headspace aldehyde concentrations (Tsuduki *et al.,* 2011), specifically, that increases in omega-3 fatty acid consumption would lead to increased propanal concentrations in tissue (Shen et al., 2007), while decreasing hexanal concentrations through a decrease in dietary omega-6 fatty acid content (Jaar et al., 1999).

Liver

According to ANOVA analysis, only liver butanal headspace concentrations were found to be significantly affected ($p<0.05$) by omega-3 dietary content (Tables 10, 11, 12). However, the variability in my aldehyde concentration data precludes any conclusion that dietary omega-3 PUFA supplementation significantly affected aldehyde concentrations. The use of a Bonferroni's correction to correct for the multiple analyses performed, may have eliminated statistical significance found.

No trend was found when looking at liver propanal concentrations. In fact, total omega-3 PUFA content did not significantly correlate with propanal concentrations in liver (Figure 25) or brain, and the same can be said for tissue acetaldehyde concentrations. Total omega-6 PUFA content also did not significantly correlate with hexanal concentrations in either liver (Figure 26) or brain. These findings suggest that omega-3 PUFA dietary intake has no significant effect on aldehyde concentrations in the liver and brain tissue of rats at dietary concentrations used in my experiment. My findings differ from those of previous studies using fish meal as a source of omega-3 PUFA, which consistently found a positive correlation between dietary omega-3 PUFA supplementation and brain aldehyde and plasma acetaldehyde concentrations (Tampier *et al.,* 1985; Quintanilla *et al.,* 1993; Tampier and Quintanilla, 2002; Quintanilla *et al.,* 2002). This leads to the hypothesis that the 95% pure omega-3 PUFA used in our study, might not only allow us to investigate the effects of individual omega-3 PUFA, but may also produce different effects than combination omega-3 fish sources. It is possible that the positive correlations made in previous studies may have been due to something other

than the omega-3 PUFA provided in the fish meal diets. As with the TBARS analysis, it is also possible that the lack of significant in headspace tissue aldehyde concentrations between dietary groups is due to the relatively short feeding period (Barbosa *et al.,* 2003), which may coincide with an initial increase in aldehyde metabolism in the short-term by the body, to defend against the toxic aldehydes.

Brain

As with liver butanal concentrations, I propose that the significant differences found at room temperature and 60°C (Figures 21, 22), are more due to a statistical anomaly, and are not indicative of any physiological significance, and may have been eliminated by using a Bonferroni's correction.

Interestingly enough, DHA and EPA supplementation did not significantly increase brain propanal concentrations (Figure 25) or decrease brain hexanal concentrations (Figure 26) as hypothesized. Brain acetaldehyde concentrations did not follow any significant pattern either (Tables 7, 8, 9). These results suggest no effect of purified omega-3 supplementation on tissue aldehyde concentrations in brain, and along with the results found in liver, indicate that aldehydes may indeed be a stable biomarker of oxidative stress (Fuchs *et al.,* 2009). Further studies may want to use larger sample sizes, and longer feeding periods to test for a potential short-term increase in aldehyde metabolism leading to misleading results in shorter feeding periods.

E) Conclusion

Liver and brain lipid compositions are not only directly affected by total omega-3 PUFA consumption, but also by which types of omega-3 FAs are consumed. My results support previous works (Gerster, 1998; Goyens *et al.,* 2006) showing that ALA consumption alone is insufficient in increasing concentrations of the long-chained omega-3 PUFA, EPA and DHA, when compared to EPA and DHA supplementation themselves. ALA also appears incapable of increasing EPA concentrations, or decreasing AA concentrations in my study, enough to affect competition between these two substrates for COX and LOX enzymes. This may help explain why dietary ALA supplementation has not shown the same cardiovascular benefits as fish oil supplementation (Anderson and Ma, 2009). Interestingly enough, my results suggest that the inclusion of EPA alone in the diet may not be able to produce sufficiently high DHA concentrations to maintain proper brain structure (Holman, 1998; Fitzpatrick, 2002), while dietary DHA supplementation appears unable to produced EPA concentrations as high as consuming EPA itself does.

Interestingly, ALA and EPA supplementation seems to have less of an effect on ALA and EPA concentration in brain than in liver, while DHA concentrations appear to be maintained in brain regardless of dietary composition in my study. These results will need to be reproduced to determine whether this consistently occurs, and if this is a sign that the brain has a mechanism for limiting EPA concentration, and if so, is this to limit competition with arachidonic acid. DHA concentrations are probably maintained due to their importance in maintaining proper brain development and function (Holman, 1998).

It appears as though, EPA and DHA consumption is necessary to gain the cardiovascular benefits found in various studies on omega-3 PUFA in liver, but that DHA is the only omega-3 PUFA that is important structurally in brain, as it appears to be conserved in brain tissue even under low omega-3 PUFA consumption.

My results did not show any significant effects of omega-3 PUFA dietary consumption on headspace aldehyde or TBARS concentrations, indicating that diet may not affect the concentrations of either. On the basis of my work, it seems as though aldehydes may provide a reliable biomarker for oxidative stress, as they appear to be unaffected by dietary composition. Certain aldehydes also appear to be a good marker for oxidative stress, which is associated with various diseases, such as cancer (Fuchs et al., 2009). It is important to note that in my study rats were fed for 8 weeks, and that based on the work of other studies, I may have found different results if dietary omega-3 PUFA supplementation was carried on for a greater period of time. I cannot rule out an initial boost in the body's natural antioxidant defense system (Barbosa *et al.* 2003) as the cause for the lack of significant differences in tissue aldehyde levels between dietary groups. It is possible that this defense was able to counter the increased production of LP-DA resulting from the increased lipid peroxidation of the PUFA through increased aldehyde metabolism, or that this boost was able to limit lipid peroxidation itself by neutralizing ROS.

Future studies will need to focus on using highly purified omega-3 PUFA, as used in my study, to test the effect of dietary omega-3 PUFA supplementation on aldehyde

concentrations over a period greater than 4 months. This will help to determine whether my findings are an indication of the individual effects of the highly purified omega-3 PUFA used (instead of the crude oils used in the past), or whether they are due to a short-term boost in the body's endogenous defense system.

Figures and Tables

Table 1: List of chemical structure, scientific and common names for each fatty acid analyzed.

Chemical Formula	Scientific Name	Common Name
C14:0	tetradecanoic acid	Myristic acid
C14:1	N/A	Physeteric acid
C15:0	N/A	N/A
C16:0	hexadecanoic acid	Palmitic acid
C16:1	9-hexadecenoic acid	Palmitoleic acid
C18:0	octadecanoic acid	Stearic acid
C18:1	9-octadecenoic acid	Oleic acid
C18:2 n-6	9,12-octadecadienoic acid	Linoleic acid (LA)
C18:3 n-6	6,9,12-octadecatrienoic acid	Gamma-linoleic
C18:3 n-3	9,12,15-octadecatrienoic acid	Alpha-linoleic acid
C18:4 n-3	N/A	Stearidonic acid
C20:0	eicosanoic acid	Arachidic acid
C20:1	9-eicosenoic acid	Gadoleic acid
C20:2 n-6	N/A	N/A
C20:3 n-6	N/A	Dihomo-gamma-
C20:4 n-6	5,8,11,14-eicosatetraenoic acid	Arachidonic acid
C20:3 n-3	N/A	N/A
C20:4 n-3	N/A	N/A
C20:5 n-3	5,8,11,14,17-eicosapentaenoic acid	EPA
C22:0	docosanoic acid	Behenic acid
C22:1	13-docosenoic acid	Erucic acid
C22:2 n-6	N/A	N/A
C22:4 n-6	N/A	N/A
C22:5 n-6	N/A	N/A
C22:5 n-3	N/A	DPA
C22:6 n-3	4,7,10,13,16,19-docosahexaenoic	DHA
C24:0	tetracosanoic acid	Lignoceric acid
C24:1	N/A	Nervonic acid

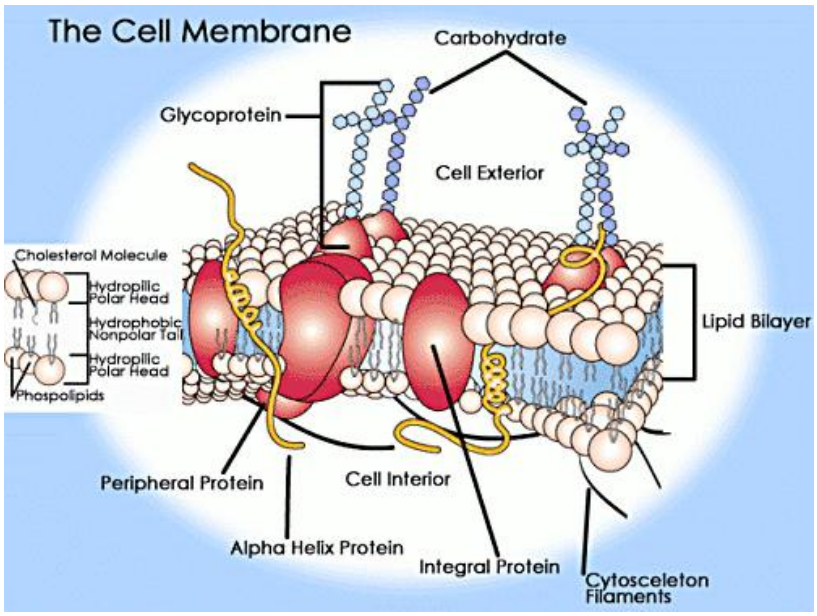
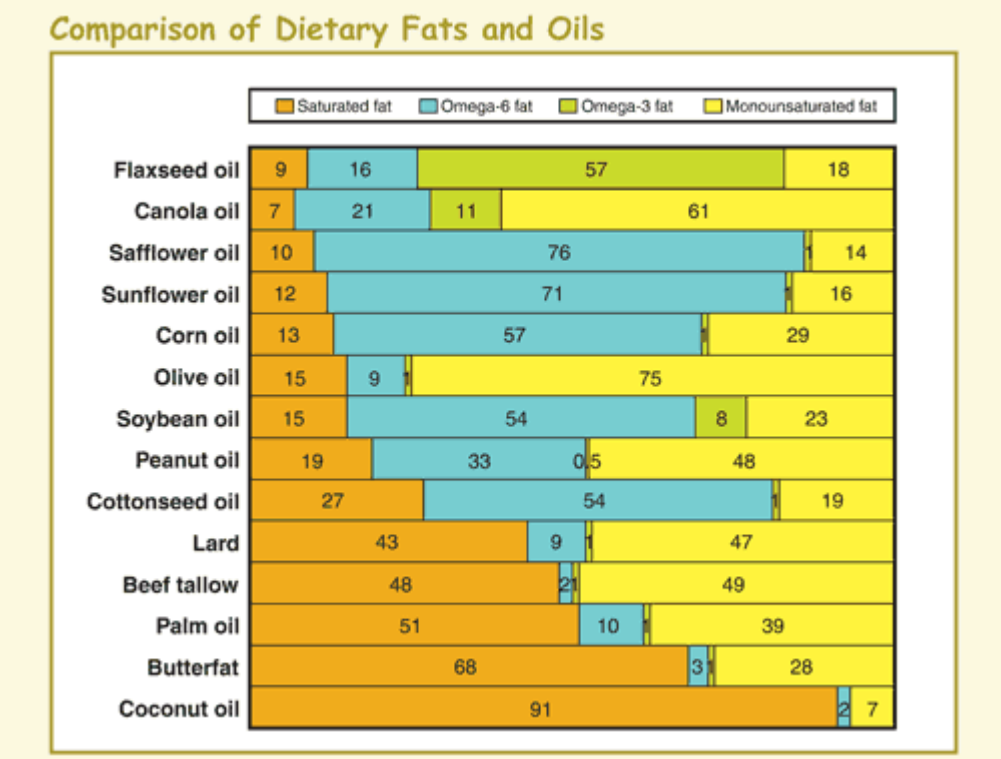


Figure 1: Lipid bilayer of the cell membrane showing integral membrane proteins responsible for transportation across membrane bilayer (Sparksnotes 2012)

Table 2: Fatty acid composition (%) of some common sources of dietary oils (Edwards, 2007).



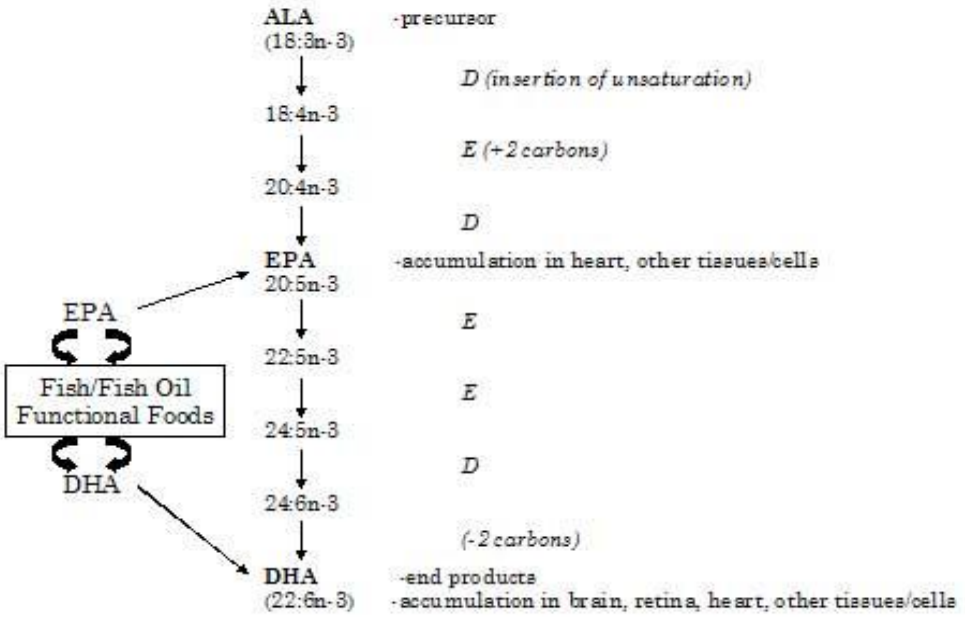


Figure 2: Synthesis of EPA and DHA from 18-carbon ALA through desaturation and elongation events (Possick 2010)

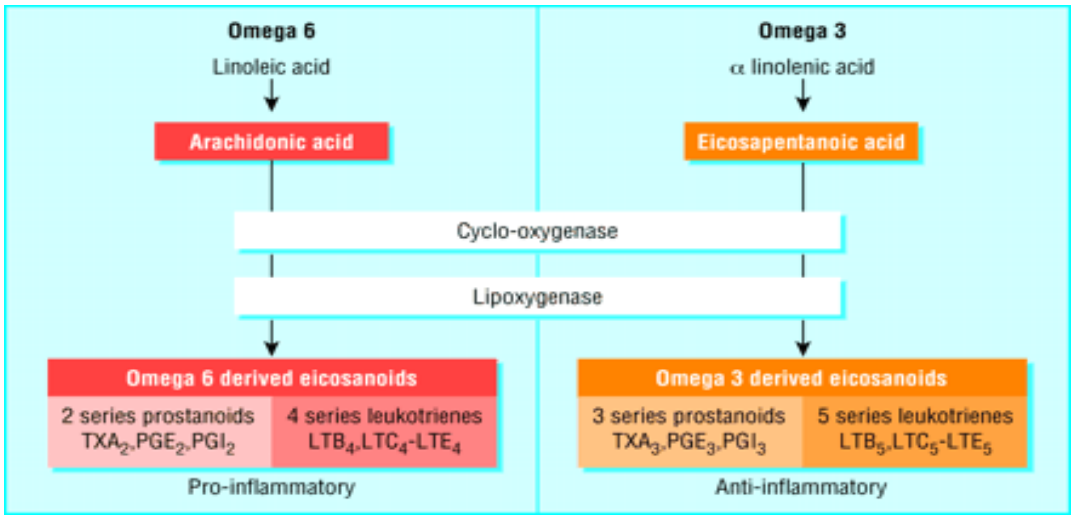


Figure 3: Conversion of different PUFA into different eicosanoids, done by same COX and LOX enzymes. Depending on the precursor fatty acid present, different types of eicosanoids are generated. (Anonymous, 2011)

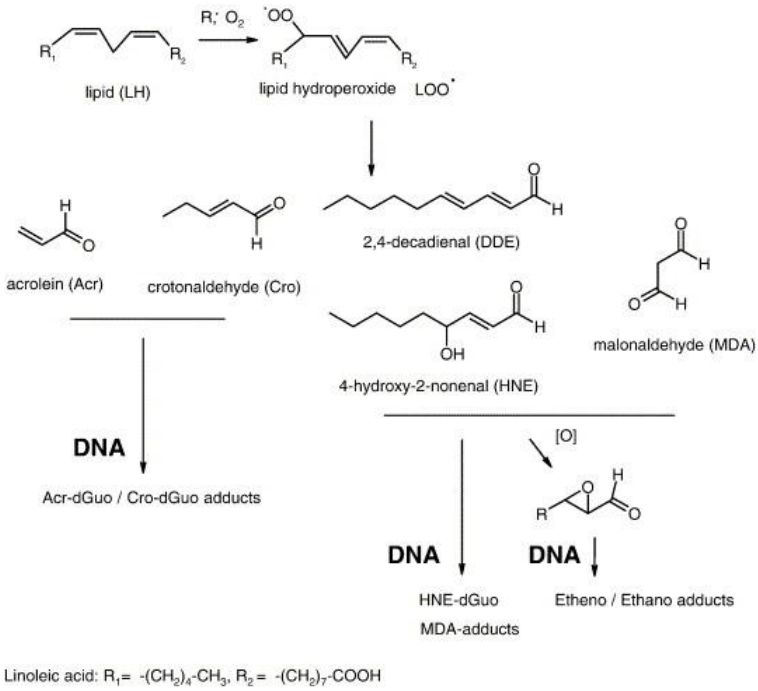


Figure 4: Degredation of lipid peroxides, producing malondialdehyde, 4-HNE and other aldehydes (Alves de Almeida *et al.,* 2007).

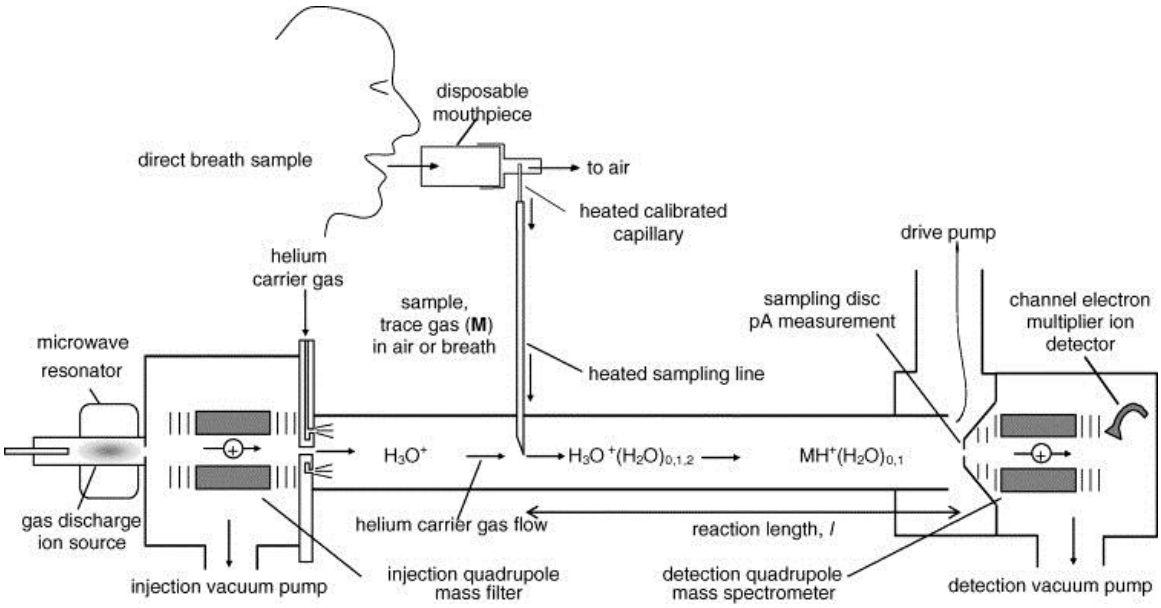


Figure 5: Schematic diagram of a SIFT-MS machine showing sample microwave resonator ion source, which produces precursor ions that react with samples introduced downstream either through breath or headspace analysis. Ions react and are carried along the flow tube by helium gas, and finally sampled by the downstream quadrupole mass spectrometer (Spanel *et al.* 2005).

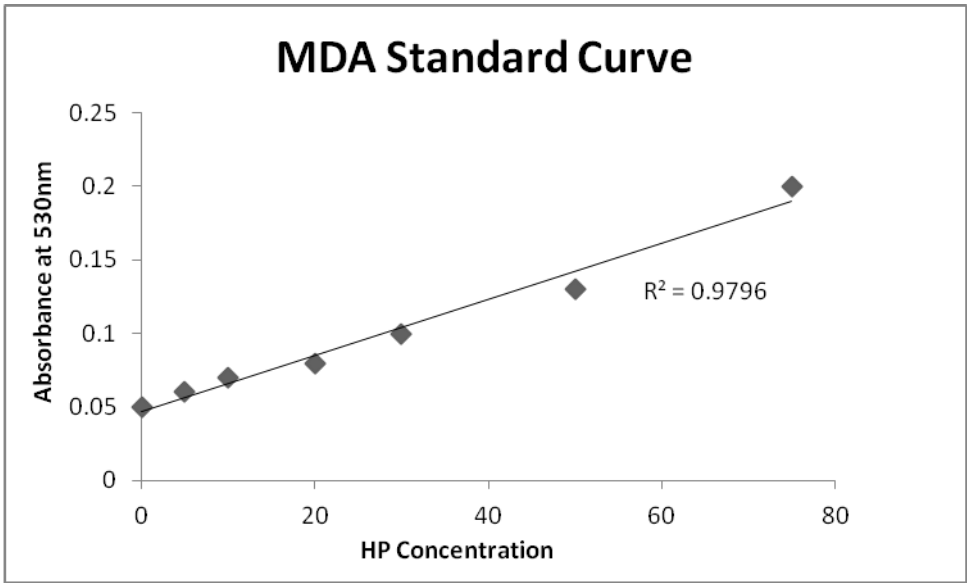


Figure 6: Malondialdehyde (MDA) standard curve. Prepared using varying concentrations of MDA standard and distilled water. HP concentration indicates final hydroperoxide concentration of MDA + water mixture.

Table 3: ANOVA results testing for significant differences in liver lipid compositions between dietary groups. F-values are used to find significant differences between dietary groups. *indicates a statistically significant difference was found between groups ($p<0.05$). Tukey Tests were then performed to determine which dietary groups produced the differences. LA – linoleic acid, ALA – alpha-linolenic acid, AA – arachidonic acid, EPA – eicosapentaenoic acid, DHA – docosahexaenoic acid

Fatty Acid	Df	MS	F	P
C14:0*	3	0.155	7.76	0.001
C14:1*	3	0.004	3.93	0.019
C15:0*	3	0.004	3.94	0.019
C16:0	3	3.620	0.80	0.505
C16:1	3	4.268	2.29	0.101
C18:0	3	7.266	2.78	0.061
C18:1*	3	103.6	7.73	0.001
C18:2 n-6 LA	3	5.813	2.04	0.132
C18:3 n-6*	3	0.019	3.42	0.032
C18:3 n-3* ALA	3	2.476	44.1	<0.001
C18:4 n-3*	3	0.001	3.91	0.019
C20:0	3	<0.001	0.40	0.754
C20:1	3	0.004	1.27	0.305
C20:2 n-6	3	0.001	1.65	0.202
C20:3 n-6*	3	0.298	10.6	<0.001
C20:4 n-6 AA*	3	150.2	42.4	<0.001
C20:3 n-3*	3	0.002	22.5	<0.001
C20:4 n-3*	3	0.003	9.96	<0.001
C20:5 n-3 EPA*	3	85.55	36.9	<0.001
C22:0	3	0.001	2.40	0.090
C22:1	3	<0.001	0.99	0.410
C22:2 n-6*	3	0.002	7.52	0.001
C22:4 n-6	3	0.001	0.77	0.522
C22:5 n-6*	3	1.424	74.8	<0.001
C22:5 n-3*	3	10.38	14.6	<0.001
C22:6 n-3 DHA*	3	162.1	52.8	<0.001
C24:0	3	0.940	0.85	0.477
C24:1	3	0.001	0.53	0.660

Table 4: Mean liver lipid composition (%) +/- one standard deviation of each fatty acid for each dietary group (EPA, DHA, ALA, CTRL). *indicates a significant at $p<0.05$, ** indicates a significance at $p<0.01$, and *** indicates a difference at $p<0.005$, as compared to palm oil control groups from Tukey Test analysis. CTRL = palm oil control, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid.

	EPA	**DHA**	**ALA**	**CTRL**
C14:0*	0.6 +/- 0.1*	0.52 +/- 0.11*	0.68 +/- 0.13	0.86 +/- 0.2
C14:1*	0.03 +/- 0.02	0.02 +/- 0.01*	0.03 +/- 0.02	0.07 +/- 0.05
C15:0*	0.1 +/- 0.07	0.13+/- 0.05*	0.11 +/- 0.07	0.09 +/- 0.07
C16:0	27.1 +/- 2.06	27+/- 2.4	26.08 +/- 1.4	25.69 +/- 2.44
C16:1	3.35 +/- 1.31	2.8 +/- 1.1	3.56 +/- 1.03	4.59 +/- 1.84
C18:0	10.8 +/- 1.6	13 +/- 1.2	11.62 +/- 2.09	10.66 +/- 1.45
C18:1*	22.75 +/- 2.36*	20+/- 2.8*	24.9 +/- 5.61	28.51 +/- 3.27
C18:2 n-6	8.63 +/- 1.83	8.5 +/- 1.1	9.04 +/- 1.17	7.04 +/- 2.24
C18:3 n-6*	0.1 +/- 0.03*	0.15 +/- 0.12	0.1 +/- 0.03*	0.21 +/- 0.08
C18:3 n-3 *	0.18 +/- 0.05	0.24 +/- 0.27	1.34 +/- 0.41*	0.1 +/- 0.04
C18:4 n-3*	0.02 +/- 0.01	0.03 +/- 0.01	0.03 +/- 0.02*	0 +/- 0
C20:0	0.06 +/- 0.01	0.06 +/- 0.01	0.06 +/- 0.01	0.06 +/- 0.01
C20:1	0.25 +/- 0.06	0.22 +/- 0.03	0.26 +/- 0.04	0.28 +/- 0.08
C20:2 n-6	0.1 +/- 0.02	0.11 +/- 0.01	0.13 +/- 0.04	0.12 +/- 0.03
C20:3 n-6*	0.57 +/- 0.14	0.87 +/- 0.18*	0.8 +/- 0.22*	0.44 +/- 0.12
C20:4 n-6 *	6.1 +/- 0.85**	5.7 +/- 0.74**	10.13 +/- 2.33*	15.17 +/- 2.7
C20:3 n-3*	0.01 +/- 0.01	0 +/- 0	0.04 +/- 0.01*	0 +/- 0.01
C20:4 n-3*	0.04 +/- 0.02	0.04 +/- 0.01	0.06 +/- 0.03*	0.01 +/- 0.01
C20:5 n-3 *	7.81 +/- 2.72***	4.2 +/- 1.1**	2.14 +/- 0.44	0.11 +/- 0.05
C22:0	0.07 +/- 0.01	0.01 +/- 0.01	0.07 +/- 0.02	0.06 +/- 0.01
C22:1	0 +/- 0	0.02 +/- 0.01	0 +/- 0	0 +/- 0
C22:2 n-6	0.02 +/- 0.01	0.05 +/- 0.03	0 +/- 0	0.02 +/- 0.01
C22:4 n-6	0.07 +/- 0.02	0.06 +/- 0.01	0.08 +/- 0.04	0.07 +/- 0.02
C22:5 n-6*	0.01 +/- 0.02***	0.03 +/- 0.01***	0.03 +/- 0.02***	0.87 +/- 0.26
C22:5 n-3*	2.97 +/- 1.53*	1 +/- 0.25	1.27 +/- 0.48	0.27 +/- 0.17
C22:6 n-3 *	7.14 +/- 0.77*	15.3 +/- 2.6***	7.07 +/- 2.22*	4.33 +/- 0.88
C24:0	0.93 +/- 2.02	0.27 +/- 0.05	0.24 +/- 0.07	0.22 +/- 0.03
C24:1	0.17 +/- 0.07	0.16 +/- 0.01	0.14 +/- 0.04	0.15 +/- 0.02

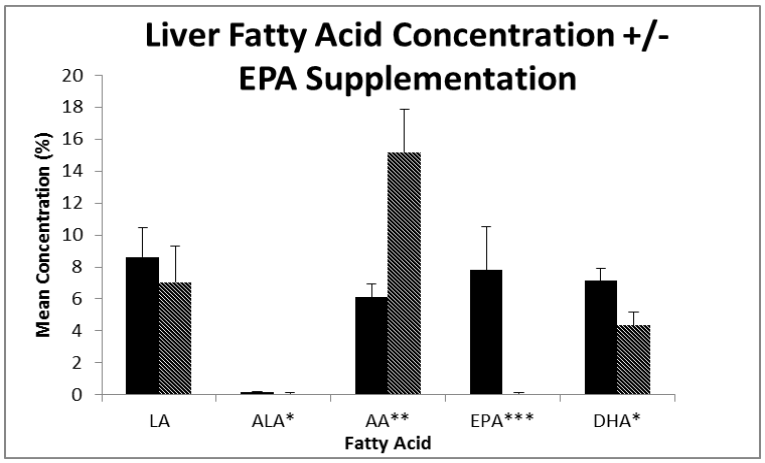


Figure 7: Effect of dietary EPA supplementation on LA, ALA, AA, EPA, DHA concentrations in brain, as compared to palm oil control groups. Solid black columns represent concentrations with EPA supplementation, while striped columns represent fatty acid concentrations of the palm oil control group. Error bars indicate one standard deviation. Statistical significance of difference between EPA and CTRL groups was analyzed using ANOVA and Tukey Tests with a significance at $p<0.05$ indicated by *, a significance at $p<0.01$ indicated by **, and a significance at $p<0.005$ indicated by ***. LA- linoleic acid, ALA- alpha-linolenic acid, AA- arachidonic acid, EPA- eicosapentaenoic acid, DHA- doccosahexaenoic acid.

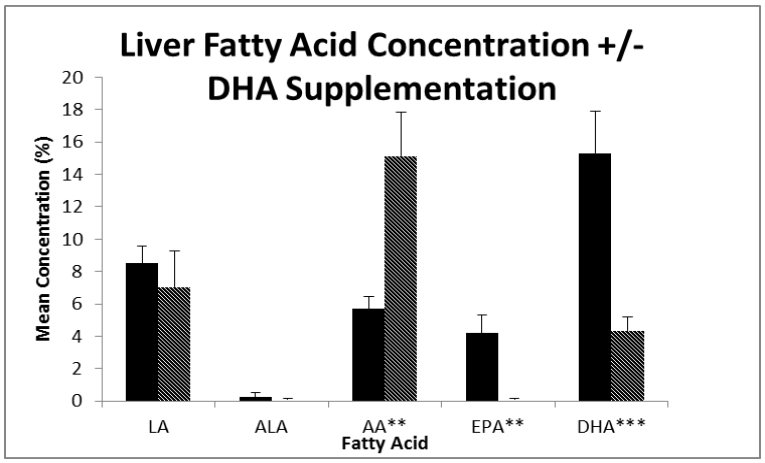


Figure 8: Effect of dietary DHA supplementation on LA, ALA, AA, EPA, DHA concentrations in brain, as compared to palm oil control groups. Solid black columns represent concentrations with DHA supplementation, while striped columns represent fatty acid concentrations of the palm oil control group. Error bars indicate one standard deviation. Statistical significance of difference between DHA and CTRL was analyzed using ANOVA and Tukey Tests with a significance at $p<0.05$ indicated by *, a significance at $p<0.01$ indicated by **, and a significance at $p<0.005$ indicated by ***. LA- linoleic acid, ALA- alpha-linolenic acid, AA- arachidonic acid, EPA- eicosapentaenoic acid, DHA- doccosahexaenoic acid.

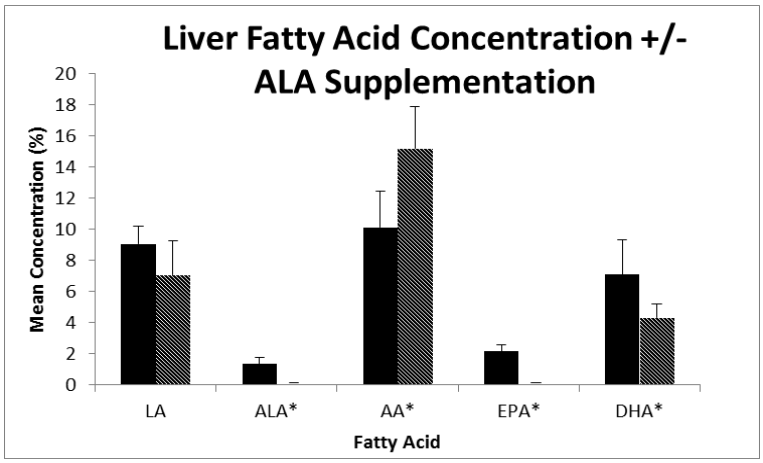


Figure 9: Effect of dietary ALA supplementation on LA, ALA, AA, EPA, DHA concentrations in liver, as compared to palm oil control groups. Solid black columns represent concentrations with ALA supplementation, while striped columns represent fatty acid concentrations of the palm oil control group. Error bars indicate one standard deviation. Statistical significance of difference between ALA and CTRL was analyzed using ANOVA and Tukey Tests with a significance at $p<0.05$, and represented in the figure with a *. LA- linoleic acid, ALA- alpha-linolenic acid, AA- arachidonic acid, EPA- eicosapentaenoic acid, DHA- doccosahexaenoic acid.

Table 5: ANOVA results testing for significant differences in brain lipid compositions between dietary groups. F-values are used to find significant differences between dietary groups. *indicates a significant difference was found between groups ($p<0.05$). LA = linoleic acid, AA = arachidonic acid, ALA = alpha-linolenic acid, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid

Fatty Acid	Df	MS	F	P
C14:0	3	0.001	0.657	0.586
C14:1	3	0.040	1.287	0.299
C15:0	3	0.001	0.107	0.955
C16:0	3	0.150	0.130	0.940
C16:1	3	0.003	0.741	0.537
C18:0	3	0.190	0.950	0.431
C18:1	3	0.190	0.180	0.907
C18:2 n-6 LA	3	0.059	3.458	0.031
C18:3 n-6	3	0.004	1.471	0.245
C18:3 n-3 ALA	3	0.001	1.828	0.166
C18:4 n-3	3	<0.001	2.176	0.114
C20:0	3	0.007	0.895	0.456
C20:1	3	0.269	0.632	0.600
C20:2 n-6	3	0.075	0.990	0.412
C20:3 n-6*	3	0.072	17.76	<0.001
C20:4 n-6 AA*	3	4.233	9.695	<0.001
C20:3 n-3	3	0.003	0.994	0.378
C20:4 n-3	3	0.003	1.102	0.365
C20:5 n-3 EPA*	3	0.069	42.85	<0.001
C22:0	3	0.006	1.53	0.452
C22:1	3	0.008	0.533	0.663
C22:2 n-6	3	0.001	0.892	0.457
C22:4 n-6*	3	0.948	41.82	<0.001
C22:5 n-6*	3	0.330	91.82	<0.001
C22:5 n-3*	3	0.487	170.5	<0.001
C22:6 n-3 DHA*	3	4.587	7.427	<0.001
C24:0	3	0.010	0.233	0.872
C24:1	3	0.259	0.620	0.608

Table 6: Mean brain lipid composition (%) +/- one standard deviation of each fatty acid for each dietary group (EPA, DHA, ALA, CTRL). *indicates a significant at p<0.05, ** indicates a significance at p<0.01, and *** indicates a difference at p<0.005, as compared to palm oil control groups from Tukey Test analysis. CTRL = palm oil control, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid.

	EPA	**DHA**	**ALA**	**CTRL**
C14:0	0.22 +/- 0.02	0.23 +/- 0.05	0.21 +/- 0.02	0.21 +/- 0.02
C14:1	0.05 +/- 0.03	0.19 +/- 0.34	0.04 +/- 0.03	0.04 +/- 0.02
C15:0	0.12 +/- 0.09	0.11 +/- 0.05	0.13 +/- 0.12	0.13 +/- 0.07
C16:0	22.41 +/- 1.01	22.46 +/- 1.03	22.6 +/- 1.05	22.24 +/- 1.17
C16:1	0.52 +/- 0.08	0.55 +/- 0.06	0.54 +/- 0.08	0.5 +/- 0.03
C18:0	19.36 +/- 0.55	19.28 +/- 0.4	19.47 +/- 0.21	19.09 +/- 0.51
C18:1	24.3 +/- 1.42	24.21 +/- 0.37	23.9 +/- 0.79	24.13 +/- 1.16
C18:2n6	0.82 +/- 0.22	0.84 +/- 0.07	0.79 +/- 0.06	0.65 +/- 0.09
C18:3n6	0.02 +/- 0.03	0 +/- 0	0 +/- 0	0.05 +/- 0.11
C18:3n3	0.03 +/- 0.04	0 +/- 0.01	0.01 +/- 0.02	0.02 +/- 0.03
C18:4n3	0.01 +/- 0.02	0 +/- 0	0 +/- 0	0 +/- 0
C20:0	0.53 +/- 0.07	0.51 +/- 0.08	0.51 +/- 0.08	0.58 +/- 0.11
C20:1	3.37 +/- 0.36	3.35 +/- 0.4	3.32 +/- 0.6	3.72 +/- 1.02
C20:2n6	0.71 +/- 0.48	0.53 +/- 0.2	0.51 +/- 0.09	0.5 +/- 0.05
C20:3n6*	0.54 +/- 0.06*	0.62 +/- 0.05*	0.52 +/- 0.02*	0.39 +/- 0.09
C20:4n6 *	8.22 +/- 0.89*	8.19 +/- 0.49*	9.34 +/- 0.54	9.61 +/- 0.62
C20:3n3	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0
C20:4n3	0.04 +/- 0.1	0 +/- 0	0 +/- 0	0 +/- 0.01
C20:5n3 *	0.2 +/- 0.06*	0.09 +/- 0.05*	0.01 +/- 0.02	0 +/- 0
C22:0	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0
C22:1	0.3 +/- 0.1	0.31 +/- 0.13	0.28 +/- 0.15	0.36 +/- 0.11
C22:2n6	0.02 +/- 0.02	0 +/- 0.01	0.02 +/- 0.03	0.02 +/- 0.03
C22:4n6*	1.98 +/- 0.18**	1.88 +/- 0.16**	2.3 +/- 0.13*	2.64 +/- 0.11
C22:5n6*	0.12 +/- 0.08*	0.02 +/- 0.05**	0.11 +/- 0.06*	0.48 +/- 0.05
C22:5n3*	0.71 +/- 0.09**	0.35 +/- 0.02*	0.31 +/- 0.04*	0.12 +/- 0.03
C22:6n3*	12.64 +/- 0.98	13.71 +/- 0.61*	12.76 +/- 0.49	11.86 +/- 0.89
C24:0	0.89 +/- 0.21	0.96 +/- 0.25	0.9 +/- 0.12	0.95 +/- 0.22
C24:1	1.86 +/- 0.92	1.6 +/- 0.62	1.4 +/- 0.42	1.71 +/- 0.44

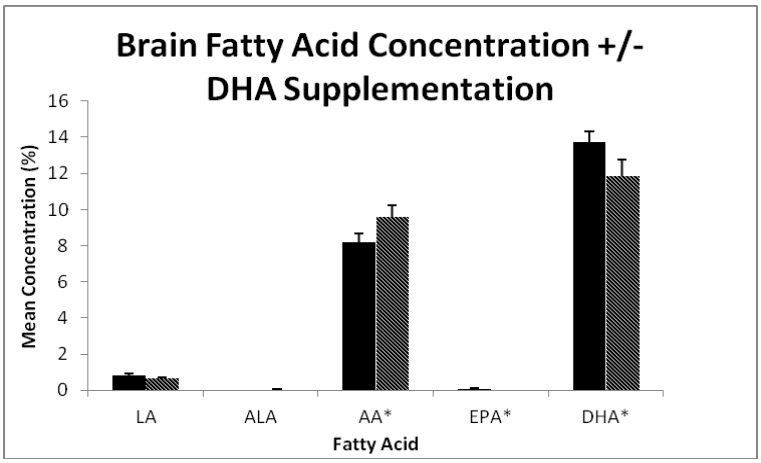


Figure 10: Effect of dietary DHA supplementation on LA, ALA, AA, EPA, DHA concentrations in brain, as compared to palm oil control groups. Solid black columns represent concentrations with DHA supplementation, while striped columns represent fatty acid concentrations of the palm oil control group. Error bars indicate one standard deviation. Statistical significance of difference between DHA and CTRL was analyzed using ANOVA and Tukey Tests with a significance at $p<0.05$ indicated by *. LA = linoleic acid, ALA = alpha-linolenic acid, AA = arachidonic acid, EPA = eicosapentaenoic acid, DHA = doccosahexaenoic acid.

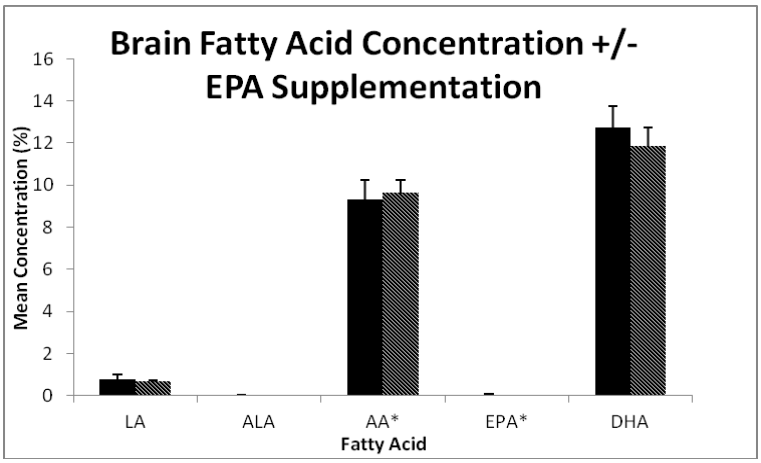


Figure 11: Effect of dietary EPA supplementation on LA, ALA, AA, EPA, DHA concentrations in brain, as compared to palm oil control groups. Solid black columns represent concentrations with EPA supplementation, while striped columns represent fatty acid concentrations of the palm oil control group. Error bars indicate one standard deviation. Statistical significance of difference between EPA and CTRL was analyzed using ANOVA and Tukey Tests with a significance at $p<0.05$ indicated by *. LA = linoleic acid, ALA = alpha-linolenic acid, AA = arachidonic acid, EPA = eicosapentaenoic acid, DHA = doccosahexaenoic acid.

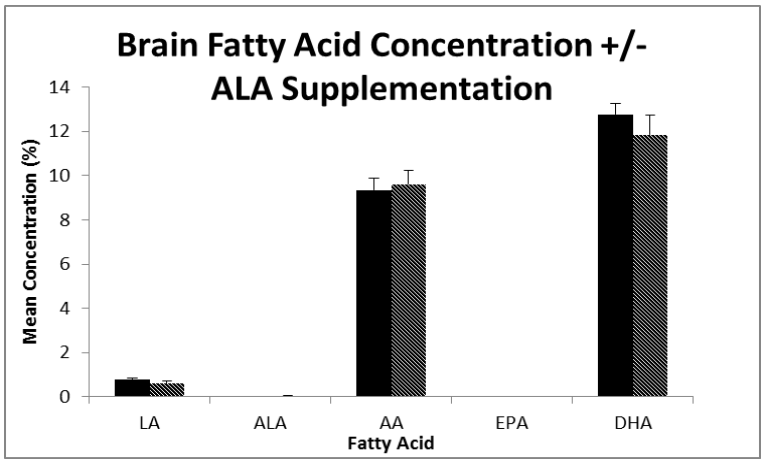


Figure 12: Effect of dietary ALA supplementation on LA, ALA, AA, EPA, DHA concentrations in brain, as compared to palm oil control groups. Solid black columns represent concentrations with ALA supplementation, while striped columns represent fatty acid concentrations of the palm oil control group. Error bars indicate one standard deviation. Statistical significance of difference between ALA and CTRL was analyzed using ANOVA and Tukey Tests with a significance at $p<0.05$, with no significant differences found. LA = linoleic acid, ALA = alpha-linolenic acid, AA = arachidonic acid, EPA = eicosapentaenoic acid, DHA = doccosahexaenoic acid.

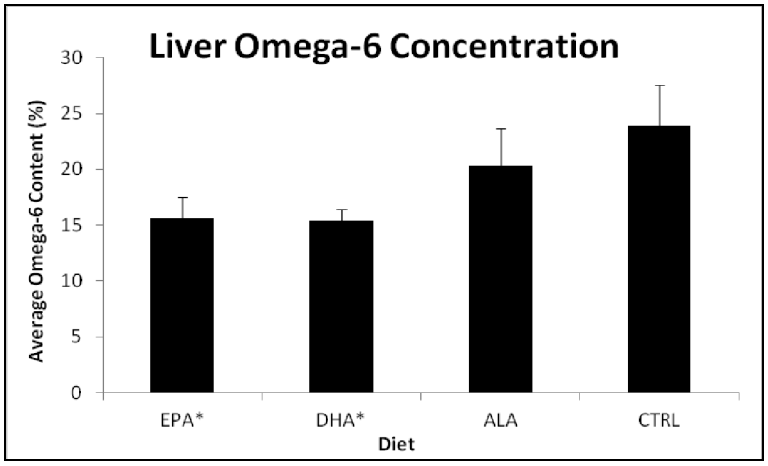


Figure 13: Effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on total omega-3 PUFA concentrations in liver. Error bars indicate standard deviation. Average values are a expressed as a percentage of total lipids. * indicates a statistically significant difference was found by ANOVA and Tukey Tests ($p<0.05$) from palm oil control group. EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control.

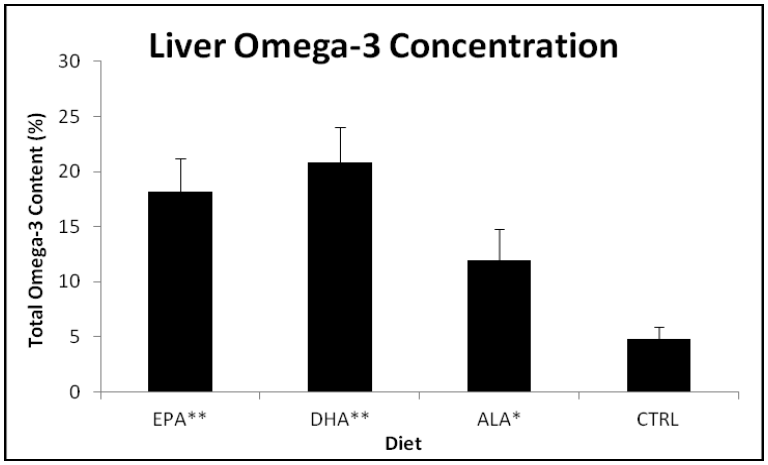


Figure 14: The effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on total omega-3 PUFA concentrations in liver. Error bars indicate standard deviation. Average values are expressed as a percentage of total lipids. * indicates a statistically significant difference was found by ANOVA and Tukey Tests ($p<0.05$) from palm oil control group. ** indicates a statistically significant difference at $p<0.01$. EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control.

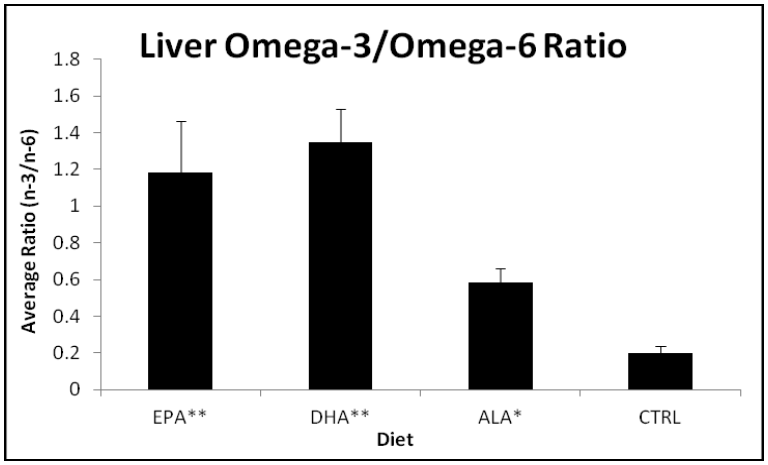


Figure 15: The effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on the omega-3/omega-6 PUFA ratio in liver. Error bars indicate standard deviation. Average values are expressed as a percentage of total lipids. * indicates a statistically significant difference was found by ANOVA and Tukey Tests ($p<0.05$) from palm oil control group. ** indicates a statistically significant difference at $p<0.01$. EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control.

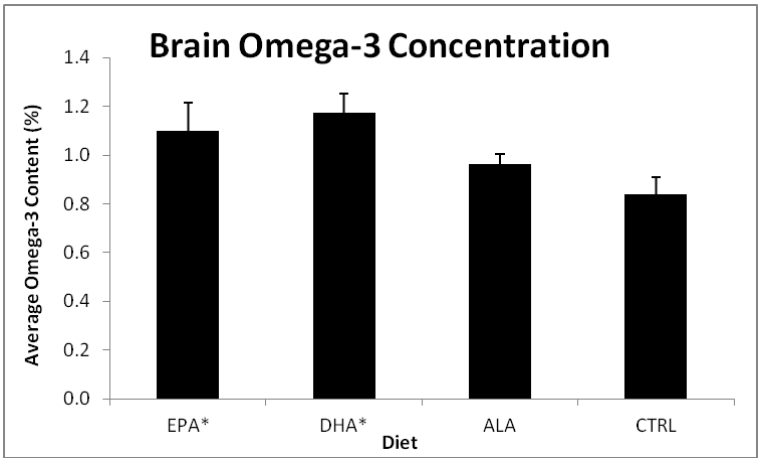


Figure 16: The effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on total omega-3 PUFA concentrations in brain. Error bars indicate standard deviation. Average values are expressed as a percentage of total lipids. * indicates a statistically significant difference was found by ANOVA and Tukey Tests ($p<0.05$) from palm oil control group. EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control.

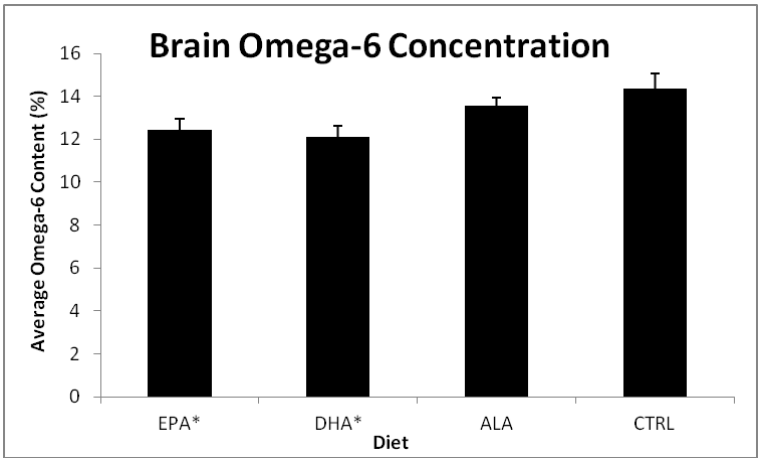


Figure 17: The effect of dietary omega-3 polyunsaturated (PUFA) fatty acid supplementation on total omega-6 PUFA concentrations in brain. Error bars indicate standard deviation. Average values are expressed as a percentage of total lipids. * indicates a statistically significant difference was found by ANOVA and Tukey Tests ($p<0.05$) from palm oil control group. EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control.

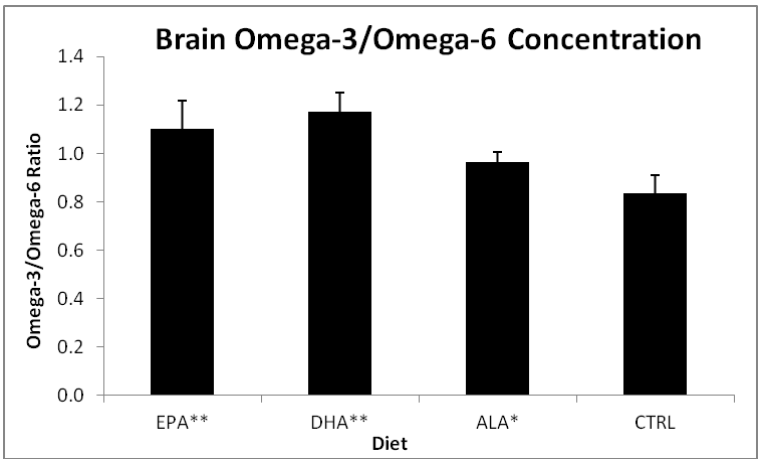


Figure 18: The effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on the omega-3/omega-6 PUFA ratio in brain. Error bars indicate standard deviation. Average values are expressed as a percentage of total lipids. * indicates a statistically significant difference was found by ANOVA and Tukey Tests ($p<0.05$) from palm oil control group. ** indicates a significant difference at $p<0.01$. EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control.

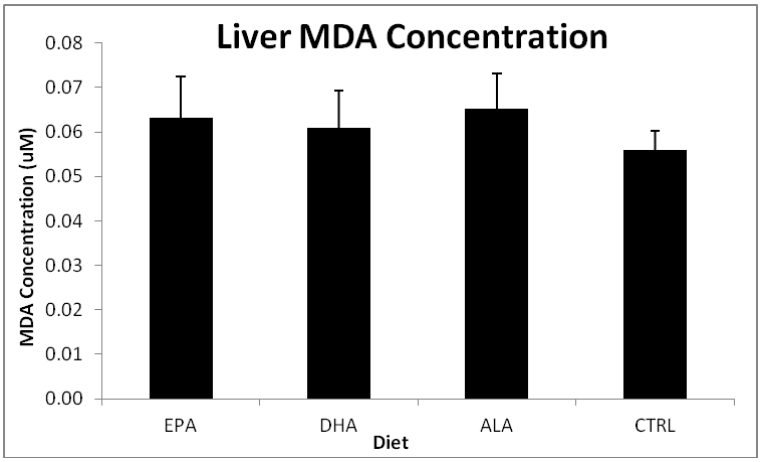


Figure 19: The effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on liver malondialdehyde concentrations in male wistar rats. Error bars indicate standard deviation. No statistically significant differences were found. EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control, MDA = maldondialdehyde.

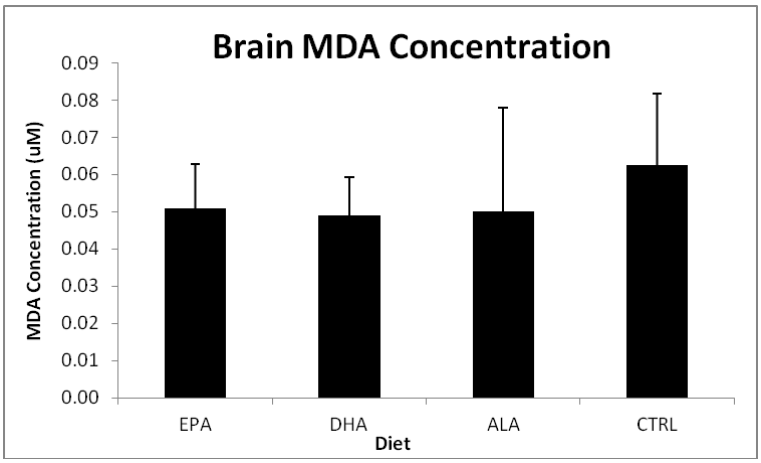


Figure 20: The effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on brain malondialdehyde levels in male wistar rats. Error bars indicate standard deviation. No statistically significant differences were found. EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control, MDA – maldondialdehyde.

Table 7: ANOVA results testing for significant differences in brain headspace aldehyde concentrations at room temperature (22°C). F-values are used to find significant differences between dietary groups. df of 3 indicates that there were 4 dietary groups analyzed (CTRL, EPA, DHA, and ALA). *indicates a significant difference was found between groups ($p<0.05$).

RT-22C	**BRAIN**		
Aldehyde	**df**	**F**	**P**
Propanal	3	0.1858	0.9051
Hexanal	3	0.3047	0.8217
Butanal	3	0.5102	0.6788
Pentanal*	3	3.5268	0.0288
Heptanal	3	1.0056	0.4060
Octanal	3	0.1184	0.9485
Formaldehyde	3	1.2910	0.2984
Acetaldehyde	3	0.5354	0.6622

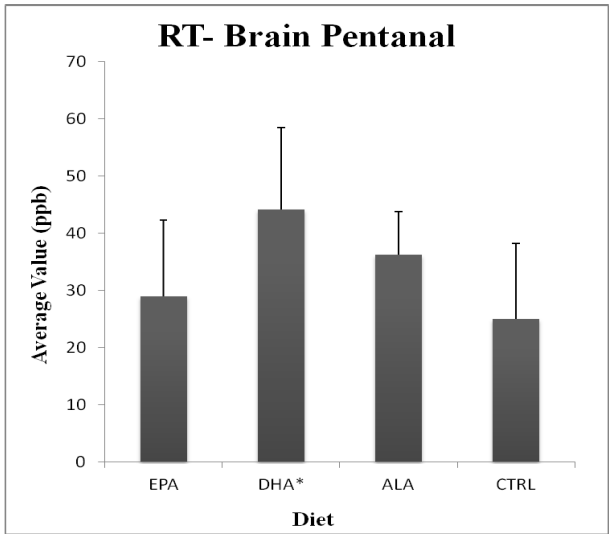


Figure 21: The effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on brain pentanal concentrations at room temperature (22°C). Error bars indicate standard deviation. * indicates that a significant difference was found by ANOVA and Tukey Test analyses vs the palm oil control group ($p<0.05$). EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control.

Table 8: ANOVA results testing for significant differences in brain headspace aldehyde concentrations at 40°C. F-values are used to find significant differences between dietary groups. df of 3 indicates that there were 4 dietary groups analyzed (CTRL, EPA, DHA, and ALA). No significant differences were found between groups ($p>0.05$).

40C	**BRAIN**		
Aldehyde	**df**	**F**	**P**
Propanal	3	0.0922	0.9637
Hexanal	3	0.3276	0.8054
Butanal	3	1.1460	0.3491
Pentanal	3	1.0257	0.3974
Heptanal	3	0.1327	0.9397
Octanal	3	0.1850	0.9056
Formaldehyde	3	1.0811	0.3744
Acetaldehyde	3	1.3735	0.2729

Table 9: ANOVA results testing for significant differences in brain headspace aldehyde concentrations at 60°C. F-values are used to find significant differences between dietary groups. df of 3 indicates that there were 4 dietary groups analyzed (CTRL, EPA, DHA, and ALA). *indicates a significant difference was found between groups ($p<0.05$).

60C	**BRAIN**		
Aldehyde	**df**	**F**	**P**
Propanal	3	1.5716	0.2201
Hexanal	3	0.8385	0.4851
Butanal	3	0.3164	0.8134
Pentanal*	3	3.5089	0.0293
Heptanal	3	2.2582	0.1054
Octanal	3	0.3506	0.7890
Formaldehyde	3	0.9992	0.4089
Acetaldehyde	3	2.7750	0.0614

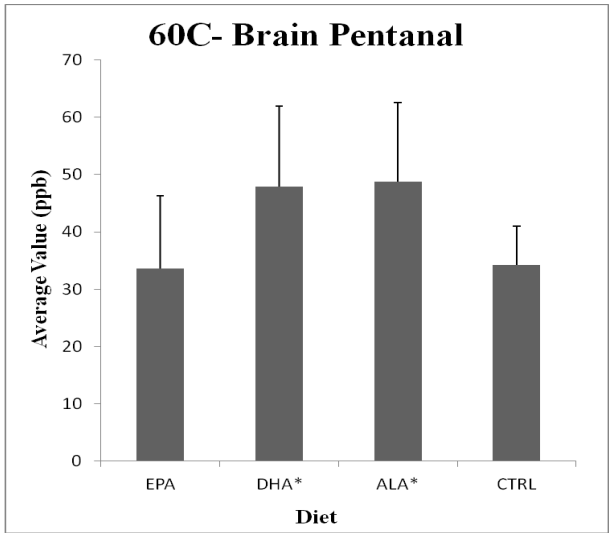


Figure 22: The effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on brain pentanal concentrations at 60°C. Error bars indicate standard deviation. * indicates that a significant difference was found by ANOVA and Tukey Test analyses vs the palm oil control group ($p<0.05$). EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control.

Table 10: ANOVA results testing for significant differences in liver headspace aldehyde concentrations at room temperature (22°C). F-values are used to find significant differences between dietary groups. df of 3 indicates that there were 4 dietary groups analyzed (CTRL, EPA, DHA, and ALA). No significant differences were found ($p>0.05$).

RT-22C	**LIVER**		
Aldehyde	**df**	**F**	**P**
Propanal	3	0.4344	0.7302
Hexanal	3	0.2597	0.8537
Butanal	3	0.2602	0.8534
Pentanal	3	1.8422	0.1643
Heptanal	3	0.5081	0.6801
Octanal	3	1.2197	0.3224
Formaldehyde	3	0.0582	0.9811
Acetaldehyde	3	0.8078	0.5010

Table 11: ANOVA results testing for significant differences in brain headspace aldehyde concentrations at 40°C. F-values are used to find significant differences between dietary groups. df of 3 indicates that there were 4 dietary groups analyzed (CTRL, EPA, DHA, and ALA). *indicates a significant difference was found between groups ($p<0.05$).

40C	**LIVER**		
Aldehyde	**df**	**F**	**P**
Propanal	3	0.0322	0.9920
Hexanal	3	0.1164	0.9497
Butanal*	3	3.3991	0.0326
Pentanal	3	0.4184	0.7413
Heptanal	3	0.3290	0.8044
Octanal	3	1.1207	0.3588
Formaldehyde	3	0.2618	0.8523
Acetaldehyde	3	0.7570	0.5284

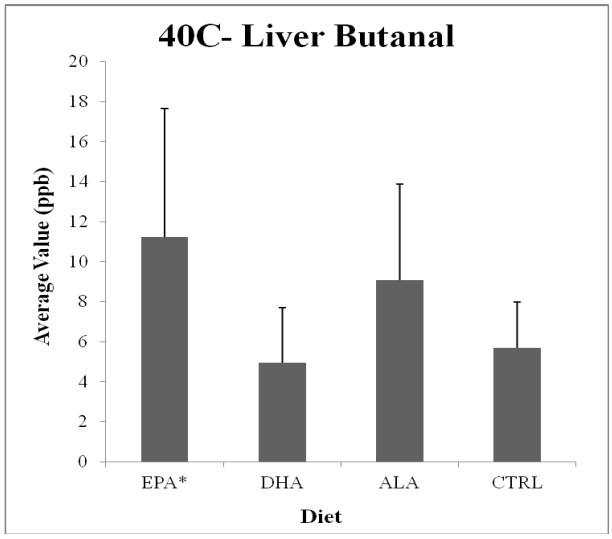


Figure 23: The effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on liver butanal concentrations at 40°C. Error bars indicate standard deviation. * indicates that a significant difference was found by ANOVA and Tukey Test analyses vs the palm oil control group ($p<0.05$). EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control.

Table 12: ANOVA results testing for significant differences in brain headspace aldehyde concentrations at 60°C. F-values are used to find significant differences between dietary groups. df of 3 indicates that there were 4 dietary groups analyzed (CTRL, EPA, DHA, and ALA). *indicates a significant difference was found between groups ($p<0.05$).

60C	**LIVER**		
Aldehyde	**df**	**F**	**P**
Propanal	3	0.3669	0.7775
Hexanal	3	1.9683	0.1435
Butanal*	3	3.5154	0.0291
Pentanal	3	0.0713	0.9748
Heptanal	3	1.0717	0.3782
Octanal	3	0.6351	0.5991
Formaldehyde	3	0.2291	0.8753
Acetaldehyde	3	0.2374	0.8695

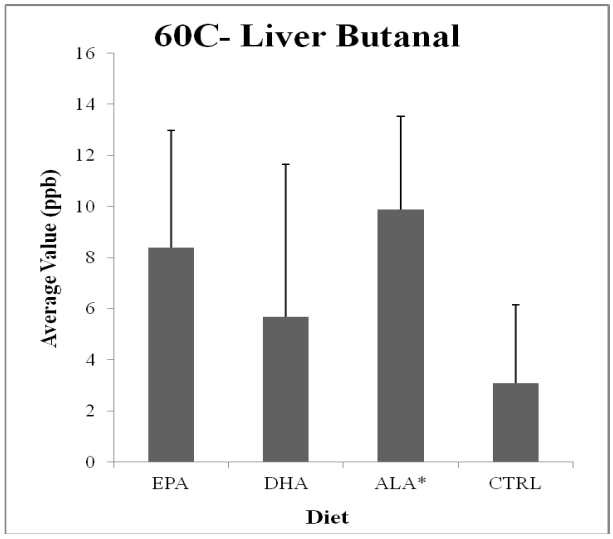


Figure 24: The effect of dietary omega-3 polyunsaturated fatty acid (PUFA) supplementation on liver butanal concentrations at 60°C. Error bars indicate standard deviation. * indicates that a significant difference was found by ANOVA and Tukey Test analyses vs the palm oil control group ($p<0.05$). EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, ALA = alpha-linolenic acid, CTRL = palm oil control.

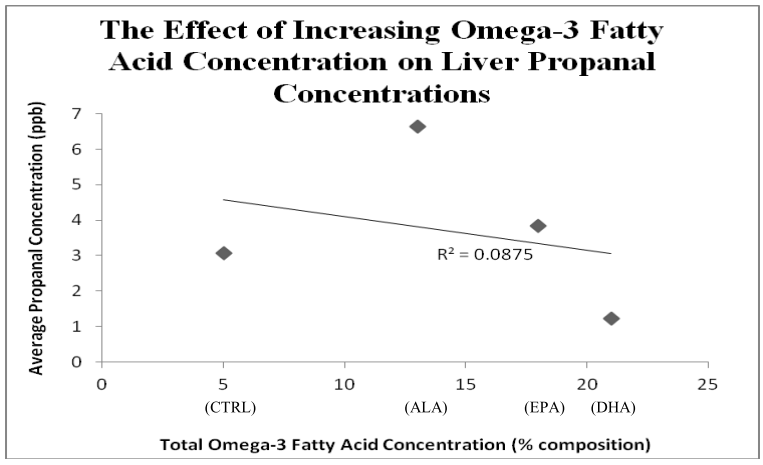


Figure 25: Showing the effect of diet on total omega-3 polyunsaturated fatty acid (PUFA) lipid composition, and how that lipid composition affects tissue propanal concentrations in liver. Correlation matrices indicate no significant relationship between total omega-3 PUFA composition and propanal concentrations in either liver or brain ($p>0.05$). Line represents regression line, with R^2 value indicating no significant relationship ($p>0.05$).

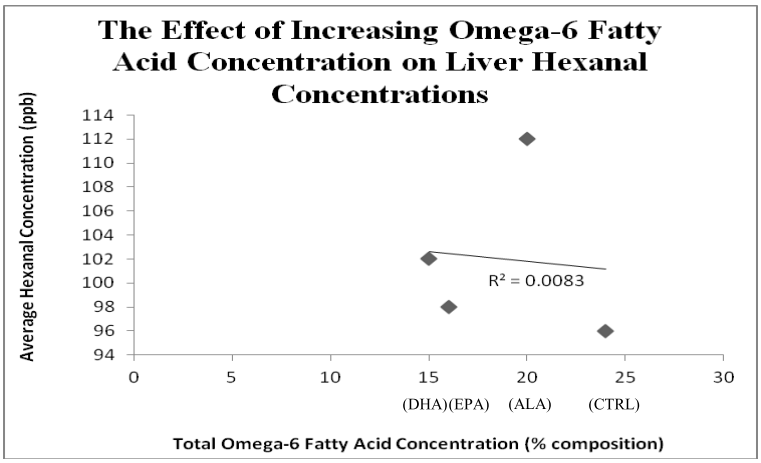


Figure 26: Showing the effect of diet on total omega-6 polyunsaturated fatty acid (PUFA) lipid composition, and how that lipid composition affects tissue hexanal concentrations in liver. Correlation matrices indicate no significant relationship between total omega-6 PUFA composition and hexanal concentrations in either liver or brain ($p>0.05$). Line represents regression line, with R^2 value indicating no significant relationship ($p>0.05$).

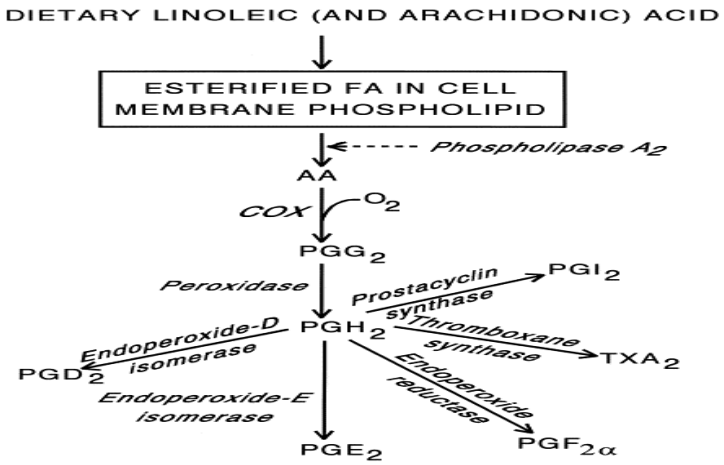


Figure 27: The metabolism of AA to the 2-series prostanoid subfamily of eicosanoids (Rose and Connolly, 1990).

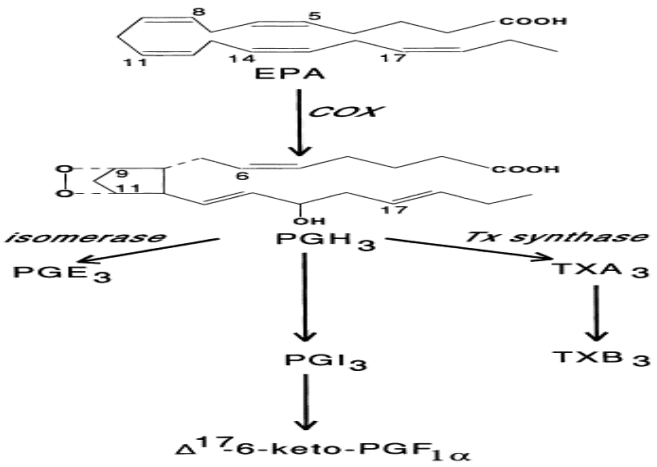


Figure 28: The metabolism of EPA to 3-series prostanoids. EPA follows a similar pathway to that of AA, using the same enzymes for conversion into its eicosanoids (Rose and Connolly, 1990).

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