IMPACT OF FISH OIL REPLACEMENT ON THE EXPRESSION OF ANTIOXIDANT GENES AND GENES INVOLVED IN THE SYNTHESIS OF OMEGA-3 LONG CHAIN POLYUNSATURATED FATTY ACIDS IN YELLOWTAIL KINGFISH (*Seriola lalandi*)

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DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Nathan Rout-Pitt

9th November, 2010

I believe that this thesis is properly presented, conforms to the specifications for the thesis and is of sufficient standard to be, *prima facie*, worthy of examination.

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Assoc. Prof. Kathy Schuller

9th November, 2010

ABSTRACT

Omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA) such as eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) are beneficial for human health to help brain and neural development, decrease the risk of cardiovascular disease and help various other aspects of human lives. Fish are the main source of these n-3 LCPUFA in the human diet as humans like most vertebrates are unable to synthesise EPA and DHA themselves. EPA and DHA are synthesised via the LCPUFA synthesis pathway from α-linolenic acid (ALA) which is abundant is some plant oils which are totally devoid of n-3 LCPUFA.

LCPUFA has large numbers of double bonds in thei hydrocarbon tail, which are prone to oxidative attack by reactive oxygen species (ROS). This means that by feeding fish on fish oil-based diets, they are more at risk of oxidative stress and this can decrease their shelf life *post-mortem.*

Captured fish are used as a source of fish oil to feed aquaculture fish, but capture fisheries are currently at their limit and using captured fish stocks as aquaculture feed is unsustainable and therefore alternative lipid sources to replace fish oil are required to allow the aquaculture industry to continue growing.

This research investigated dietary fish oil replacement with plant or terrestrial animal oils and its impact on the seafood product quality of yellowtail kingfish (*Seriola lalandi*), an important aquaculture species in South Australia. It investigated how this replacement affected the expression of the fatty acid synthesis genes Δ6 fatty acyl desaturase (Δ6 fads)

and fatty acyl elongase (elovl), and the antioxidant genes peroxiredoxin 1 and 4 (Prx1 and 4) and glutathione peroxidase 1 and 4 (GPx1 and 4).

It was found that fish oil substitution with canola oil and poultry oil did not significantly change the expression of the fads or elovl genes. The antioxidant genes also showed no significant changes in gene expression except for GPx1 which showed an increased expression in fish fed the canola oil-based diet compared to fish fed the fish oil-based diet.

Hepatosomatic indices were significantly higher for fish fed the canola oil-based diet compared to fish fed the fish oil-based diet and the fatty acid profiles of fillets from fish fed the canola oil-based diet showed a low omega-3/omega-6 ratio below 1. This compared with the high omega-3/omega-6 ratio of ???? for fish fed the fish oil-based diet. Thus, it is proposed that GPx1 expression may be up-regulated in fish fed the canola oil-based diet due to increased immune system activity as a result of the relatively high concentration of omega-6 fatty acids in their flesh.

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ABBREVIATIONS

PUFA- Polyunsaturated fatty acids

LCPUFA- Long chain polyunsaturated fatty acids

LA- Linoleic acid

AA- Arachidonic acid

ALA- α-linolenic acid

EPA- Eicosapentaenoic acid

DHA- Docosahexaenoic acid

Prx- Peroxiredoxin

GPx- Glutathione peroxidase

fads- Fatty acyl desaturase

elovl- Fatty acyl elongase

n-3- omega-3

n-6- omega-6

ROS- Reactive oxygen species

ETC- Electron transport train

YTK- Yellowtail Kingfish

TPx- Thioredoxin peroxidase

GSH- Glutathione

Cys- Cysteine

HSI- Hepatosomatic index

Chapter 2: MATERIALS AND METHODS

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CHAPTER 4: DISCUSSION

CHAPTER 4: DISCUSSION

Out of the 6 genes analysed for differences in gene expression when fish oil was substituted with either canola or poultry oil, significant differences were found for the antioxidant genes Prx4 and GPx1. Prx4 gene expression was found to be significantly higher in fish fed the fish/poultry oil-based diet compared to fish fed the canola oil-based diet whereas GPx1 expression was found to be higher in fish fed the canola oil-based diet compared to fish fed the fish oil-based diet. This is contrary to our original hypothesis which stated that the expression of all antioxidant genes will be up-regulated in fish fed the fish oil-based diet. Several studies have shown that fish fed fish oil-based diets (which contain higher concentrations of n-3 LCPUFA and therefore a greater number of unsaturated chemical bonds) are more at risk of oxidative stress due to lipid peroxidation than fish fed plant oil-based diets (Ng et al., 2007; Grubinko and Leus, 2002). Other studies have shown that an increase in dietary n-6 PUFA can increase the immune response and thereby promote oxidative stress. Kinsella et al. (1990) discussed how n-6 PUFA such as arachidonic acid (AA) can be converted into eicosanoids and prostaglandins which can increase the initiation and progress of atherogenesis and become involved in thrombosis. Different plant oils are characterised by different fatty acid profiles. Miller et al. (2008) highlighted the different fatty acids present in a range of plant oils. In particular linseed oil contained 53.3 g/100 g of n-3 PUFA which was almost all made up of ALA whereas it contained only 15.7 g/100 g of n-6 PUFA giving an n-3/n-6 ratio of 3.4. This was compared to palm oil which contained no n-3 PUFA but 11.4 g/100 g of n-6 PUFA. Canola oil which was used in the study reported in this thesis has been shown to contain 7.3 g/100 g of n-3 PUFA compared to 21.6 g/100 g of n-6 PUFA giving an n-3/n-6 ratio of 0.3. This n-3/n-6 ratio for canola oil is reflected in the n-3/n-

6 ratio for the YTK fillet when the 5% lipid from fish oil in the fish meal is taken into account. The n-3/n-6 ratio in the fillet for the fish fed the canola oil-based diet was 0.85. In addition, the hepatosomatic index of these fish was significantly higher than that of the fish fed the fish oil-based diet. The YTK liver as a result of feeding the canola oil-based diet was yellow, indicating lipid droplets and it was much larger than the liver from fish fed the fish oil-based diet which was a red colour indicative of a healthy liver. These data suggest that the health of the fish fed the canola oil-based diet was compromised. The low n-3/n-6 ratio indicates that the n-6 PUFA could be inhibiting the n-3 PUFA from entering the LCPUFA synthesis pathway. The n-6 PUFA may out-compete the n-3 PUFA for the use of the fads and elovl enzymes leading to increased production of the pro-inflammatory products prostaglandins and eicosanoids from the n-6 fatty acid AA (Hata and Breyer 2004). These molecules are able to cause inflammation which results from an immune response. ROS are a by-product of macrophages and monocytes which secrete various cytokines which are pro-inflammatory molecules which are then able to oxidise lipids (Singh et al., 2005). With this increasing pro-inflammatory response, ROS could accumulate, providing a possible reason for the observed increases in GPx1 and Prx4 expression.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) is a highly sensitive technique which is able to determine how many copies of a gene are present in a sample. There are several ways of determining the expression of genes using either relative or absolute methods. The similarity which both methods have is the use of a reference gene or genes. Some of the most common reference genes are housekeeping genes such as β-actin, GAPDH, 18S rRNA, ELF1α and 2βMG. Under certain circumstances, these genes may vary in their expression, rendering them unusable as reference genes. β-actin expression has

been found to be unstable in the presence of some hormones (Schroder et al., 2009) while 18S rRNA has been criticised due to its abundance within cells making its expression much higher relative to the genes of interest (Vandesopele et al., 2002). The present study has used the housekeeping genes β-actin, ELF1α and 2βMG as reference genes. These are involved in different cellular functions and therefore any one particular treatment is less likely to impact on the expression of all three genes, providing a more stable baseline when the expression of all three genes is averaged together to normalise the expression of the gene of interest.

The raw data obtained for the reference genes showed that there was a large amount of variation between the different fish samples. The expression pattern between the diets appeared to be consistent for all three reference genes with increasing expression with increasing saturation of the diet. This could possibly be due to the increased amount of lipid in the liver causing damage to the cells and leading to decreased cellular metabolism. Another plausible reason for the large variation in expression between samples could be variations in RNA input or in the efficiency of cDNA synthesis. For this reason, reference genes are used to normalise gene expression.

The inclusion of vegetable oils in fish feeds has been shown to increase the expression of genes involved in EPA and DHA synthesis from ALA in fresh water fish species (Zheng et al., 2005) but not as much in marine fish species (Geay et al., 2010). For example, Geay et al. (2010) showed that fads gene expression was up-regulated in European sea-bass with the substitution of linseed oil for fish oil, but the fads protein expression level was unaffected.

Gonzalez-Rovira et al. (2009) also found increases in Δ6 fads gene expression in European sea-bass and Atlantic salmon when the fish were fed diet blends of fish oil with linseed oil and canola oil however no significant increase was observed with and olive oil blend with fish oil. Tocher et al. (2006) found an increase in Δ6 fads in Atlantic cod fed a diet containing canola, linseed and palm oil in a 2:2:1 ratio, although this increase wasn't significant due to the large variation between replicates. Considering the n-3 PUFA and n-6 PUFA levels in linseed oil, canola oil and olive oil, it can be seen that there is an n-3/n-6 ratio above 1 for linseed oil, below 1 for canola oil and 0 for olive oil which contains no n-3 PUFA. In the studies cited above, the blend of fish oil with the vegetable oils would have raised the n-3/n-6 ratio above the ratio for the pure vegetable oils and this could have result in the n-3 PUFA out-competing the n-6 PUFA for the Δ6 fads resulting in increased Δ6 fads gene expression. Olive oil however, is devoid of n-3 PUFA and as a result the fish oil added may not have been sufficient to increase the n-3/n-6 ratio above 1. The study presented in this thesis also showed a 2.46-fold increase in Δ6 fads expression in fish supplied a canola and fish oil blend compared with much lower expression for fish supplied a 100% canola oil-based diet, however, due to the large variation between replicates, the difference was not statistically significant. The canola oil-based diet showed a slight decrease in expression relative to the fish oil-based diet. This indicates that fatty acyl desaturase expression may be dependent on the ratio of fish oil (rich in n-3 LCPUFA) to vegetable oil containing ALA, the substrate for the LCPUFA synthesis pathway. . The mechanisms of this await further investigation.

Due to a lower level of unsaturation in the canola oil as compared to the fish oil it was hypothesised that the expression of antioxidant genes would decrease as there would be lower amounts of ROS and lipid hydroperoxides. This was expected to be especially the case

for GPx4 as it is the only enzyme capable of acting on peroxidised phospholipids. However this was not seen as there was no significant decrease in expression of Prx1, Prx4 and GPx4 in fish fed the canola oil-based diet compared to the fish fed the fish oil-based diet.

Due to the lack of *S. lalandi* sequence data in the GenBank database, many of the PCR primers were designed based on conserved regions of genes from closely related fish species such as *S. quinqueradiata.* Despite the lack of data, the consensus sequence primers were still able to amplify the correct fragments. However, one set of primers was not successful; the elongase primers (elo-a) were not able to amplify the expected product effectively without producing primer dimers which could be seen in the melt curve from the qPCR experiment. The elo-a standard curve showed a product melting point of about 86-89°C which equated approximately to the correct temperature, however when cDNA was used as the template, the major peak had a lower melting temperature of about 81°C while the product at a melting temperature at 86-89°C showed only very a small peak. To design better matching primers, a larger fragment of a YTK fatty acyl elongase gene was amplified using primer elo-564. The nucleotide sequence of this fragment was then used to design more specific primers which were called elo-b. These primers were successful in amplifying the expected product and there was no sign of primer dimers.

Conclusion

This study identified an increase in GPx1 gene expression. This result was contrary to our original hypothesis which stated that there would be a decrease in the expression of antioxidant genes in fish fed a canola oil based diet compared to fish fed a fish oil based

diet. Fatty acid profile data of the fish fillets showing a low n-3 / n-6 ratio and the hepatosomatic indices indicated that the fish fed the canola oil-based diet may have been under stress and that their health may have been compromised. This then could have resulted in oxidative stress due to increased inflammation due to the production of prostaglandins and eicosanoids from n-6 PUFA. Overall the data presented in this thesis indicate that 100% fish oil substitution with canola oil is not effective in up-regulating the LCPUFA synthesis pathway and could cause more oxidative stress to the fish then the fish oil based diet.

Future directions

This study has focused on the effects of dietary fish oil substitution at the transcriptional level which may not necessarily be correlated with effects at the protein expression or enzyme activity level due to post-transcriptional or post-translational modifications. This provides a basis for further research to analyse variation in protein expression for both the fatty acid synthesis enzymes and antioxidant enzymes.

Furthermore, cloning and characterization of the fatty acyl elongase and fatty acyl desaturase genes from yellowtail kingfish could be carried out to determine how these genes fit phylogenetically amongst those from other fish species. This characterization would also help determine the substrate specificities of the enzymes these genes encode and add to our understanding of the LCPUFA synthesis pathway in marine fish species.

Research at CSIRO (Glencross, 2009) is currently investigating transgenic plants transformed with algal fads and elovl genes. Algae are at the bottom of the marine food chain and they

have the enzymes capable of synthesising EPA and DHA which ultimately bioaccumulate in large carnivorous fish species at the top of the marine food chain (Tocher, 2003). This means that canola plants expressing these genes can synthesize EPA and DHA from ALA (Robert et al., 2005). These transgenic canola plants could be a promising new source of EPA and DHA both for fish feeds and possibly also for human consumption.

Further research could analyse what kinds of immune responses are occurring in fish fed canola oil as compared with fish oil and the mechanisms involved in inducing inflammation to determine whether or not the high concentrations of n-6 PUFA are responsible.

1.1 Fatty acid structure and function

Omega-3 and omega-6 long chain polyunsaturated fatty acids (n-3 LCPUFA, n-6 LCPUFA) are fatty acids which contain an unsaturated double bond at the omega-3 (n-3) or omega-6 (n-6) carbon in the hydrocarbon tail counting from the methyl ($-CH_3$) end. Furthermore, in order for a fatty acid to be recognised as long chain, there should be 20 or more carbon atoms (Hastings et al., 2005). Fig. 1.1 shows the monounsaturated fatty acid *cis*-9-Octadecenoic acid [18:1n-9] and the long chain polyunsaturated fatty acid eicosapentaenoic acid (EPA) [20:5n-3]. The degree of unsaturation of a fatty acid depends on the number of double bonds within the hydrocarbon tail and these double bonds also alter the shape of the tail. Double bonds cause the tail to bend at an angle at the unsaturated carbon. The more unsaturated a fatty acid is the more bends it has in its hydrocarbon tail. This results in a change from the straight tail found in saturated fatty acids which enables the fatty acids to group much closer to form a solid structure, to polyunsaturated fatty acids which are able to bend causing a more fluid structure due to the increased distance between the molecules. Fatty acids are primarily found in the lipid bilayer making up the cell membrane (Tocher et al., 2003). In animals which live at low ambient temperatures, such as fish, the lipid membrane must contain larger amounts of polyunsaturated fatty acids in order to maintain its fluidity (Mourente et al., 2007).

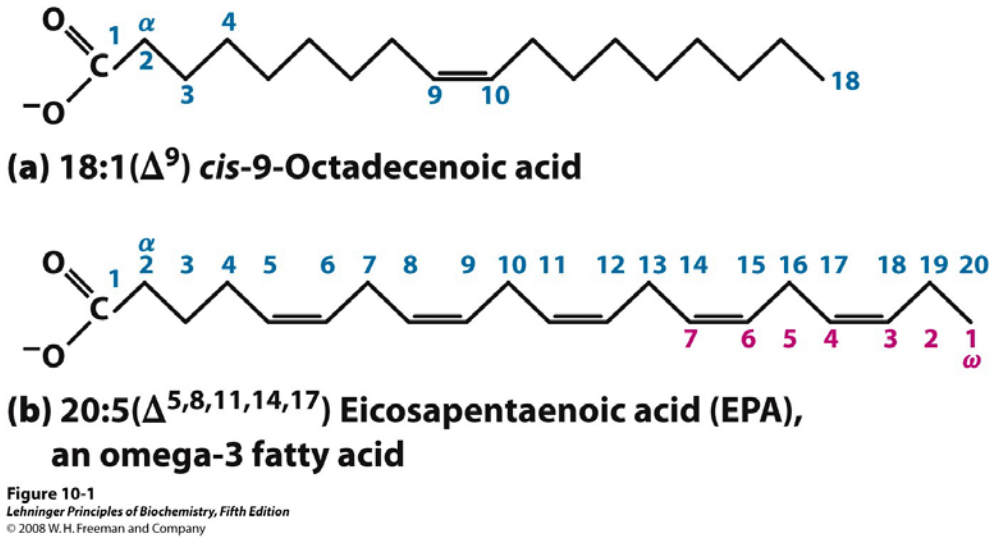

Figure 1.1: The structure of fatty acids showing the carbon numbering and the unsaturated bonds. (a) *cis*-9-Octadecenoic acid [18:1n-9] is a monounsaturated fatty acid with 1 double bond at the 9th carbon (b) eicosapentaenoic acid [20:5n-3] is a polyunsaturated fatty acid with 5 unsaturated bonds at the 5th, 8th, 11th, 14th and 17th carbons and with the 17th unsaturated bond being at the omega-3 carbon (ω-3) making it an n-3 LCPUFA. This figure was taken from Lehninger Principles of Biochemistry, 5th edition, edited by David L. Nelson and Michael M. Cox, published by W.H. Freeman and Company, New York.

1.2 Long chain polyunsaturated fatty acid synthesis pathway

The LCPUFA synthesis pathway in vertebrates, including fish, can be seen in Fig. 1.2. Both the n-3 PUFA and the n-6 PUFA utilise this pathway using the same enzymes to convert linoleic acid (LA) [18:2n-6] to arachidonic acid (AA) [20:4n-6] and α-linolenic acid (ALA) [18:3n-3] to eicosapentaenoic acid (EPA) [20:5n-3] and docosahexaenoic acid (DHA) [22:6n-3]. These conversions use fatty acyl desaturase (fads) enzymes to desaturate a bond in the hydrocarbon tail and fatty acyl elongase enzymes (elovl) to elongate the hydrocarbon tail by two carbons. The final step in the formation of DHA has been shown to proceed via a C_{24} intermediate synthesized from EPA by two successive elongase reactions followed by a Δ6 desaturase reaction. This C_{24} intermediate then undergoes β-oxidation in the peroxisomes

to reach the final DHA product. Due to the n-6 fatty acids utilising the same pathway, this means that the n-3 and n-6 fatty acids compete for the use of the same enzymes.

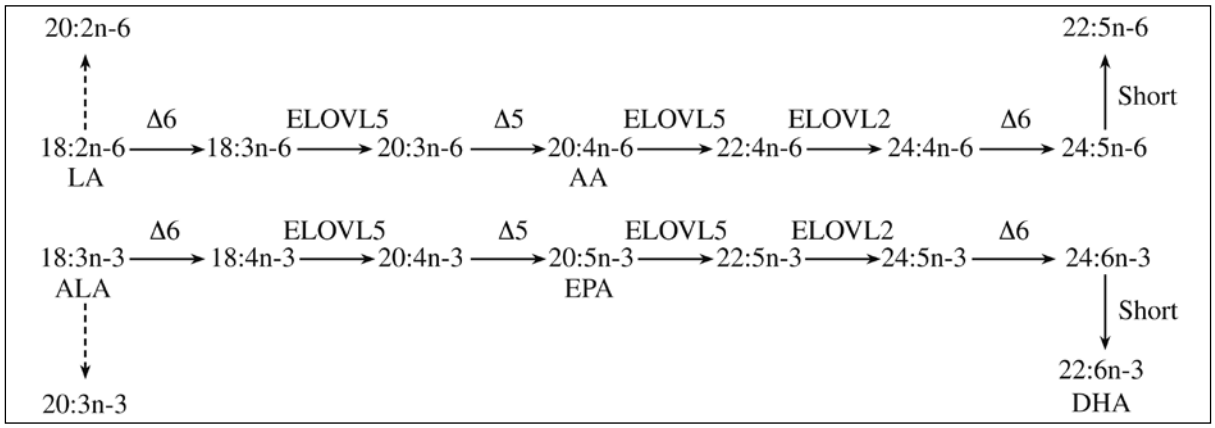

Figure 1.2: The LCPUFA synthesis pathway in vertebrates including fish. Abbreviations: LA, linoleic acid; ALA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ELOVL5 and ELOVL2, fatty acyl elongase 5 and 2, respectively; Δ6 and Δ5, Δ6 and Δ5 fatty acyl desaturase, respectively. This figure was adapted from Miller et al. (2008).

1.2.1 Fatty acyl desaturases

Fatty acyl desaturase (fads) enzymes are characterised by the presence of 3 histidine boxes, 2 trans-membrane regions and an N-terminal cytochrome b_5 domain containing a heme binding motif for electron transfer (Hastings et al. 2005). These regions are highly conserved among species. The role of desaturase enzymes is to desaturate a C-C single bond to a C=C double bond in the fatty acid tail. These double bonds provide more fluidity to the lipid membranes which is important particularly for animals in cold ambient temperatures (Mourente et al., 2007). Desaturase enzymes are named based on the carbon at which they desaturate. Animals possess Δ5, Δ6 and Δ9 desaturases (Los et al., 1998) which means they can act on the 5^{th}, 6^{th} and 9^{th} carbons, therefore limiting animals to the synthesis of the

omega-3 fatty acids starting from ALA. ALA is obtained from plants which contain the Δ6, Δ9, Δ12 and ω3 desaturases (Los et al., 1998) which enables them to synthesise the important ALA from oleic acid, however the lack of a Δ5 desaturase prevents them from making any longer chain omega-3 fats such as arachidonic acid, EPA and DHA.

1.2.2 Fatty acyl elongases

Fatty acyl elongase enzymes are characterized by the presence of a histidine box, endoplasmic reticulum retention signal and 2 trans-membrane regions which are conserved among all kingdoms of life (Hastings et al., 2005; Morais et al., 2009). Fatty acyl elongases act by extending the hydrocarbon tail of fatty acids by two carbons with each reaction. Seven different elongases have so far been identified in mammals (Morais et al., 2009) with two of them, termed elovl2 and elovl5 found in fish which are homologous to the mammal forms (Morais et al., 2009). These two elongases in particular are active towards different length substrates within the LCPUFA synthesis pathway. The elovl5 enzymes have been found to be most active towards C_{18} and C_{20} PUFA whereas the elovl2 enzymes have been found to be most active towards C_{20} and C_{22} PUFA and they have very low activity towards C_{18} PUFA (Morais et al., 2009). Recently a second *elovl5* has been discovered which is now called *elovl5b*. This is in addition to the original *elovl5a*. Fish oil substitution with vegetable oil in feeds for Atlantic salmon increased the expression of *elovl5b* and *elovl2* but not *elovl5a* in the liver (Morais et al., 2009). In addition, phylogenetic comparisons have shown that Acanthopterygian fish species which are marine lack *elovl2* which may explain why marine fish species are less efficient than freshwater species or salmonids in utilising the LCPUFA synthesis pathway to produce DHA (Morais et al., 2009).

1.3 Human health benefits of n-3 LCPUFA

The n-3 LCPUFA are essential in vertebrate nutrition as vertebrates are unable to synthesise n-3 LCPUFA themselves (Miller et al., 2008). Finding the right balance between different fatty acids is essential for good health. In particular the n-3:n-6 ratio plays an important role in the health of both fish and humans (Simopoulos., 1999). A high n-3:n-6 ratio has been shown to be associated with good health and development while a low ratio is often associated with poor health including increased risk of cancer, atherosclerosis, muscle necrosis and thinning of ventricular walls (Miller et al., 2008). Over the last several thousand years and in particular since the beginning of the agricultural revolution, there has been a shift in the Western diet from low fat/low energy density to high fat/high energy density (Simopoulos., 1999) which is causing more and more health problems in society such as the current obesity epidemic.

The two most important n-3 LCPUFA for human health are EPA and DHA. A review by Calder and Yaqoob (2009) has described the health benefits of EPA and DHA in the human diet and they are summarized in Table 1.1. In particular, increasing dietary EPA and DHA increases their concentration in both white and red blood cells as well as in cardiac tissue and many other cells. This increase eicosanoid generation from n-3 PUFA and increases membrane fluidity which then aids in the activity of membrane bound proteins such as receptors and signalling molecules. Furthermore, EPA and DHA have been found to decrease blood pressure and inflammation, while at the same time improving immune function, visual signalling and brain development and ameliorating the symptoms of rheumatoid arthritis.

Table 1.1: A summary of the human health benefits of n-3 LCPUFA (reproduced from Calder and Yaqoob, 2009). Abbreviations: CVD, cardiovascular disease

Summary of the physiological roles and potential clinical benefits of very long chain ω-3 fatty acids

Physiological role of very long chain ω-3 fatty acids	Potential clinical benefit	Target
Regulation of blood pressure	Decreased blood pressure	Hypertension; CVD
Regulation of platelet function	Decreased likelihood of thrombosis	CVD
Regulation of blood coagulation	Decreased likelihood of thrombosis	CVD
Regulation of plasma triglyceride concentrations	Decreased plasma triglyceride concentrations	Hypertriglyceridemia; CVD
Regulation of vascular function	Improved vascular reactivity	CVD
Regulation of cardiac rhythm	Decreased arrhythmias	CVD
Regulation of inflammation	Decreased inflammation	Inflammatory diseases (arthritis, inflammatory bowel diseases, psoriasis, lupus, asthma, cystic fibrosis, dermatitis, neurodegeneration . . .); CVD
Regulation of immune function	Improved immune function	Compromised immunity
Regulation of bone turnover	Maintained bone mass	Osteoporosis
Regulation of insulin sensitivity	Improved insulin sensitivity	Type-2 diabetes
Regulation of tumor cell growth	Decreased tumor cell growth & survival	Some cancers
Regulation of visual signaling (rhodopsin)	Optimized visual signaling	Poor infant visual development (especially pre-term)
Structural component of brain and central nervous system	Optimized brain development – cognitive and learning processes	Poor infant and childhood cognitive processes and learning

1.4 Oxidation of LCPUFA

Due to the greater number of unsaturated bonds in DHA and EPA, they are more susceptible to oxidative attack by reactive oxygen species (ROS) than saturated fatty acids or shorter chain unsaturated fatty acids (Girotti, 1985). The oxidation of these unsaturated bonds causes the fatty acids to become lipid peroxyl radicals. LCPUFA are primarily incorporated into the cellular membrane and therefore due to their close proximity to one another, the formation of a lipid peroxyl radical can lead to a chain reaction of lipid peroxidation leading to damage to the membrane.

The peroxidation of fish lipids causes the flesh to become rancid and no longer consumable. Determining ways in which to extend the shelf life of fish by minimising the peroxidation of fatty acids in the edible portion of the flesh is a key to increasing profitability for fish farmers.

1.5 Oxidative stress and reactive oxygen species

Reactive oxygen species (ROS) are molecules which oxidise cellular components such as lipids, proteins and DNA thereby damaging cellular systems. ROS are formed as a result of normal cellular pathways. For example, they are produced by monocytes and macrophages in the immune system (Singh et al., 2005) and they are byproducts of respiration in the mitochondrial electron transport chain (ETC) (Mourente et al. 2007). The ETC reduces molecular oxygen to water through the addition of electrons at the various complexes of the ETC. When it is operating inefficiently, the ETC can produce ROS including the superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) (Fig. 1.3). The hydroxyl radical is the most highly reactive but it is only short-lived.

Oxidative stress results when ROS are generated quicker than they can be removed causing damage to cellular contents such as proteins, DNA and lipid membranes. As described above, lipid peroxidation can cause a chain reaction in the cellular membrane, resulting in widespread damage. This peroxidation is often initiated by the highly reactive hydroxyl radical which accepts a hydrogen atom from a methylene group of the PUFA, creating a PUFA radical (Mourente et al., 2007). However there are dietary and cellular components that control the concentration of ROS and these are known as antioxidants.

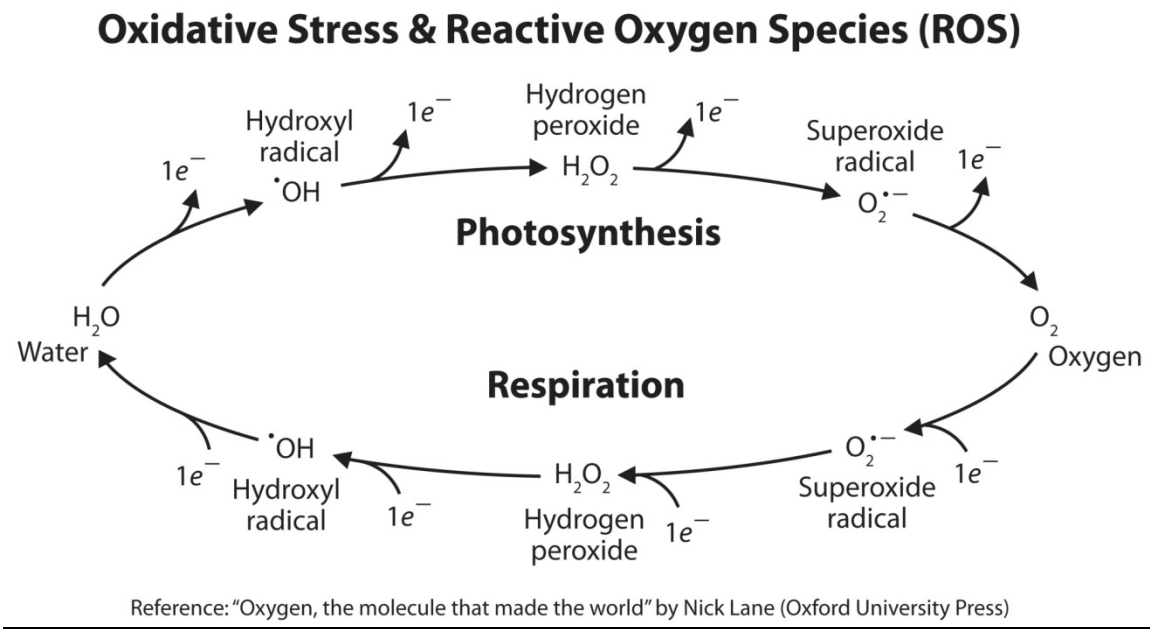

Figure 1.3: Reactive oxygen species (ROS) production during respiration in animals and photosynthesis in plants.

1.6 Antioxidants

Antioxidants are enzymes and small molecules such as vitamin C and E which collectively protects cells from becoming damaged by reactive oxygen species (ROS) by converting them to more stable compounds (Mourente et al., 2007). Enzymes such as peroxiredoxins (Prx) and glutathione peroxidases (GPx) reduce ROS to a more stable and un-reactive state. Each of these two antioxidant families contains multiple isoforms, and each one has a specific role to play. The GPx family has six isoforms identified in mammals (GPx1-6) with each having different cell and tissue distribution (described further in Section 1.6.1). Prx has six subclasses identified in mammals (Prx1-6) which are predominantly found in the cytosol, mitochondria and nucleus (described further in Section 1.6.2).

1.6.1 Glutathione peroxidases

The glutathione peroxidases are a family of antioxidant enzymes, usually containing a selenocysteine at the active site (Herbette et al., 2007). They detoxify hydrogen peroxide, small organic peroxides and, in the case of GPx4, fatty acid hydroperoxides. Six isoforms of GPx have been identified in mammals, with all but GPx5 and 6 containing the selenocysteine at the active site. GPx1 is a cytosolic GPx found ubiquitously in all cells. GPx2 is a gastro-intestinal GPx found in the stomach and intestine. GPx3 is secreted into the plasma in many different organs. GPx4 is a phospholipid hydroperoxide GPx which is found ubiquitously in all cells protecting phospholipids in the cellular membranes. GPx5 is an epididymal GPx found in skin cells and spermatozoa as an inactive form until secreted. GPx6 is an olfactory-metabolizing protein (OMP) found in olfactory epithelium cells (Herbette et al., 2007)

Detoxification of ROS by GPx is achieved by converting them to more stable compounds such as water or alcohols (Herbette et al., 2007). Lipid hydroperoxides are formed when cellular lipids undergo peroxidative attack. Due to the close proximity of lipids in the cellular membrane, the peroxidation of one lipid can create a chain reaction causing damage to the cellular membrane. GPx genes and proteins have not been studied in great detail in fish. However, Thompson et al. (2006) found that a GPx1 protein was expressed at high levels in the liver of southern blue fin tuna (SBT, *Thunnus maccoyii*) and also at lower levels in SBT muscle and plasma. It has also been determined that glutathione peroxidases and other antioxidants are often more highly expressed in farmed fish which are in more stressful environments due to reduced space and increased handling compared to wild fish (Murata et al., 1996; Ferrante et al., 2008). This stress can induce inflammatory responses leading to

increased oxidative stress and resulting in an increased risk of developing diseases or morbidity.

1.6.2 Peroxiredoxins

Peroxiredoxins (Prx) are also known as thioredoxin peroxidases (TPx) as they were originally identified in yeast as peroxidases reduced by thioredoxin (Rhee et al., 1999). Later Prx5 was found to be a selenocysteine-containing Prx reduced by glutathione (GSH) and therefore the family was given the more general name peroxiredoxin (Rhee et al., 1999). Prx are distinct from other antioxidant proteins in that they contain no prosthetic groups such as selenium or heme but have a catalytic site containing a conserved cysteine (Cys) residue coordinated with an arginine residue and either a threonine or serine residue (Flohé et al., 2003). They can be further split into two subgroups, i.e., 1-Cys or 2-Cys peroxiredoxins. The 2-Cys peroxiredoxins are again further separated depending on whether they are typical or atypical peroxiredoxins (Wood et al., 2003). In the 2-Cys peroxiredoxins, one Cys residue is located at the C-terminus and the other at the N-terminus of the protein. The N-terminal Cys is known as the peroxidatic Cys and this is where H_2O_2 and organic hydroperoxides attack and oxidise the peroxidatic Cys residue while themselves becoming reduced (Wood et al., 2003). The 1-Cys Prx enzymes contain one conserved Cys residue and this is the peroxidatic Cys at the N-terminal end. The 2-Cys Prx enzymes which include Prx1, 2, 3 and 4 when oxidised form a sulfenic acid and this then causes them to form homodimers creating disulphide bridges between the peroxidatic Cys of one monomer and the second Cys of the other monomer. This dimer then allows for the thioredoxin reductase to reduce the oxidised peroxidatic Cys resiude back to its original active form (Flohé et al., 2003) so that the whole catalytic process can be repeated. Research has tended to show that Prx proteins act as a

back up to other antioxidant proteins such as GPx enzymes in selenium deficient situations (Flohé et al., 2003). This assumption is based on the common observation the GPx enzymes have greater catalytic efficiency than Prx enzymes.

1.7 South Australian aquaculture

Yellowtail Kingfish (YTK) is a marine fish species found mostly in temperate to warm waters and it is commercially and recreationally fished worldwide (Aquaculture SA, 2002). After southern bluefin tuna (SBT, *Thunnus maccoyii*), YTK is the second most valuable marine fish farmed in South Australia. Aquaculture is a $468 million industry in South Australia alone (Australian Fisheries Statistics, 2008). More than 40 aquaculture species are produced commercially throughout Australia (Primary industries and resources SA, 2009) and aquaculture is currently the fastest growing primary industry in the world (Miller et al., 2009). Fish is the primary source of food for over 1 billion people around the world (NSW Department of Primary Industries, 2004) showing how important it is for the industry to continue growing.

1.8 LCPUFA, where do they come from?

Marine fish such as YTK obtain omega-3 LCPUFA from fish oils from lower trophic level fish such as sardines from capture fisheries (Miller et al., 2008). These lower trophic level fish are high in EPA and DHA which bioaccumulate in the flesh of fish from algae at the bottom of the food chain. The fatty acids become incorporated into the muscle (fillet), liver and other organs as part of the lipid membranes (Geay et al., 2010). The current production of capture fisheries is about 85-95 million tonnes per year worldwide and these capture fisheries are the source of fish oil used in aquaculture feeds (Ng et al., 2007). Over the last

20 years, the production from capture fisheries has reached a plateau, while aquaculture production has increased exponentially (Fig. 1.4). As a result, capture fisheries are currently at their sustainable limits (Miller at al., 2008; Ng et al., 2007). Thus, the continued use of capture fisheries to supply fish oil for aquaculture feeds is unsustainable and this has led to new ways of feeding farmed fish.

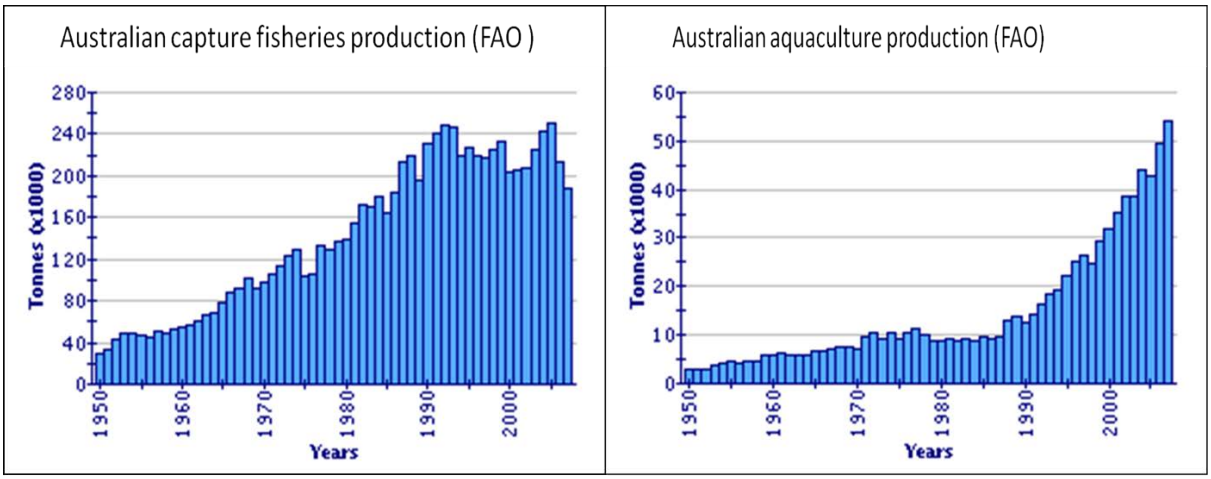

Figure 1.4: Australian capture fisheries and aquaculture production between 1950 and 2007 showing production in kilotonnes per year. Figures obtained from the Food and Agricultural Organization of the United Nations, Fisheries and Aquaculture Department.

Currently, the most popular alternative to fish oils from capture fisheries is vegetable oils from terrestrial plants (Turchini et al., 2009). The problem is though that vegetable oils are devoid of the n-3 LCPUFA EPA and DHA found in fish oils and instead they are rich in the C_{18} PUFA α-linolenic acid (ALA) or linoleic acid (LA) (Geay et al., 2010). Marine fish are poor at converting ALA to EPA and DHA (Tocher, 2003) and in addition ALA and LA compete with 24:5n-3 for Δ6 desaturase activity required to convert 24:5n-3 to 24:6n-3 which is the immediate precursor of DHA (Regost et al., 2003; Park et al., 2009).

1.9 Fatty acid compositions in different oils

Oils obtained from different plants and animals contain different fatty acid compositions. Fish oils are rich in n-3 LCPUFA and low in n-6 PUFA. In comparison, other animal oils are high in saturated fatty acids and n-6 PUFA but very low in n-3 PUFA. Plant oils vary greatly in their fatty acid compositions but are devoid of n-3 and n-6 LCPUFA and they contain different levels of n-3 and n-6 PUFA. Table 1.2 shows the fatty acid compositions of several animal fats, fish oils and plant oils. The Table shows that all plant oils listed except linseed oil contain larger amounts of n-6 PUFA than n-3 PUFA. Linseed oil is the opposite however and contains more n-3 PUFA than n-6 PUFA.

A benefit of switching to plant oils which are devoid of the LCPUFAs is the reduction of the number of double bonds which are prone to oxidative damage. This can increase the health and quality of the fish and research has shown that the health and biochemistry of the fish pre-mortem can also directly be associated with the quality of the fish post-mortem (Ferrante et al., 2008).

1.10 Marine fish *vs*. fresh water fish

The capacity of organisms to synthesize DHA from its C_{18} precursor ALA varies from species to species and fish are no exception to this rule. Fresh water fish have been found to very effectively use the LCPUFA synthesis pathway to produce LCPUFA from shorter n-3 fatty acids (Owen et al., 1975). By substituting the fish oil for plant oils, the LCPUFA synthesis pathway is up regulated, primarily in fresh water fish. Marine fish however have so far been unsuccessful in experimental studies to increase the rate of LCPUFA synthesis (Geay et al., 2010; Owen et al., 1975). Geay et al. (2010) found that the gene expression of fatty acyl

desaturases can be up regulated with the inclusion of vegetable oils rich in PUFAs, however protein expression tests have show that the protein levels remain unchanged possibly due to post transcriptional modifications. Alternatively Mourente et al. (2005) have shown that the inclusion of vegetable oils has no effects on the expression of both fads and elovl.

Table 1.2: Typical fatty acid compositions (% total fatty acids) of fish oils, vegetable oils and animal fats. Reproduced from Turchini et al. (2009)

Oils/fats	Iodine value‡	SFA	MUFA	LA	AA	ALA	EPA	DHA	n-6 PUFA	n-3 PUFA	n-3/n-6 ratio
Fish oils											
Anchovy oil	180–200	28.8	24.9	1.2	0.1	0.8	17.0	8.8	1.3	31.2	24.0
Capelin oil	95–160	20.0	61.7	1.7	0.1	0.4	4.6	3.0	1.8	12.2	6.8
Menhaden oil	150–200	30.5	24.8	1.3	0.2	0.3	11.0	9.1	1.5	25.1	16.7
Herring oil	115–160	20.0	56.4	1.1	0.3	0.6	8.4	4.9	1.4	17.8	12.7
Cod liver oil	n.a.§	19.4	46.0	1.4	1.6	0.6	11.2	12.6	3.0	27.0	9.0
Vegetable oils											
Crude palm oil	44–58	48.8	37.0	9.1	–¶	0.2	–	–	9.1	0.2	0.0
Soybean oil	120–141	14.2	23.2	51.0	–	6.8	–	–	51.0	6.8	0.1
Canola/rapeseed oil	110–126	4.6	62.3	20.2	–	12.0	–	–	20.2	12.0	0.6
Sunflower oil	110–143	10.4	19.5	65.7	–	–	–	–	65.7	0.0	0.0
Cottonseed oil	99–113	45.3	17.8	51.5	–	0.2	–	–	51.5	0.2	0.0
Groundnut oil	n.a.	11.8	46.2	32.0	–	–	–	–	32.0	0.0	0.0
Corn oil	103–128	12.7	24.2	58.0	–	0.7	–	–	58.0	0.7	0.0
Linseed oil	177	9.4	20.2	12.7	–	53.3	–	–	12.7	53.3	4.2
Animal fats											
Beef tallow	41–52	47.5	40.5	3.1	0.4	0.6	–	–	3.1	0.6	0.2
Pork lard	52–74	38.6	44.0	10.2	–	1.0	–	–	10.2	1.0	0.1
Poultry fat	80–85	28.5	43.1	19.5	–	1.0	–	–	19.5	1.0	0.0

†Data compiled from National Research Council (1993), Gunstone *et al.* (1994) and Hertrampf and Piedad-Pascual (2000).
‡Expressed as grams of iodine absorbed per 1000 g of fat/oil, which is a measure of the chemical saturation of the oil/fat.
§Data not available.
¶Not detectable.
AA, arachidonic acid, 20:4 n-6; ALA, α-linolenic acid, 18:3 n-3; DHA, docosahexaenoic acid, 22:6 n-3; EPA, eicosapentaenoic acid, 20:5 n-3; LA, linoleic acid, 18:2 n-6; MUFA, monounsaturated fatty acids; n-3 PUFA, polyunsaturated fatty acids with the first double bond at the 3rd carbon atom; n-6 PUFA, polyunsaturated fatty acids with the first double bond at the 6th carbon atom; SFA, saturated fatty acids.

Vegetable oils have so far been the main focus to develop ways of increasing sustainability and productivity of aquaculture. Studies have looked at how fish oil substitution with plant oils such as linseed oil, canola oil and palm oil rich in n-3 PUFAs affects the growth and development of fish as well as how it affects the fatty acid profiles (Geay et al., 2010; Owen et al., 1975; Zheng et al., 2004). Numerous fish species from both marine and fresh water environments have been studied looking at fish oil substitution diets including Trout and Sea

Bream (Zheng et al, 2004), European sea bass (Geay et al, 2010) and Atlantic salmon (Zheng et al., 2004).

One way that has been suggested to maintain the high n-3 LCPUFA levels in fish fillets is to place the fish on a finishing diet high in n-3 LCPUFAs just prior to harvest compared to the grower diets which they are fed beforehand which contains the plant oils low in these n-3 LCPUFAs (Ng et al., 2007).

1.11 Aims

The overall aim of this project is to investigate dietary fish oil replacement with plant or terrestrial animal oils and its impact on the seafood product quality in yellowtail kingfish (*Seriola lalandi*), an important aquaculture species in South Australia.

Five diets were tested:

1. a fish oil-based diet high in the n-3 LCPUFA EPA and DHA.
2. a canola oil-based diet devoid of LCPUFA but containing high proportions of C_{18} n-3 and n-6 PUFA and high proportions of monounsaturated fatty acids
3. a poultry oil-based diet devoid of the n-3 LCPUFA EPA and DHA but rich in saturated fatty acids, monounsaturated fatty acids and n-6 PUFA
4. a fish/canola oil-based diet
5. a fish/poultry oil-based diet

For fish fed each of these diets, the expression of 6 genes was investigated using qPCR:

1. Peroxiredoxin 1 (Prx1), an antioxidant enzyme which reduces hydrogen peroxide and small organic hydroperoxides.

2. Peroxiredoxin 4 (Prx4), also an antioxidant enzyme which also reduces hydrogen peroxide and small organic hydroperoxides.

3. Glutathione peroxidase 1 (GPx1), another antioxidant enzyme which reduces hydrogen peroxide and small organic hydroperoxides.

4. Glutathione peroxidase 4 (GPx4) which as well as reducing hydrogen peroxide and organic hydroperoxides also reduces lipid hydroperoxides.

5. Δ6 fatty acyl desaturase (Δ6 fads) which is involved in the synthesis of LCPUFA from the C_{18} precursors ALA and LA which are abundant in vegetable oils

6. fatty acyl elongase (elovl) which is involved in the synthesis of LCPUFA from the C_{18} precursors ALA and LA which are abundant in vegetable oils

Two main hypotheses were tested and they were that the replacement of fish oil (highly unsaturated) with canola oil (less highly unsaturated) or poultry oil (highly saturated) would:

1. decrease the expression of the antioxidant genes (GPx1, GPx4, Prx1, Prx4) because of the decreased unsaturation of the fish flesh reflecting the decreased unsaturation of the diet and
2. increase the expression of the LCPUFA synthesis genes (fads and elovl) because of the decreased abundance of LCPUFA in the fish flesh reflecting their decreased abundance in the diet

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APPENDIX 1

Sequencing data and blast searches of PCR amplicons

β-actin

ATCGGCATGAGAGGTTCCGTTGCCCAGAGGCCCTCTTCCAGCCTTCCTTCCTGAAACAAACAAAAAACCCCGA
AGCTACCYWWARAACCMAGGMYAARGTGATAGAGTCCCATTTTTTTTTCCCTGGAGAAGAGCTACGAGCTG
CCTGACGGACAGGTCACACCATCGG

Blast results

HM047109.1	Stegastes partitus beta-actin mRNA, partial cds	91.6	177	59%	1e-15	98%
GU188683.1	Lates calcarifer beta-actin mRNA, partial cds	91.6	91.6	30%	1e-15	98%
GQ223283.1	Menidia estor beta-actin mRNA, partial cds	91.6	91.6	30%	1e-15	98%
FJ966346.1	Menidia peninsulae isolate Mpen2 beta-actin gene, partial cds; and intron	91.6	91.6	30%	1e-15	98%
FJ966345.1	Menidia peninsulae isolate Mpen1 beta-actin gene, partial cds; and intron	91.6	91.6	30%	1e-15	98%

ELF1a

CCTTTGTCATCATCCTGAACCACCCTGGCCAGATCAATGAGGGTTATGCCCCAGTGCTGGATTGCCACACCGCT
CACATTGCCTGCAAGTTCAGAGGAGCTCATCGAGAAGATTGACCGTCGTTCCGGCAAGAAGAGAGAG

Blast results

AB032900.1	Seriola quinqueradiata mRNA for elongation factor 1 alpha, complete cds	233	233	92%	2e-58	99%
AB326302.1	Solea senegalensis SseEF1A1 mRNA for elongation factor 1 alpha isoform 1, complete cds	211	211	92%	7e-52	96%
EU561358.1	Hippoglossus hippoglossus elongation factor 1 alpha gene, partial cds	206	206	92%	3e-50	95%
EU561357.1	Hippoglossus hippoglossus elongation factor 1 alpha mRNA, complete cds	206	206	92%	3e-50	95%
EU412621.1	TSA: Hippoglossus hippoglossus all_halibut.1599.C1 mRNA sequence	206	206	92%	3e-50	95%

ELO-564

TGCTGCTGGACAACTACCCACCAACCTTTGCATTCACAGTCATGTACCTTCTGATCGTGTGGAWGGGGCCCAA
GTACATGAAACACAGGCAGCCGTACTCCTGCAGAGGCCTCCTGGTGCTCTACAATCTGGGCCTCACACTCTTG
TCTTTCTACATGTTCTATGAGCTTGTTACTGCTGTGTGGCACGGTGGCTACAACTTCTACTGCCAGGACACTCA
CAGTGCACAGGAAGTGGATAACAAGATTATAAATGTCCTGTGGTGGTATTACTTCTCCAAGCTCATCGAATTC
ATGGACACATTTTTCTTCATACTACGAAAGAATAATCATCAGATCACGTTTCTTCACATCTACCACCACGCTACC
ATGCTGAATATCTGGTGGTTCGTTATGAACTGGATACCCTGCGGCCATTCGTACTTCGGTGCGTCCCTAAACAG
CTTCGTCCACGTCGTGATGTATTCTTACTAC

Blast results

GU047382.1	Lates calcarifer fatty acid elongase (Elovl) mRNA, complete cds	754	754	100%	0.0	95%
GQ214180.1	Lates calcarifer fatty acyl elovl5 elongase mRNA, complete cds	749	749	100%	0.0	95%
FJ440239.1	Rachycentron canadum polyunsaturated fatty acid elongase (elovl5) mRNA, complete cds	743	743	100%	0.0	94%
FJ156735.1	Thunnus maccoyii isolate SBTElo putative fatty acyl elongase mRNA, complete cds	741	741	99%	0.0	94%
GQ204105.1	Thunnus maccoyii isolate sbtElovl5 fatty acyl elongase mRNA, complete cds	730	730	99%	0.0	94%

fads

CTTTTTCCCCCTAATTATGTTCATCTTGGTTAATGAAGAGTCACGGTTCGTGTGGGGACTCAGAGACTTCCCTTT
CCTTGACWCGACCATGAGAAGCACCTRGACTKGCTAACCATGCAGCTACGGAAAAACCGGTAATATCGAGCA
GTCCTTCTTCAACGACTGGTTCTCTTGACATCAGA

Blast results

AB069727.1	Oreochromis niloticus Fadsd6 mRNA for putative delta-6 fatty acyl desaturase, complete cds	89.8	89.8	71%	5e-15	78%
GQ214179.1	Lates calcarifer fatty acyl delta6 desaturase mRNA, complete cds	84.2	84.2	73%	2e-13	78%

GPx1

TGATCGTTATATAGAGGGACGGGCAAGCTAATGGAACGGGCGATAGTAAATGATGTGGCGTCTCTCTGAGGT
ACGACCGCCAGGGATTACACCCAGATGAACGAGCTCCACCAGCGGTACTCCGGCAAGGGGCTKRTSATCCTG
GGAGTGCCCTGCAACCAGTTCGGACATCAGGAGAAGCCTTATTTACTGCGCTCCGTAATGACCCCTCGAAAAA
AAGATGGACT

Blast results

GQ376155.1	Cyprinus carpio glutathione peroxidase 1 (Gpx1) mRNA, partial cds	128	128	57%	1e-26	84%
EF139089.1	Carassius auratus glutathione peroxidase 1 mRNA, partial cds	128	128	55%	1e-26	84%

NM_001007281.2	Danio rerio glutathione peroxidase 1a (gpx1a), mRNA	124	124	55%	2e-25	83%
BC083461.1	Danio rerio glutathione peroxidase 1a, mRNA (cDNA clone MGC:103683 IMAGE:7261884), complete cds	124	124	55%	2e-25	83%
BC076215.1	Danio rerio cDNA clone IMAGE:7079552	124	124	55%	2e-25	83%

GPx4

AAAGAAGCCTTAAATTGTTTACACGGTGTTCAACACCACCTGCAAATGTGCTCCCCAGTAAACTACTCTCAGTT
TGATCAGATGCACGCCAAGTATGCTGAGAGAGGTTTACGCATCCTTGCCTTCCCCTCAAACCAGTTTGGGAAC
CAGGAGCCTGGCAATGAATCTCAGATCAAACAGTKTGCCCAGTCTTACAACGCTCAGTTCGACATGTTCAGCA
AGATCGAA

Blast results

EF452498.3	Thunnus maccoyii phospholipid hydroperoxide glutathione peroxidase mRNA, complete cds	259	259	76%	4e-66	93%
CR692381.2	Tetraodon nigroviridis full-length cDNA	243	243	76%	4e-61	91%
AY309440.1	Micropterus salmoides glutathione peroxidase-4 mRNA, partial cds	233	233	74%	3e-58	91%

Prx1

AAAAAGGCGTGCTCTGTGGTGAGACTCTGTGCTTGGTGCAGGCTTTCCTGTATATATATCTTTTTTTTTTTTTTG
GAAAAAGGGGGGGTTTTGTTTTTCTTTTTTGGTGTRTYCTTGKKKTTTSTAACCTTCCCTCTTCGAAAGAGGTTA
TGAGGCAGATCACCATCAATGACTGCCCGTGGTCGCTCTTGACGAATTTGTTATA

This resulted in no blast results, only 1 nucleotide match between forward and reverse primers

Prx4

CCCCCCGGGTGGACTTGGAGAAATGAAAATCCCTCTGTTGTCTGACCTCACTCATCAGATTCAAAAGACTATG
ATCAACATCGTTTCTAATGTTGTTGGGGGAGACGGATCTGACTCCACGAATTCGTTCACTTGTGGCGATTACG
GGCGTTTAATTAAAACATCCGACTTTTAAAGGA

Blast results

FJ013222.1 Seriola lalandi 2-Cys peroxiredoxin 4 (PRDX) mRNA, complete cds 119 119 37% 6e-24 98%