TELEOST FATTY ACYL DESATURASE GENES: A COMPARATIVE STUDY.

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Declaration

I hereby declare that this thesis was composed by myself and is the result of my own investigations. It has neither been accepted nor submitted for any other degree. All sources of information have been duly acknowledged.

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A Ph.D. is not just the obtaining of a degree but also a right of passage into the world of science and as a right of passage it is full of tormentuous times which without the help and support of others it would be impossible to successfully pass through.

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ABSTRACT

Marine teleosts, unlike their freshwater counterparts, have a repressed ability to synthesise long chain highly unsaturated fatty acids (HUFA). In competent species, the Δ6 and Δ5 fatty acid desaturases are critical in the biosynthetic pathway that produces the HUFA's arachidonic acid (20:4*n*-6; AA), eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA) from the C_{18} polyunsaturated fatty acids (PUFA), linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3). The deficiency in HUFA biosynthesis in marine fish is of considerable practical significance because, in consequence, farmed marine species require a dietary source of presynthesised HUFA. This is provided by processed products from "industrial" species of marine fish such as sand eel, sardine, capelin and anchovies which themselves obtain HUFA through the food chain. Indicators suggest that the wild fishery supporting the aquaculture feed industry is unsustainable at current levels of exploitation. This has consequential effects on human health as fish, especially marine fish, are the predominant dietary source of HUFA that are crucial for maintaining cell membrane integrity as well as being central to eicosanoid metabolism.

Therefore, the primary aims of this project were to further our understanding of the molecular differences in HUFA biosynthesis between marine and freshwater teleosts. This was achieved by comparing the fatty acid desaturase genes of representative marine and freshwater fish. The desaturases are enzymes involved in the biosynthesis of HUFA from PUFA and have been considered as one of the steps that may be compromised in marine fish. The desaturase genes were studied with a view to relating structural, and potential functional differences with different HUFA synthesis phenotypes.

During the course of this project sequences of putative desaturase genes were cloned from two freshwater (zebrafish and carp), two marine (turbot and cod) and one anadromous fish species (Atlantic salmon). Once translated, the protein sequences of all the gene products contained all the necessary domains and motifs shown to be required for efficient desaturase function including an N-terminal cytochrome b_5 domain, and three catalytically important histidine boxes conserved in all members of the gene family. They all included the variant third histidine box that

seems typical of Δ5 and Δ6 desaturase genes described to date. All of the protein sequences from the fish species had greatest homology to the mammalian desaturases, specifically the human Δ6 desaturase.

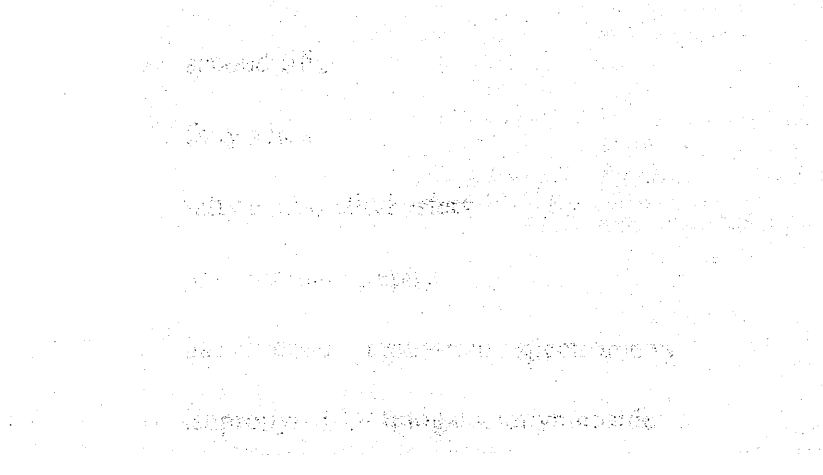
The cDNAs of salmon, carp and zebrafish were functionally characterised in *Saccharomyces cerevisiae*. Three carp transcripts were sequenced and functionally characterised. Two had no Δ5 or Δ6 desaturase activity, while the third efficiently desaturated 18:3*n*-3 at the Δ6 position. Of the two functionally characterised salmon transcripts one had no Δ5 or Δ6 activity whereas the third efficiently desaturated 20:4*n*-3 at the Δ5 position. The transcripts that had no desaturase activity were considered either non-functioning alleles or pseudogenes acquired as a result of a genome doubling event. It is believed that other Δ5/6 like desaturases probably exist for both carp and salmon as salmon is known to have high levels of Δ6 desaturase activity. However, neither the cod nor the turbot cDNAs were functionally characterised in yeast.

The most significant result of the functional characterisation study concerned the zebrafish (*Danio rerio*). The 1590 bp transcript has close similarity to mammalian Δ6 desaturase. However, the clone encodes a novel desaturase. When expressed in yeast the zebrafish gene confers the ability to convert 18:2*n*-6 and 18:3*n*-3 to their corresponding Δ6 desaturase products, 18:3*n*-6 and 18:4*n*-3. In addition, it confers the ability to convert 20:3*n*-6 and 20:4*n*-3 to their Δ5 desaturase products, 20:4*n*-6 and 20:5*n*-3, respectively. Therefore, the zebrafish gene encodes a bi-functional enzyme having both Δ6 and Δ5 desaturase activity. This was the first report of a functionally characterised desaturase of fish, and, in particular, of a fatty acid desaturase with both Δ6 and Δ5 activity.

The structure of the primary sequences of the fish desaturases were analysed in relation to function and some interesting and potentially highly significant relationships were discovered. However, it was not possible to determine which residue or residues were responsible for the differing substrate specificities between the transcripts.

In summary, the results presented in this thesis indicate that (i) all the fish species used in this study possessed desaturase-like sequences (ii) the zebrafish contains a novel, unique desaturase enzyme with both Δ6 and Δ5 desaturase activity (iii) marine fish possess Δ5/6 desaturase-like transcripts (iv) there is some evidence that fish species that have undergone tetraploidy or recent genome duplication appear to have duplicated genes, possibly pseudogenes and/or non-functioning alleles (v) significant differences in primary structure which may have important consequences for function were observed although unequivocal identification of residues responsible for determining function or specificity was not possible.

In conclusion, this study has produced results that not only further our understanding of the fatty acid genes of fish but which also furthered our knowledge of the fatty acid desaturases in general. The data will facilitate studies of how fatty acid desaturase primary structures relate to function. Information from this and other studies will lead to complete knowledge of how sequence and structure contribute to confer substrate specificity and how the fatty acid desaturase gene family has evolved.



LIST OF ABBREVIATIONS

AA	arachidonic acid
ALA	alpha linolenic acid
BSA	bovine serum albumin
cDNA	complimentary DNA
CHD	coronary heart disease
Chr	chromosome
CNS	central nervous system
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EFA	essential fatty acids
EPA	eicosapentaenoic acid
F1	first filial
F2	second filial
FA	fatty acids
FAME	fatty acid methyl esters
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
IPTG	isopropyl-B-D- thiogalactopyranoside
LA	linoleic acid
LC-HUFA	long chain highly unsaturated fatty acids
LDL	low density lipoprotein

LT	leukotrienes
MFO	mixed function oxidase
MMLV	molony murine leukaemia virus
MUFA	monounsaturated fatty acids
OFN	oxygen free nitrogen
ORF	open reading frame
PCR	polymerase chain reaction
PG	prostaglandin
PKS	polyketide synthase
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
TBE	Tris-boric acid-EDTA
TLC	thin layer chromatography
TX	thromboxane
UTR	untranslated region
UV	ultraviolet
XGAL	5-bromo-4-chloro-3-indoyl-beta-D-galactopyranoside

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Chapter 1

General Introduction

Chapter 1 – Introduction

Global wild fish stocks have been in decline for many years largely due to over-fishing, particularly during the last decades. The importance of this is two fold. Firstly, fish are an essential part of the human diet. They contain high levels of specific essential fatty acids (EFA) only found in abundance in fish or seafood and are often the major source of protein for rural people in the developing countries around the world. Secondly, the industrial wild fisheries of so-called "trash" species have been a source of high quantity fish meal and fish oil that have traditionally been used as a cheap feed source for the aquaculture industry. However, during the past decade worldwide aquaculture production has more than doubled and continues to rise steadily at over 10% per year. The benefits to the global economy from aquaculture in 2000, based on the total value of worldwide aquaculture production, was over $50 billion (FAO statistics).

The following chapter describes the importance of fish to human health as a source of EFA and discusses the benefits and requirements of fish in the human diet. It also summarises the EFA requirements of fish and humans and defines why, in relation to the fatty acid biosynthetic pathway, both have an absolute requirement for certain polyunsaturated fatty acids (PUFA) and/or highly unsaturated fatty acids (HUFA). This chapter will discuss, with reference to the study of the PUFA biosynthetic pathway in fish, how furthering our understanding of the biosynthesis of HUFA may aid the alleviation of fishing pressures on wild fish stocks and improve human and fish nutrition.

Before the relationship between EFA and human health can be made some definitions and background on mans diet and where PUFA and HUFA originate are required.

1.1 Essential Fatty Acids

Fatty acids are essential to life and are major components of many lipid classes and to date over 100 fatty acids have been isolated from lipids of various different organisms. There are two major characteristic features of fatty acids. The first is a hydrocarbon chain, usually linear but can be branched, which may possess one or more carbon-to-carbon double bonds ("unsaturated") or no double bonds ("saturated"). The second characteristic is a terminal carboxyl group. The fatty acids differ primarily in the number of carbon atoms in the hydrocarbon chain and in the number and position of their unsaturated carbon-to-carbon bonds. Saturated and monounsaturated (one double bond) fatty acids can be synthesised by all plants and animals. However, only plants and a few lower invertebrate animals can synthesise PUFA with 2 or more double bonds. Therefore, PUFA are essential dietary components for most animals, including all vertebrates such as mammals and fish.

1.1.1. Unsaturated fatty acids

This project is concerned with unsaturated fatty acids, which contain carbon-to-carbon double bonds as opposed to saturated fatty acids which do not contain any double bonds. There are three classes of unsaturated fatty acids, monounsaturated fatty acids (MUFAs), PUFAs and HUFAs. The MUFAs are defined as fatty acids that contain only 1 carbon to carbon double bond in their hydrocarbon backbone. The PUFAs contain 2 or more double bonds, whereas the HUFA is a term for a subgroup of PUFA with 20 or more carbon atoms in the hydrocarbon chain and with 3 or more double bonds (Sargent *et al.*, 2002).

1.1.2. Fatty acid nomenclature

The unsaturated fatty acids have their own nomenclature symbolised by shorthand notation that designates the length of the carbon chain and the number, position and

the configuration of the double bonds. Thus, lauric acid which contains no double bonds and 12 carbon atoms in the hydrocarbon backbone is 12:0, whereas oleic acid, which is an 18 carbon unsaturated fatty acid containing 1 double bond between carbons 9 (from the methyl end) and 10, is 18:1*n*-9. Therefore, linoleic acid (LA), 18:2*n*-6 is an 18 carbon unsaturated fatty acid with 2 double bonds with the first located at carbon 6 from the methyl end and α-linolenic acid (ALA), 18:3*n*-3 has 3 double bonds with the first located on carbon 3 from the methyl end.

1.2. Food Webs and Mans Changing Diet

1.2.1. Our ancestral diet

The human diet today is very different to our ancestors', whose diet contained fewer calories and was higher in fibre, fruits, vegetables, meat and fish. As a result of this, our ancestral diet was lower in total fat, particularly saturated fat and contained roughly equal amounts of *n*-6 and *n*-3 PUFA. The major *n*-6 PUFAs are LA and arachidonic acid (20:4*n*-6; AA) and the major *n*-3 PUFAs are ALA, eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA). The ratio of *n*-6 to *n*-3 PUFA of our ancestors was probably about 1:1 with a maximum of 2:1 with relatively higher levels of HUFA, such as EPA, DHA, and AA than our diet today. It is believed that our own species (*Homo sapiens*) are unlikely to have evolved such a large, complex and metabolically expensive brain in an environment which did not provide abundant dietary LC-PUFA (Broadhurst *et al.*, 2002). It is thought that most of this HUFA was provided by exploitation of river and estuarine stranded and spawning fish rather than organised, sophisticated hunting or fishing trips (Broadhurst *et al.*, 2002). Therefore, the human body probably evolved a poor ability to metabolise LA to AA and ALA to EPA and DHA. The *n*-6/*n*-3 PUFA ratio today in Western societies ranges from 10:1 to 25:1 indicating that Western diets are

relatively deficient in *n*-3 PUFA compared with the diet on which the human lineage evolved (Simopoulos, 2000). This is a crucial point since the *n*-3 and *n*-6 fatty acids are not interconvertible in the human body, have differing physiological functions or efficacies and, as described later, are important components of cell membranes as well as being central to eicosanoid metabolism. The fatty acid composition of cell membranes is, to a large extent, dependent on dietary intake. Thus, receiving the correct amounts and ratio of dietary *n*-6 and *n*-3 fatty acids is crucial. Western societies have now been recommended to decrease their intake of *n*-6 fatty acids and increase their *n*-3 PUFA consumption (Simopoulos, 1999, 2000).

1.2.2. Food webs

The primary producers in the terrestrial food production systems are the green plants which are rich in short chain C_{18} PUFA. Unlike the terrestrial environment the primary producers of the food webs underlying the major fisheries are unicellular phytoplanktonic algae. These phytoplankton consist of a number of species. These include the silicaceous diatoms, various flagellates, the most important of which are the dinoflagellates, and haptophyceans including, *Emiliania huxleyi* and *Phaeocystis pouchetii* (Sargent *et al.* 1995). The diatoms contain large quantities of 20:5*n*-3 and C_{16} *n*-3 PUFA but negligible amounts of 22:6*n*-3 whereas the dinoflagellates contain large amounts of 22:6*n*-3 and 18:5*n*-3. These species differ from other groups of algae such as the green algae which do not produce 22:6*n*-3 but instead produce various C_{16} to C_{20} *n*-3 PUFA (Sargent *et al.* 1995). The phytoplankton are harvested by the zooplanktonic crustaceans. It is not known if the zooplankton can chain elongate and desaturate the shorter chain *n*-3 PUFA to their longer-chain homologues. However, given the amount of HUFA in their diet they probably do not require to form significant amounts of the longer chain homologues. The

zooplankton are in turn consumed by the zooplanktonivorous fish (Sargent *et al.* 1995). Thus, HUFA are transferred up the marine food web from phytoplankton to fish.

1.3. Why are PUFA important?

1.3.1. Health and essential fatty acids

1.3.1.1. Essential fatty acids in early life

Essential fatty acids are structural components of all tissues, being indispensable for cell membrane synthesis. N-3 HUFA, especially DHA, are found in abundance in the brain, retina and other neural tissues (Uauy *et al.*, 1999). As such, there have been many studies that have shown the importance of DHA to brain development particularly in foetal and newly born infants. The DHA for developing foetuses comes entirely from the maternal supply, moreover, the maternal EFA status decreases during pregnancy (Hornstra *et al.*, 1995). The maternal EFA status is also significantly lower in multiparous as compared to primiparous women. However, significant increases in AA and DHA in foetal brain tissue have been observed during the last trimestor of gestation and initial postnatal months (Clandinin *et al.*, 1980). This would suggest a specific requirement for HUFA, particularly DHA, during pregnancy.

One study by Innis and colleagues showed that the progression of language development is related to DHA status (Innis *et al.* 2001). Their results reveal a significant relationship between DHA and speech perception performance. Infants with higher DHA status at 2 months of age were better able to discriminate a non-native phonetic contrast at 9 months of age. Other workers have observed a significant difference in cognitive scores in term infants fed formulas supplemented with DHA (Birch *et al.*, 2000).

22:6*n*-3 is present in large quantities in the retina where it comprises about 50% of the total esterified fatty acids (Neuringer *et al.*, 1998). The exact function of 22:6*n*-3 is unclear however recent reviews have concluded that the experimental evidence supports a recommendation to add 20:4*n*-6 and 22:6*n*-3 to preterm infant formulas (Laurtizen *et al.*, 2001). The studies have examined the effect of adding 22:6*n*-3 to formula on different aspects of the visual and cognitive abilities of healthy preterm infants with a gestational age from 28-31 weeks. In summary the studies with preterm infants have all shown a positive effect of dietary 22:6*n*-3 on the visual development. Carlson's randomised clinical studies in preterm infants supplemented with HUFA demonstrated better visual acuity in infants up to 4 months of age when supplemented with DHA compared to infants without supplementation (Carlson *et al.*, 1993, 1996,a,b; Werkman and Carlson, 1996). The investigators also reported more rapid visual processing, as measured by the Fagan test of visual recognition, at 6-12 months of age in DHA supplemented infants (Fagan, 1984). Another experiment investigated the dietary restriction of *n*-3 PUFA through 2 or more generations in rats and discovered that it resulted in a loss of brain DHA which was associated with poorer performance in spatial tasks (Salem *et al.*, 2001).

Fatty acids, particularly PUFA and HUFA or their metabolites also regulate cellular gene expression, including during retinal and central nervous system development. This can be done at the second messenger level as regulation of gene expression by fatty acids occurs at the transcriptional level and is mediated by nuclear transcription factors activated by fatty acids. There are two types of PUFA responsive transcription factors, the peroxisome proliferator-activated receptors (PPARs) and the hepatic nuclear factor 4α (Uauy, 1999). PUFA can also have an effect on structural function. Significant effects on photoreceptor membranes

involved in the signal transduction process, rhodopsin activation and rod and cone development within the eye are also caused by DHA (Uauy, 1999).

1.3.1.2. Essential fatty acids and human health

Eicosanoids, a term coined by Corey and colleagues (Corey *at al.*, 1980) is a generic name for a range of oxygenated metabolites of 20-carbon polyunsaturated fatty acids, including dihomo-γ-linolenic acid (20:3*n*-6), AA and EPA. They are formed enzymatically via membrane-bound cyclooxygenases or specific lipoxygenases primarily from AA, the most abundant direct precursor fatty acid in mammals. Prostaglandins, prostacyclins and thromboxanes (prostanoids), leukotrienes, lipoxins, and other hydroxy fatty acids belong to this extensive family of eicosanoids (Fischer, 1989).

In blood vessel endothelium, AA is mainly transformed into prostaglandin (PG) I_2 which is a highly potent vasodilator and inhibitor of thrombocyte aggregation (Moncada *et al.*, 1976). It also prevents the shape change of platelets and reduces adherence of platelets to the endothelium (Weiss & Turitto, 1979). In contrast the main cyclooxygenase product of AA in thrombocytes is thromboxane (TX) A_2 which is a strong vasoconstrictor and stimulator of platelet aggregation. The balance between synthesis of PGI_2 by the endothelium and synthesis of TXA_2 by platelets is one of the major factors maintaining vascular integrity (Moncada & Vane, 1979).

The leukotrienes (LT) are thought to have a proinflammatory role which may be of importance in the human cardiovascular disease (Fischer, 1989). LTC_4 and LTD_4 influence vascular tone and enhance vascular permeability, whereas, LTB_4 exerts a chemotactic activity on neutrophils and may cause their adherence to the arterial endothelium.

N-3 and n-6 fatty acids and coronary heart disease.

There is extensive evidence in the literature that demonstrates beneficial health effects of substituting saturated fatty acids with PUFA, particularly in reducing coronary and arterial disease risk in humans (Keys *et al.,* 1986; Wood & Oliver, 1992). It has been observed that human populations with relatively high levels of PUFA in their adipose tissue have less mortality from coronary heart disease (CHD). In the ISIS (Indian Study of Infarct Survival), 360 patients with suspected myocardial infarction were divided into 3 groups, in one group, 122 patients were given fish oil containing 1.8 g/day EPA plus DHA (Singh *et al.*, 1998). Another group of 120 patients took mustard oil containing 2.9 g/day ALA, and 118 were given an aluminium hydroxide-containing placebo. Further cardiac events such as sudden death, fatal and non-fatal myocardial infarction occurred in 24.5% of fish oil patients and in 47.4% of placebo patients (Singh *et al.*, 1998). Another similar study treated 11,324 survivors of myocardial infarction with either a 1g/day capsule of 85% EPA and DHA, 300 mg of vitamin E, both, or neither. Treatment with EPA and DHA, but not with vitamin E, significantly lowered the risk of fatal myocardial infarction and stroke by 10 to 15% depending on the statistical analysis (GISSI, 1999). It seems that in both these studies the observed benefit was a reduction in myocardial infarction, mostly fatal myocardial infarctions and not to a reduction in non-fatal myocardial infractions. It is believed that most sudden deaths are due to myocardial infarctions complicated by severe arrhythmia and/or pump failure (von Schaky, 2001). Some, therefore, consider the benefit of *n*-3 fatty acids to be mainly due to their antiarrhythmic action. Dietary HUFA also have beneficial effects on blood lipid levels by decreasing triacylglcerol and cholesterol by decreasing low density lipoprotein (LDL). Moreover, *n*-3 PUFA have a greater effect than *n*-6 PUFA

and HUFA greater than C_{18} PUFA (von Schaky, 2001). If this is the case then it is possible that a combination of LDL-lowering statins with *n*-3 PUFAs seems a promising approach towards the prevention of atherosclerosis (Nordoy *et al.*, 2000). In the trials mentioned the dose of *n*-3 PUFA that was found to effective, safe and free from troublesome side effects were 0.85 to 1.5 g/day. However, studies have found it more difficult to establish the absolute optimal *n*-6 to *n*-3 ratio for good health.

In the 60's, the Seven Countries Study showed that cohorts of middle-aged men from Italy, Greece and former Yugoslavia had a daily fatty acid intake in the range of 24-27% of total energy (~9% saturated fatty acid, 11% MUFA and 3% PUFA) (Keys *et al.*, 1986). These figures were much lower than populations from Northern Europe such as Finland and the Netherlands whose total fat intake was 34-38% (~20% saturated fatty acid, 12% MUFA and 3-5% PUFA). Correlated with fat and PUFA levels the death rates from CHD were lower in populations from the Mediterranean area (Keys *et al.*, 1986).

There is, of course, a balance, as in some circumstances very high dietary PUFA may enhance arterial damage and atherosclerotic progression (Rubba & Iannuzzi, 2001). The possible mechanism responsible for this could be the oxidation of PUFA carried by LDL (Diaz *et al*, 1997). It was established from epidemiological studies on Eskimos that there was a low incidence of atherosclerotic disease (Kromann & Green, 1980). The Eskimos are known to eat a large amount of marine food rich in HUFA. However, there is also a prolonged bleeding time accompanied by a reduction in ex vivo platelet aggregability (Dyerberg & Bang, 1979).

The effect of n-3 and n-6 PUFA on immune and inflammatory disease.

Autoimmune disorders and dietary fatty acids are linked. Multiple sclerosis, an autoimmune disorder of the CNS was one of the first to be linked to dietary fatty acids epidemiologically and to be treated with changing the intake of *n*-6 and *n*-3 PUFA (Belluzzi, 2001). In 1978, 116 patients with acute remitting multiple sclerosis were treated by Bates (Bates *et al.*, 1978). The patients received either linoleic or γ-linolenic acid with oleic acid as a control group and although the rate of deterioration and frequencies of attacks were not significantly different between treated and control group, the exacerbations were shorter and less severe in patients receiving a high dose of LA than those in controls (Bates *et al.*, 1978). Subsequent more recent studies support this (Swank and Grimsgaard 1988; Norvik *et al.*, 2000).

Rheumatoid arthritis is a chronic inflammatory disorder. Belch and colleagues (1988) administered 49 rheumatoid arthritis patients with γ-linolenic acid (18:3*n*-6; GLA) alone (450 mg/day) or in association with EPA (240 mg/day plus GLA 450 mg/day) on a stable dose of nonsteroidal anti-inflammatory drugs (NSAIDS). 73% of the patients taking GLA alone and 80% of the GLA/EPA group were able to reduce and/or stop their NSAIDS intake compared with only 33% in the placebo group (Belch *et al.*, 1988). A similar beneficial effect of GLA and EPA was observed by Leventhal (Leventhal *et al.*, 1993). Several other studies have looked at *n*-3 PUFA supplementation. One study by Geunsens demonstrated that supplementation of 90 rheumatoid arthritis patients with 2.6 g of *n*-3 HUFAs significantly reduced the use of NSAIDs and/or anti-inflammatory drugs (Geunsens *et al.*, 1994). In a similar way Faarvang and co-workers were able to demonstrate a small but significant improvement in morning stiffness and joint tenderness by

giving 51 active rheumatoid arthritis patients 3.6 g of *n*-3 HUFAs (Faarvang *et al.*, 1994).

Other inflammatory diseases in which an imbalance in the ratio of *n*-6 to *n*-3 PUFA may be implicated include inflammatory bowel disease, ulcerative colitis and Crohn's disease (Belluzzi, 2001). It appeared that the increasing incidence of Crohn's disease was strongly correlated with the increasing ratio of *n*-6 to *n*-3 fatty acid intake. Thus increased dietary intake of *n*-6 with less *n*-3 PUFAs may contribute to the development of Crohn's disease. Some epidemiological studies indicate that the level of dietary fat intake and the types of fatty acids consumed influence cancer risk and disease progression. Research such as that on breast cancer by Bougnoux *et al.* (1994), and on colon cancer (Caygill *et al.*, 1995), show that increasing *n*-3 PUFA in the diet can help prevent and/or inhibit progression of these cancers. Increasing the proportion of *n*-3 to *n*-6 fatty acid in the diet by either taking fish oil supplements or by eating oily fish may be beneficial in many other diseases and conditions such as asthma (Hodge *et al.*, 1998), and depression (Edwards *et al.*, 1998).

The effect of n-3 and n-6 PUFA on depression

As previously mentioned DHA is essential for the growth and development of the brain of infants but it is also required for maintenance of normal brain function in adults (Horrocks & Yeo, 1999). Depression has been linked to depletions of *n*-3 fatty acids, particularly DHA, levels and/or an increase of the *n*-6/*n*-3 fatty ratio in adipose tissue (Mamalakis *et al.*, 2002) and in erythrocyte phospholipids (Mischoulon and Fara, 2000; Peet *et al.*, 1998). Depressive symptoms during the postpartum period has also been linked with low levels of DHA in the mothers' milk (Hibbeln, 2002). Increasing the *n*-3 fatty acids, in particular DHA, has resulted in some cases to a longer period of remission (Colin *et al.*, 2003).

Therefore, *n*-3 PUFA are beneficial to human health, from early foetal development in the womb through to adulthood and old age. Fish are the only major food that contain *n*-3 HUFA in sufficient amounts to supply human *n*-3 HUFA requirements. The paradox is this; the worldwide fishing industries have been over-exploiting the wild fish stocks, therefore, in order to maintain fish as a source of *n*-3 HUFA, future fish for human consumption must come from aquaculture. However, in order to produce these fish the aquaculture industries use fish oils in their fish feed, but the decline in the world fish stocks brings with it a decline in the supply of fish oils. There have been many attempts to replace fish oil with vegetable oil from a variety of plants but doing so is difficult and problematic as vegetable oils contain no *n*-3 HUFA. Mosses, ferns and liverworts are the only terrestrial plants which contain any EPA and DHA (Tinoco, 1982). Since few plants contain *n*-3 HUFA, and the only potential feeds that contains high levels of *n*-3 HUFA are fish themselves, replacing fish oils with vegetable oils results in many problems for the aquaculture industry. The following paragraphs discuss why the replacement of fish oils with vegetable oils produces major problems for the aquaculture industry.

1.3.2. Essential fatty acids and fish

1.3.2.1. Fatty acid composition of fish

The dietary lipid and fatty acid requirements of fish came under investigation in the 1960's with the beginnings of the aquaculture industry. Artificial diets for fish were originally formulated without consideration for the fatty acid composition of the oil component (March, 1992). Oil rather than fat was chosen for the inclusion in salmonid diets on the basis that a low melting point would be more conducive to utilisation at the lower environmental temperature of these species. However, it was soon discovered that corn oil, a popular oil component in experimental diets for

avian and mammalian species, produced a number of adverse effects on the growth and behaviour of fish (March, 1992). The reason for this was ultimately discovered to be due to the overly high *n*-6 to *n*-3 PUFA ratio. The lipids of terrestrial mammals including humans are comprised mainly of *n*-6 fatty acids (Fischer, 1989), whereas fish lipids are composed mainly of *n*-3 fatty acids. Freshwater fish contain relatively high levels of *n*-3 PUFA but marine fish generally contain even higher ratios of *n*-3 to *n*-6 PUFA. The muscle of capelin (*Mallotus villosus*), a marine fish, contains an *n*-3 to *n*-6 PUFA ratio of 18.9, whereas muscle from freshwater fish such as carp (*Cyprinus carpio*) and green snakehead (*Ophiocephalus punctatus*) have *n*-3/*n*-6 PUFA ratios of 2.0 and 1.6 (Henderson & Tocher, 1987). In the anadromous fish, Atlantic salmon (*Salmo salar*) the ratio of *n*-3 to *n*-6 in whole body samples is 5-10 (Henderson & Tocher, 1987).

1.3.2.2. Symptoms of EFA deficiency in fish

There are observable signs of EFA deficiency in the rainbow trout (*Oncorhynchus mykiss*) including fin erosion, swollen pale livers, heart myopathy and shock syndrome (Castell *et al.*, 1972, March, 1992). The shock or fainting syndrome occurred when they were stressed by handling or by a blow to the side of the tank (March, 1992). Castell published some of the first work reporting the dietary requirement for essential fatty acids in trout and the effects of EFA deficiency in fish (Castell *et al.*, 1972). He reported that symptoms such as fin erosion, heart myopathy and a shock syndrome were cured or prevented by linolenic acid. The effects of *n*-3 PUFA deficiency in trout are similar to those of *n*-6 PUFA deficiency in rats. Burr and Burr observed that rats fed a diet deficient in essential EFA consumed excessive amounts of water without excreting large amounts of urine (Burr & Burr, 1930). They observed that the condition of the flesh suggested the accumulation of water

within the muscle tissue and a role for EFAs in membrane permeability. A similar effect is observed in trout, whereby the trout flesh is soft and flaccid but is only marginally higher in water content (March, 1993). The fin erosion in EFA deficient trout may be caused by loss of integrity of the skin, conducive to bacterial infection in a similar way to EFA deficiency leading to tail erosion in rats. In turbot (*Scophthalmus maximus*), the destruction of the gill epithelium also provided an important sign of EFA deficiency (Bell *et al.*, 1985). Gilthead sea bream (*Sparus aurata*) swim bladders are affected by EFA deficiency, resulting in the bladder being unable to inflate (Koven *et al.*, 1990). It is believed that 20:5*n*-3 and 22:6*n*-3 fulfil the same basic structural functions in cell membrane phospholipids in fish as 20:4*n*-6 in terrestrial mammals (Sargent *et al.*, 1995). Therefore, EFA functions can be divided into two broad areas, firstly, a general role for HUFA in maintaining the normal structure and function of cell membranes and secondly, a role for certain specific HUFA as precursors of the eicosanoids (Sargent *et al.*, 1995).

The effects of the incorrect dietary intake and ratio of PUFA in fish has been observed in many studies and effects are found at all life stages from the young fry to fully matured adults, and within many species (Watanabe, 1982). When rainbow trout are fed an EFA deficient diet for three months prior to spawning there was a decline in egg production, hatching was poor and a proportion of the larvae were deformed (Watanabe, 1982). Survival of asian seabass (*Lates calcarifer*) larvae at metamorphosis is also severely affected. PUFA deficiencies cause a significant increase in mortality rates (Dhert *et al*, 1990). Larvae that have sufficient PUFA in their diet perform better in grow-out conditions compared to PUFA-deficient larvae and are more resistant to starvation, temperature or oxygen stresses, and are consequently less susceptible to disease (Dhert *et al*, 1990). Tuncer and Harvell

(1992) obtained a similar result when studying larval striped bass (*Morone saxatilis*) and palmetto bass (*Morone saxatilis* X *Morone chrysops*) fed PUFA-enriched versus unenriched *Artemia*. They found that the higher the concentration of HUFA's in the *Artemia*, the better the survival and growth of the fish larvae (Tuncer and Harvell, 1992).

When fingerling rainbow trout were fed on corn oil as the only dietary lipid source they grew poorly and had only 75% survival after 12 weeks compared to trout fed lipids that contributed *n*-3 PUFA to the diet. The fish fed *n*-3 PUFA grew faster with better feed efficiency and lower mortality (Lee *et al.*, 1967).

Other symptoms of deficiency in young fish include impaired vision, as shown in juvenile herring (*Clupea harengus*) (Bell *et al.*, 1995). The herrings ability to capture prey at low light intensities when rod cells are operative, is hampered considerably in a manner which is consistent with impaired vision. Thus, insufficient HUFA in marine fish larval diets is likely to impair neural and visual development, as in other experimental animals, with significant and serious consequences for a whole range of physiological and behavioural processes including those dependent on neuroendocrines.

1.3.2.3. Marine-freshwater differences

The precise EFA requirement is dependent on the fish species. It has been discovered from a number of studies that the specific PUFA requirement appears to correlate with the environment in which the fish is found. An observable division was observed between "freshwater fish" and "marine fish" or some authors prefer omnivorous/herbivorous fish and piscivorous/carnivorous fish.

The basic requirement for PUFA remains the same for both freshwater and marine fish, that is all fish require dietary PUFA, but the qualitative and quantitative

requirements vary between species. Marine fish generally receive high levels of HUFA's in their diet, as they generally consume fish and small marine organisms rich in AA, EPA and DHA. In contrast, freshwater fish do not obtain such high levels of HUFA but instead attain a higher proportion of short chain PUFA's such as 18:2*n*-6 and 18:3*n*-3. The diet of the early life stages of freshwater fish commonly features algae that contain higher levels of C_{18} PUFA and relatively deficient in C_{20} or C_{22} HUFA. Aquatic insects are a major feature in the diet of freshwater fish, particularly salmonids, and contain AA and EPA up to 7 and 25%, respectively, of the total fatty acids, although they predominately contain C_{18} PUFA (Bell *et al.*, 1994). However, freshwater fish do have the ability to bioconvert the C_{18} PUFA to HUFA's via a fatty acid desaturation and elongation pathway. A measure of the ability to bioconvert PUFA is the relative bioconversion ability or RBCA (Kanazawa *et al.*, 1979). Fresh water fish such as rainbow trout have an RBCA defined as 100, whereas marine fish such as red sea bream (*Chrysophrys major*) have an RBCA of 15, puffer fish (*Fugu rubripes*) of 13 and rockfish (*Sebasticus marmoratus*) of 7. Thus, marine fish have a much lower RBCA than freshwater fish indicating a much lower activity of the fatty acid desaturation/elongation pathway, and consequently marine fish must receive preformed C_{20} and C_{22} HUFA's in their diet (Kanazawa *et al.*, 1979). Therefore, whereas freshwater fish can bioconvert PUFA's such as 18:3*n*-3, ALA was not effective as an EFA source for marine fish such as turbot (Owen *et al.*, 1975), red sea bream (Fujii and Yone, 1976) and puffer fish (Kanazawa, *et al.*, 1979).

The reason underpinning the absolute dietary requirement of marine fish for C_{20} and C_{22} PUFA was elucidated by injecting isotopically labelled 18:2*n*-6 and 18:3*n*-3 into fish. The results showed that marine teleosts, such as red sea bream, rockfish and puffer fish, scarcely convert exogenous C_{18} PUFA to C_{20} and C_{22} HUFA

(Kanazawa *et al.*, 1979). Turbot fed 1-^{14}C-labelled 18:1*n*-9, 18:2*n*-6 or 18:3*n*-3 converted only small amounts of labelled fatty acid (3-15%) into fatty acids of longer chain lengths (Owen *et al.* 1975). However, in rainbow trout, ayu (*Plecoglossus altivelis*) and eel (*Anguilla japonica*) fed radiolabelled 18:3*n*-3, substantial amounts of radioactivity were recovered in EPA and DHA (Kanazawa *et al.*, 1979). Owen showed that 70% of the radioactivity recovered from injected ^{14}C18:3*n*-3 was recovered as DHA in trout (Owen *et al.* 1975). Tilapia (*Oreochromis nilotica)* fish are more unusual in that they require *n*-6 PUFA as found in mammals, rather than *n*-3 PUFA as in most fish species. *Oreochromis nilotica* requires 18:2*n*-6 at a level of 0.5% of total fatty acids (Takeuchi *et al.*, 1983).

However, there are exceptions and in this instance it is carnivorous freshwater fish. Northern pike (*Esox lucius)* is a fresh water species and, as a top level predator, highly carnivorous, and it does not convert 18:2*n*-6 or 18:3*n*-3 to the corresponding HUFA to any significant extent (Henderson *et al.*, 1995). However, studies of the juvenile stages of the herbivorous, freshwater, red pirhana (*Serrasalmus natteri*) when reared on mosquito larvae readily convert 18:2*n*-6 to 20:4*n*-6 and 18:3*n*-3 to 20:5*n*-3 and 22:6*n*-3. The larvae are relatively deficient in C_{20} and C_{22} PUFA. In this instance it's a omnivorous/herbivorous fish and piscivorous/carnivorous split rather than a "freshwater" and "marine" split.

The question then arises as to what causes the differences in EFA or PUFA metabolism and consequently requirements; why do humans and some fish species require HUFA and why can some species of fish survive on short chain PUFAs? It is important to understand why different fish have differing capabilities to convert C_{18} PUFA from vegetable oils into HUFA in fish. In order to do this the biosynthetic pathway must be considered.

1.4. The Biosynthetic Pathway of HUFA

One obvious possibility is that the different EFA requirements are due to differences in the biosynthetic pathway for HUFA. Although they can be obtained from the diet, saturated fatty acids can also be synthesised *de novo* by all organisms. Similarly, all organisms can synthesise *de novo* monunsaturated fatty acids. Thus, all plants and animals can synthesise *de novo* fatty acids such as stearic acid (18:0) and oleic acid 18:1*n*-9. However, only plants can synthesise the PUFA, linoleic and linolenic acids. Most animals other than a few lower vertebrates, cannot biosynthesise PUFA *de novo* and so must obtain these from their diet. Once obtained, however, they can be chain elongated and desaturated to HUFA through a biosynthetic pathway, although the extent to which this pathway operates varies enormously between different species.

The HUFA biosynthetic pathway consists of a series of elongations, additions of 2 carbon atoms, and desaturations, insertions of carbon-to-carbon double bonds. Animal tissues are able to introduce double bonds at the Δ5 and Δ6 positions of acyl chains. The desaturases are named according to the position in which they insert the double bond. In this instance the insertion carbon is counted from the carboxyl end of the fatty acid. These desaturase enzymes require oxygen and are similar to mixed function oxidases (MFO) and the desaturase reaction is simply a variation of a standard MFO. The chemistry of the reaction is below:

$$NADH^{+} + H^{+} + RCH2\text{-}CH2R + O2 \Rightarrow NAD^{+} + RCH{=}CHR + 2H_2O$$

The hydrogens and electrons are passed down an electron transport chain from NADH to the non-haem iron in cytochrome b_5 reductase to the haem iron in the

cytochrome b_5 ending up on the iron in the histidine box structures, the desaturase being the last step in this pathway. The hydrogens and electrons are eventually passed to oxygen along with the two hydrogens abstracted from the fatty acid chain to form the double bond in the fatty acid and two water molecules. However, the detailed chemical mechanism is not fully understood.

The first double bond inserted into a saturated fatty acid is at the Δ9 and once converting the carbon chain from 18:0 into 18:1*n*-9, the second double bond must be entered between the methyl end of the fatty acid and the Δ9 bond. Only plants have so-called "methyl directed" desaturases that can introduce double bonds between the Δ9 and the terminal methyl carbon. Thus, only plants contain Δ12 and Δ15 desaturase enzymes. Therefore, oleic acid (18:1*n*-9) is desaturated by Δ12 desaturase to linoleic acid (18:2*n*-6), which can then be desaturated further by Δ15 desaturase to produce α-linolenic acid (18:3*n*-3). This project concerns the so-called "front end" desaturases which introduce double bonds between the Δ9 position and the carboxyl group.

The fatty acyl desaturases are members of a family of enzymes which are found in all plants and animals and that have the function of introducing double bonds in fatty acyl chains. The fatty acyl desaturases can be classified into three main sub-families (Tocher *et al.*, 1998).

- The acyl-ACP desaturases; these introduce double bonds into fatty acids esterified to acyl carrier protein (ACP) and are found in the stroma of plant plastids.
- The acyl-lipid desaturases; these are membrane-bound enzymes associated with the endoplasmic reticulum and chloroplast (plastid) membranes of

higher plants and cyanobacterial thylakoid membranes, and desaturate fatty acids esterified in glycerolipids.

- The acyl-CoA desaturases; these introduce double bonds into fatty acids esterified to coenzyme A (CoA) and are membrane-bound in the endoplasmic reticulum of animals, plants and fungi.

This project is concerned with the latter type of desaturases, the membrane-bound, acyl-CoA desaturases.

It is apparent that some organisms are more efficient at biosynthesising HUFA than others. To understand why this is, we must first understand the pathway. However, this itself is complicated as the nature of the pathway is still under investigation and different interpretations of the pathways can be found in the literature. Until recently it was believed that long chain fatty acids were biosynthesised from a carbon 18 precursor via the enzymatic pathway shown in Figure 1. The original pathway involves LA or ALA undergoing Δ6 desaturation followed by elongation, a Δ5 desaturation, a further elongation step and finally a Δ4 desaturation step. However, there was no good experimental evidence for the existence of a Δ4 desaturase. For example, Ayala and co-workers (1973) could not detect any Δ4 desaturase activity when they incubated rat liver and testes microsomes with 22:4*n*-6. Rosenthal also suggested that there may an alternative pathway based on metabolic modification of [3-^{14}C] docosatetraenoate (22:4*n*-6) and [3-^{14}C] docosapentaenoate (22:5*n*-3) by human cells in culture (Rosenthal *et al.*, 1991). In similar experiments, Voss and colleagues were unable to detect radioactive 22:6*n*-3 when rat microsomes were incubated with [1-^{14}C] 22:5*n*-3 showing that 22:5*n*-3 is not a direct substrate for microsomal desaturation. However, they discovered that 22:5*n*-3 was efficiently chain-elongated to 24:5*n*-3 and, in addition,

this product was desaturated to 24:6*n*-3. As a result of these studies, a revised pathway in which C_{24} PUFA had roles as intermediates in the 22:6*n*-3 biosynthesis

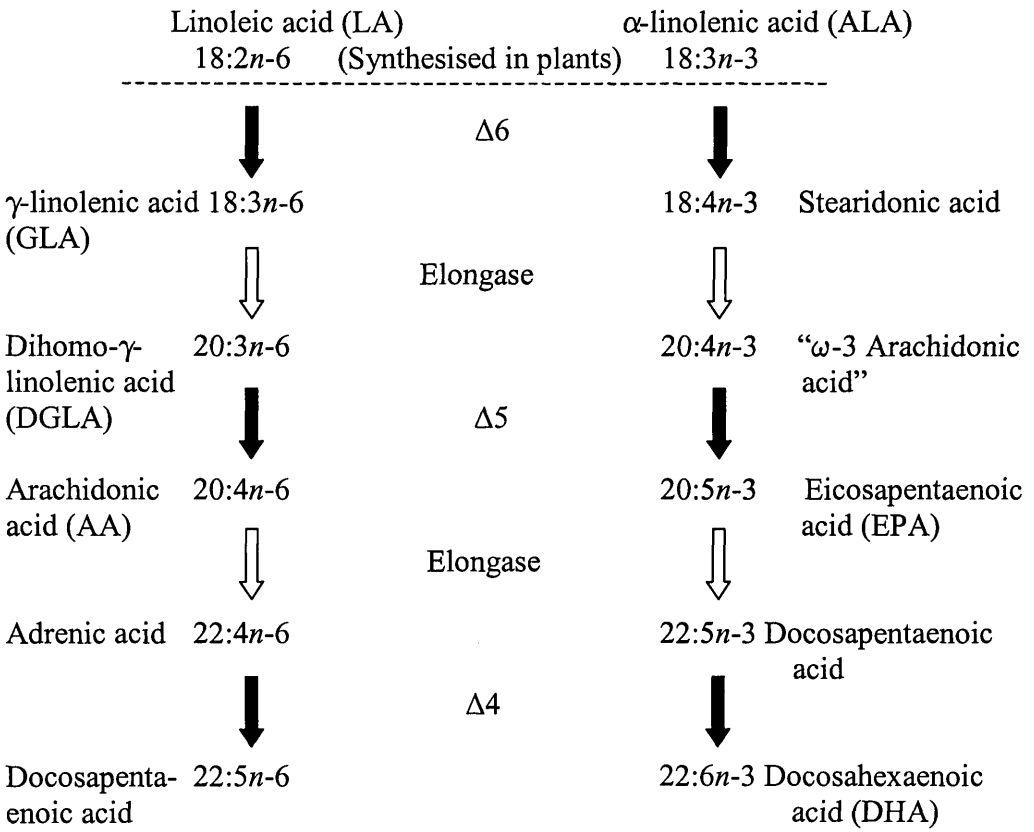


Figure 1. The original HUFA biosynthetic pathway.

pathway was suggested (Figure 2). The pathway suggests that 22:6*n*-3 is produced without the need for a Δ4 desaturase. Voss and colleagues reported that the primary metabolic fate for 24:6*n*-3 is chain shortening via limited β-oxidation to 22:6*n*-3. However, this pathway is complicated by the necessary movement of fatty acid intermediates between organelles in the cell as the partial degradation by β-oxidation appears to occur in the peroxisomes (Sprecher *et al.*, 1995, Sprecher & Chen, 1999). The evidence for this comes in part from patients with Zellweger disease, which is a cerebro-hepato-renal syndrome, specifically an autosomal recessive disorder with severe alteration in peroxisomal biogenesis (Petroni *et al.*, 1998). The Zellweger

patients had reduced levels of 22:5*n*-6 and 22:6*n*-3, a finding that implied that intact peroxisomes are required for their synthesis (Sprecher, 1996). Thus, it is believed that the C_{24} fatty acids move from the microsomes to the peroxisomes to be partially degraded to C_{22} fatty acids.

A noteworthy feature of the pathway in Figure 2 is that a Δ6 desaturation occurs twice, and there is continuing debate as to whether only one Δ6 enzyme exists or whether chain-length specific enzymes exist (Voss *et al.*, 1992). Two genes have

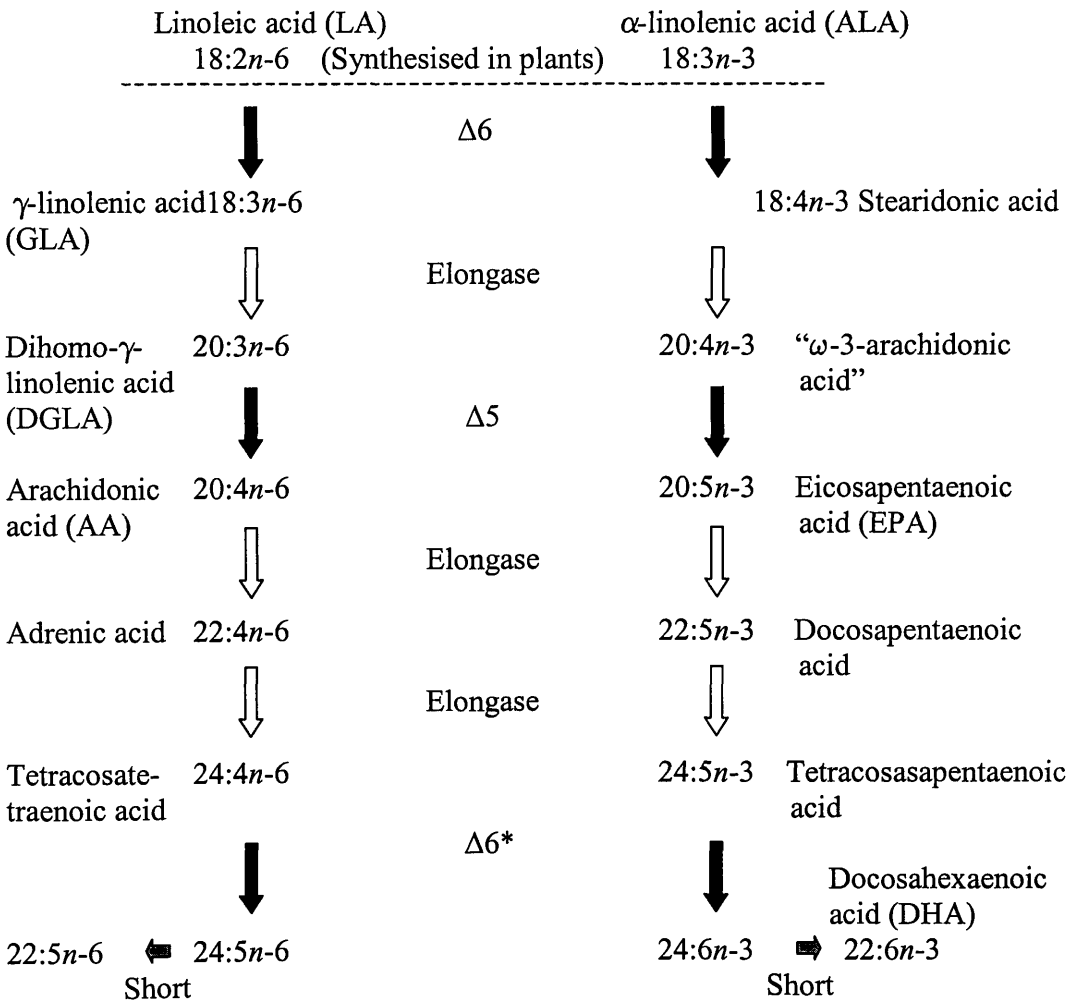


Figure 2. The alternative Sprecher pathway.

been detected for stearoyl Δ9 desaturase, and they are known to be tissue specific, but there is no evidence of chain length substrate specificity in stearoyl Δ9 desaturase (Kaestner *et al*, 1989). Marzo *et al* (1996) suggested that two different Δ6 desaturase activities may exist based on experiments using cell differentiation and culture in a serum free media. The results suggested that a distinctive Δ6-desaturase activity from that which acts on 18:2*n*-6 and 18:3*n*-3 may be involved in the synthesis of 22:6*n*-3. A situation with two distinct Δ6 desaturases could explain why the synthesis of 22:6*n*-3 appears to occur only in a restricted number of cell types, despite the large scale distribution of Δ6 and Δ5 desaturases, elongases and retroconversion enzymes in cells (Marzo *et al*, 1996). However, recent evidence points to only one Δ6 desaturase (de Antoneo *et al.*, 2001) However, several other biochemical studies by Sprecher, and Geiger gave results consistent with only one Δ6 desaturase (Sprecher *et al.*, 1994, 1995; Geiger *et al.*, 1993). Geiger and colleagues attempted to determine whether microsomes from rat liver contain chain-length-specific Δ6-desaturases (Geiger *et al.*, 1993). They made two discoveries, firstly that there was always preferential desaturation of 18:3*n*-3 over 24:5*n*-3 and secondly that competitive studies between 18:2*n*-6 and 18:3*n*-3 and between 24:4*n*-6 and 24:5*n*-3 showed that the *n*-3 fatty acid was always preferentially desaturated over the *n*-6 fatty acid. They interpreted these results as being consistent with a single Δ6-desaturase. Successful attempts have been made at the molecular level to obtain Δ6 desaturase and it has been isolated in *Borage officinalis* (Sayanova *et al.*, 1997), *Caenorhabditis elegans* (Napier *et al.*, 1998), *Mortierella alpina* (Saknurandani *et al.*, 1999, Huang *et al.*, 1999), rats (Aki *et al.*, 1999), mice and humans (Cho *et al.*, 1999). However, only one Δ6-desaturase has been isolated for each of these species. A third pathway involves a Δ8 desaturase enzyme and has also been described (see Chapter 3).

1.5. Potential Variation and Pathology of the HUFA Biosynthesis Pathway

1.5.1. The HUFA pathway in humans

A possible reason for the requirement of HUFA in the diets of certain fish species and in the human diet may be due to inherent rate limitations in the biosynthetic pathway. The first step in the pathway is the Δ6-desaturase which is believed to be the rate-limiting step (Brenner, 1966; Horrobin, 1993, Riemersma, 2001). Studies show that humans can form 22:6*n*-3 from 18:3*n*-3 but that conversion was relatively slow. When human skin fibroblasts were incubated with [1-^{14}C] linolenate or [1-^{14}C] linoleate for 24 hours, only 2.7% of the radioactivity from linolenate was recovered in 22:6*n*-3, 11.2% was in 20:3*n*-3 and 58% remained as 18:3*n*-3. Similarly, only 5.6% of the radioactivity from linoleate was recovered in 20:4*n*-6 and 86% remained as 18:2*n*-6 (Aeberhard *et al.*, 1978). It has also been reported that human liver microsomes desaturated 18:2*n*-6 to 18:3*n*-6 and 18:3*n*-3 to 18:4*n*-3, under the same experimental incubation conditions as rat and mouse liver microsomes, but at much lower rates than the rat or mouse microsomes (de Gomez Dumm and Brenner, 1975).

Some very early studies looking at EFA deficiency had also given information on the activity of the HUFA biosynthetic pathway in humans. The relationship between EFA and atopic eczema has been known since the 1930's when the Burrs, who discovered EFAs, reported that hand dermatitis improved on increasing EFA intake (Crawford, 1983). Dermatitis is an early sign of EFA deficiency in both humans and animals. In rats, dermatitis had been associated with low EFA concentrations in blood and that the condition responded to very high doses of LA (20-50g/day) (Brown & Hansen, 1937; Hansen *et al.*, 1947). However, one of the most important studies reported that LA concentrations in blood of eczema

patients treated with LA were normal, whereas AA concentrations were well below normal indicating that the conversion of LA to AA was either not efficient or impaired (Brown & Hansen, 1937). The fatty acid composition of plasma phospholipids from normal and atopic eczema patients were compared, and it was observed that LA concentrations were normal but the concentrations of GLA, DGLA and AA in patients with atopic eczema were below normal (Manku *et al.*, 1982). This suggested that either the Δ6 desaturase activity was reduced or that the consumption of the metabolites was excessive and could not be compensated by the rate-limiting enzyme (Manku *et al.*, 1982). Manku observed that by by-passing the Δ6 desaturase by giving the patients GLA in the form of evening primrose oil led to partial normalisation of the phospholipid composition (Manku *et al.*, 1982). Several studies since then have also confirmed an abnormal EFA pattern in atopic eczema patients including studies on children (Wright and Bolton, 1989), in cord-blood lymphocytes of babies at risk of atopic eczema (Galli and Simopolous, 1989) and in red cells of eczema patients (Oliewiecki *et al.*, 1989).

Diabetes mellitus patients also appear to have an impaired Δ6 desaturase activity (Horrobin, 1993). In these patients, LA concentrations are almost always normal or slightly above normal whereas concentrations of LA metabolites are consistently below normal. Other complications arise with diabetes such as long term damage to the retina, kidneys, the cardiovascular system and the peripheral nerves. It is possible that these complications are partly caused by the impaired essential fatty acid metabolism. Supplementing the diet with GLA may help to improve or overcome these conditions (Horrobin, 1993).

Stable isotopes which are ideal for in vivo studies and give a better perspective from a whole animal nutrition point of view than using hepatocytes have

been used successfully in tracer studies. The studies have shown that women may possess a greater capacity for ALA conversion than men (Burdge and Wootton, 2002). This increased capacity may be important for meeting the demands of the foetus and neonate for DHA during pregnancy and lactation. It appears that adult males ability to convert ALA to DHA was either very low or absent and uptake of DHA from the diet may be critical for maintaining adequate membrane DHA concentrations in these individuals (Burdge *et al.*, 2002).

1.5.2. The HUFA biosynthesis pathway in fish

As mentioned previously, the EFA requirement of fish depends on the species in question which may be related to their feeding habitats and/or the aquatic environment in which they are found. Generally, freshwater fish can survive on short chain C_{18} fatty acids obtained from vegetable matter in their diets by converting it to HUFA whereas marine fish must obtain HUFA in their diet in order to survive. The differences in EFA requirement are due to differing abilities to biosynthesise HUFA, believed to be caused by rate-limiting steps within the pathway possibly being deficient, and/or inefficient enzymes. The inefficient or lacking enzymic steps thought to be responsible appear to vary depending on the species of fish studied. According to the literature, the fundamental difference between freshwater fish and marine fish in fatty acid desaturation ability is the deficiency or impairment of Δ5 desaturase in marine fish (Owen *et al.*, 1975, Tocher *et al.*, 1989). Freshwater fish can metabolise α-linolenic acid (18:3*n*-3) by Δ6 desaturation to stearidonic acid (18:4*n*-3) which can then undergo elongation to "ω3-arachidonic acid" (20:4*n*-3) as demonstrated in carp (Tocher *et al.*, 1996; Tocher & Dick, 1999; Tocher & Dick, 2000) and zebrafish (*Danio rerio*) (Meinelt *et al.*, 2000; Tocher *et al.*, 2002). The 20:4*n*-3 can be subsequently converted to EPA by Δ5 desaturase. Marine fish seem

to be deficient in Δ5 desaturase and therefore require the PUFA's EPA, DHA and probably AA in their diet (Bell *et al.*,1985). Rainbow trout cells possess Δ6 and Δ5 desaturase whereas Δ4 desaturase activity is absent. Turbot were first believed to have a very active Δ6 but deficient in Δ5 activity (Bell *et al.*, 1985). However, recent data on turbot cells are more consistent with a deficiency in the C_{18} to C_{20} elongase multi-enzyme complex rather than a deficiency in Δ5 desaturase (Ghioni *et al.*, 1999). In contrast, a recent study has shown that the Δ6 desaturase, $C_{18\text{-}20}$ and $C_{20\text{-}22}$ elongases were all highly active in gilthead sea bream cells (SAF-1). Therefore, the probable explanation for the dietary requirement for C_{20} and C_{22} highly unsaturated fatty acids in sea bream was due to a deficiency in Δ5 fatty acid desaturase (Tocher and Ghioni, 1999).

Thus it has been well established that marine fish have an inability to efficiently convert C_{18} precursor PUFA into HUFA, but this raises another question as to what type of pattern do anadromous fish possess? Anadromous fish such as Atlantic salmon cannot be classified as truly freshwater or marine fish, as they spend part of their life in freshwater lochs and rivers and part in the ocean. Atlantic salmon develop into adults in the ocean but breed and spend the early part of their life in freshwater. The eggs of the Atlantic salmon hatch in freshwater as alevins then once the yolk sac has been absorbed they become fry. The fry then develop into parr and remain in freshwater for 1 to 4 years. Some of the parr, each year, develop the silver colour of adults and are then termed smolts. It is the smolt stage which migrates downstream and out to the sea where they develop into adult fish over the course of 4 years (Tchernavin, 1939; Mills, 1971). When it is time for them to spawn, the fish re-enter freshwater systems, usually the systems from where they emerged, and migrate upstream to the spawning area where eggs are laid in the gravel of the river beds

(Tchernavin, 1939; Mills, 1971). Most of the males perish at this stage whereas the females usually survive until they have returned to the sea. A few females may, if they are in good condition, return again to spawn a second time (Henderson and Tocher, 1987). The parr-smolt transformation is a particularly stressful time for the fish as they experience many energy demanding processes involving a variety of physiological, morphological and behavioural changes to prepare them for life in the ocean. However, the transition seems to cause a change in the salmons ability to synthesise HUFA. Prior to smolting, when Atlantic salmon are in their parr stage, the fatty acid patterns of lipids are typical of freshwater fish (Henderson and Tocher, 1987). During their time at sea the fatty acid patterns of salmon lipids resemble marine fish. These differences could easily arise through differences in the diet in the two environments. However, it has been reported that smolts of steelhead trout (*O. mykiss*) held in fresh water in captivity had higher levels of HUFA than the parr from which they were produced. Sheridan (1983) concluded that the smolt had assumed a marine-type pattern in their lipids while still in fresh water, before they had entered the sea (Sheridan and Allen, 1983). The fish were fed the same diet throughout the period when they changed from parr to smolts suggesting that the changes in fatty acid profiles were not solely dictated by changes in the diet. Bell and co-workers have shown that the growth of Atlantic salmon post-smolts was not affected when fed either fish oil or a mixture of corn oil and lard over a 16 week period (Bell *et al.*, 1989). DHA and total *n*-3 PUFA in the polar lipids of liver and white muscle were unaffected by the different diets suggesting that LA undergoes elongation and desaturation to AA in post-smolts. This result means that the enzymatic processes in Atlantic salmon post-smolts are as in freshwater fish. However, Ackman and Takeuchi have found that Atlantic salmon undergoing the parr-smolt change had

little or no elongation and desaturation of 18:2*n*-6 to C_{20} HUFA although substantial amounts of 18:2*n*-6 were present in the tissue (Ackman & Takeuchi, 1986). It has been postulated by Bell "that at the time of the change to smolts, when body lipids are changing to an even more preponderantly long-chain *n*-3 HUFA spectrum, some inhibition by dietary fatty acids of the desaturases that metabolise 18:2*n*-6 may occur" (Bell *et al.*, 1989).

It is reasonable then to study the genes which code the enzymes involved in this pathway in an attempt to further our understanding of the biosynthesis of HUFA.

The study of the molecular biology of fatty acid desaturases began with the cloning of the Δ9 desaturase genes from both animals and plants. These genes were sequenced early on. Following this, the Δ12 and Δ15 (ω-3) fatty acid desaturases were cloned from cyanobacteria and higher plants. The first Δ6 to be cloned was that of *Borage officinalis* (Sayanova *et al.*, 1997) which allowed the first Δ6 from an animal to be cloned, that of *Caenorhabditis elegans* (Napier *et al.,* 1998). Around this time the first Δ5 desaturase from an animal was also cloned, again from the nematode *C.elegans* (Michaelson *et al.,* 1998). This paved the way and enabled the mammalian, rat (Aki *et al.*, 1999), human and mouse (Cho *et al.*, 1999), Δ6 desaturases to be cloned followed by the human (Leonard *et al.*, 2000) and rat (Zolfaghari *et al.*, 2001) Δ5 desaturases. However, until this time no Δ6 or Δ5 desaturases had ever been cloned from fish despite the importance of such a task. The following, final, paragraphs are a testament to this and outline the rationale and objectives of this project.

1.6. Rationale and Objectives of the Study

At the initiation of this project only a single desaturase gene had been cloned from any fish species, the Δ9 desaturase gene from the common carp (Tiku *et al.*, 1996). There were no further reports of the cloning of any other desaturase or elongase genes from any fish species. However, it is important to both clone and examine the function of these PUFA desaturase genes in fish. It is possible that some of the genes responsible for enzymes involved in the pathway either do not exist or are somehow defective due to their coding sequence. However, it may be more complicated than this and may involve the interaction of other regulatory genes or be due to promoter regions or other regulatory sequences within the genomic sequence itself. Moreover, since there is no sequence information on these genes it is important to first establish their sequence and function before any other hypotheses can be made. The cloning and functional characterisation of the genes involved in the biosynthesis pathway from both marine and freshwater fish would further our understanding of why different fish species have differing essential fatty acid requirements. If information was gained as to why certain fish have greater requirements for HUFA due to "physiological" blocks in the biosynthetic pathway caused by deficient and/or inefficient enzymes it would enable scientists working in fish health and nutrition to better understand the EFA required in fish feeds for different species. This may in the future have consequences on the use of marine fish oils in aquaculture feeds and consequently alleviate the pressures on wild fish stocks.

Therefore, the objectives of this study were as follows;

1. To clone and sequence the Δ5/6 fatty acyl desaturase genes of representative marine, anadromous and freshwater fish species.

2. To functionally characterise the cloned cDNAs with a view to relating structural with functional differences.
3. To establish how closely related the fish desaturase genes were in comparison to other cloned and functionally characterised Δ5/6 desaturase genes to further our understanding of the evolution of mammalian and fish desaturases.

Together the above objectives will aid our understanding of the biosynthesis of HUFA in different species of fish and may have consequences on the future development of feeds for the aquaculture industry as well as continuing to further our knowledge of desaturase evolution.

Chapter 2

General Materials and Methods

Chapter 2 – General Materials and Methods

The following chapter contains generic descriptions of the basic protocols and procedures used in the study. More specific descriptions cataloguing details of individual procedures are given in the relevant sections.

2.1 Fish Species

2.1.1. Species chosen for study

The decision on what species to study was made on the basis of several criteria. The first was environment, as mentioned in chapter 1 the main objective of this study was to investigate the desaturase genes from representative marine and freshwater teleosts. The second criterion was how important the species in question was to the aquaculture industry, with particular reference to the UK industry, as the study aimed to be of practical relevance. The third criterion was the genetics of the species. Species with simple diploid organisation were the preferred choice, although this was not entirely possible, as often the species with the greatest commercial importance to the aquaculture industry did not have a simple chromosomal organisation. The fourth criterion, was ease of obtaining RNA from liver samples. Liver contains large amounts of RNA degradative enzymes, more than any other tissues and the longer it takes to dissect the livers from the fish the greater the risk of RNA degradation. Therefore, the fish species used in the study had to be readily available and easily accessible to allow liver samples to be removed quickly and efficiently. It was for the above reasons that the fish species named below were used in this study.

2.1.1.1. Freshwater species

Two freshwater fish species were chosen, the first being zebrafish (*Danio rerio*).

Zebrafish (*Danio rerio*): Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Cypriniformes; Cyprinidae; Danio.

Danio rerio was chosen because it is a model species with a simple diploid makeup.

The second freshwater species was the carp, *Cyprinus carpio*.

Common Carp (*Cyprinus carpio*): Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Cypriniformes; Cyprinidae; Cyprinus.

The common carp is a commercially important species in many parts of the world and it is also closely related to the zebrafish although it has a complicated chromosomal organisation.

2.1.1.2. Anadromous species

One anadromous species was chosen for the study, the Atlantic salmon (*Salmo salar*).

Altantic salmon (*Salmo salar*): Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Euteleostei; Protacanthopterygii; Salmoniformes; Salmonidae; Salmo.

The Atlantic salmon is able to survive both in the freshwater and the marine environment which alone makes it an interesting species, however, it is also a highly valuable and commercially important species in the Northern and Southern hemispheres.

2.1.1.3. Marine species

Two marine species were chosen cod, *Gadus morhua*, and turbot, *Scophthalmus maximus*.

Cod (*Gadus morhua*): Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Euteleostei; Neoteleostei; Acanthomorpha; Paracanthopterygii; Gadiformes; Gadidae; Gadus.

Turbot (*Scophthalmus maximus*): Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Actinopterygii; Neopterygii; Teleostei; Euteleostei; Neoteleostei; Acanthomorpha; Acanthopterygii; Percomorpha; Pleuronectiformes; Pleuronectoidei; Scophthalmidae; Scophthalmus

Cod and turbot are both marine fish however, they are quite different from each other, turbot is a flatfish and cod is a gadoid. Both are commercially important species and fulfil the criteria mentioned above.

2.1.2. Tissue choice

In order to gain the gene sequence a tissue had to be chosen for the extraction of RNA. One of the sites of highest HUFA production is the liver and for this reason the liver was chosen as the organ of interest.

2.2. Gene Cloning

The following sequence of procedures was required before confirmation of the gene function using yeast expression assays. RNA was extracted from the tissue of choice, cDNA prepared from the RNA, PCRs carried out, the PCR products ligated into *E. coli* cloning vectors and the constructs transformed into *E. coli*. The positive colonies containing PCR/vector constructs were then identified by α-complementation, this gave blue and white coloured colonies, the white colonies contained vectors with inserts. These were subsequently sequenced for completion of identification.

2.2.1. Total RNA extraction

RNA was extracted from liver tissue using TRIzol® reagent (GibcoBRL, NY, U.S.A.). 50 – 100 mg tissue was homogenised with 1 ml of TRIzol® in a 1.5 ml microcentrifuge tube using the UltramaxT8 S8N5G (IKA Labortechnik, Staufen, Germany). The homogenate was subsequently incubated for 5 minutes at room temperature (~15°C) before the addition of 0.2 ml of chloroform (Sigma-Aldrich, Dorset, UK). The tubes were shaken vigorously by hand for 15 seconds, incubated for a further 3 minutes at room temperature and centrifuged in a chilled centrifuge, at 12,000 x g for 15 minutes at 4°C in a cooling microcentrifuge (Eppendorf, Hamburg, Germany). The aqueous phase was transferred to a clean 1.5 ml microcentrifuge tube and 0.5 ml of isopropanol (Sigma-Aldrich, Dorset, UK) added. The mixture was then incubated for 10 minutes at room temperature after which it was centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed, the pellet washed with 1 ml of 75% ethanol, mixed by vortex and centrifuged at 7,500 x g for 5 minutes at 4°C. The ethanol was aspirated, the pellet air dried, 100 μl of diethyl pyrocarbonate-(DEPC; Sigma-Aldrich, Dorset, UK) treated H_2O added and incubated at 55-60°C for 10 minutes to aid resuspension.

2.2.2. Preparation of cDNA

Briefly, cDNA was prepared from the liver RNA of all the species studied for use in PCR. cDNA was prepared by combining 10 μl total RNA (1 μg) with 1 μl of Not1polyT primer (200ng) (appendix III) in a 0.2 ml PCR tube, mixed by vortexing and incubating at 70°C for 5 minutes in the PTC-100 thermocycler PCR machine (MJ Research, MA, U.S.A.). The reaction mixture was cooled on ice and the following components added: 4 μl MMLV (molony murine leukaemia virus) buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT), 0.4 μl

dNTPs (10 mM), and brought to 20 μl with sterile H_2O. The reagents were briefly vortexed and incubated at 42°C for 5 minutes. After incubation, 1 μl (200 units) of MMLV enzyme (Promega, Madison, WI, U.S.A.) was added, the tube vortexed once again and incubated at 42°C for a further 50 minutes. Finally, the reaction mix was heated at 70°C for 10 minutes to inactivate the enzyme.

2.2.3. Preparation of 5' RACE cDNA

In some cases only partial sequence was gained from polyT cDNA, so in order to gain the full sequence including the start codon of the open reading frame, essential for functional characterisation, 5' RACE cDNA was prepared. This allowed the 5' end of the gene to be amplified without the need for gene specific primers.

The following protocol was based on the manual supplied with the SMART™ RACE cDNA amplification kit (Clontech, NJ, U.S.A.) and involved the use of a thermocycler (PTC-100, MJ Research, MA, U.S.A.). In a 0.2 ml PCR tube, 1 μl RNA (1 μg) was combined with 1 μl 5'-CDS primer and 1 μl SMART II A oligo (appendix III), and the reagents brought to a final volume of 5 μl with sterile H_2O. The contents were vortexed briefly and incubated at 70°C for 2 minutes before being cooled on ice for 2 minutes. Then the following reagents were added: 2 μl 5X First-strand buffer (250 mM Tris-HCl (ph8.3), 375 mM KCl and 30 mM $MgCl_2$), 1 μl dithiothreitol (20 mM), 1 μl dNTP mix (10 mM) and 1 μl PowerScript reverse transcriptase (Clontech, NJ, USA). The entire reaction was gently mixed by pipetting the reaction mix up and down, and incubated at 42 °C for 1.5 hours in the PTC-100 thermocycler. When the reaction was complete 100 μl of Tricine-EDTA buffer (10mM Tricine-KOH (pH 8.5), 1 mM EDTA) was added and the reaction mixture heated at 72°C for 7 minutes in the PTC-100, after which the newly synthesised cDNA was ready for use or storage at –20°C.

2.2.4. Cloning into *E. coli* vectors

The putative genes were cloned into *E.coli* TA overhang vectors. The rationale for cloning into these vectors is founded on the ability of Taq-DNA polymerase to add an unpaired deoxyadenosine (dA) to the 3' ends of the reaction products. The vectors have conveniently unpaired deoxythymidylate (dT) ends thus allowing easy ligation of the PCR product into the vector. The PCR products were cloned into one of either three vectors; the Invitrogen™ TOPO® TA cloning vector (Invitrogen, CA, U.S.A.) the Clontech pT-Adv vector from the AdvanTAge™ PCR cloning range (Clontech, NJ, U.S.A.), or into pBluescript® II KS (+/-) (Stratagene, La Jolla, CA, U.S.A.) by first adding the TA overhangs using Taq polymerase.

2.2.4.1. Clonetech pT-Advantage

The PCR products were ligated into the pT-Adv vector as follows. First an estimation of the amount of PCR product needed was calculated using the equation given in the corresponding kit manual and stated below:

$$\text{x ng PCR product} = \frac{\text{(y base pairs product) (50ng vector)}}{\text{(size of vector: ~5, 900base pairs)}}$$

Once calculated, the PCR products were ligated into the vector as follows: ? µl (as calculated above) PCR product, 1 µl T4 DNA ligase (Promega, WI, U.S.A.) 1 µl 10x corresponding ligation buffer (300 mM Tris-HCl (pH 7.8) 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP) 2 µl vector, and the reaction mixture brought to a final volume of 10 µl with sterile H_2O. The mixture was vortexed and incubated overnight at room temperature (~15°C). Upon completion, 2 µl of the reaction was used for direct transformation into competent *E.coli* cells.

Transformation into E.coli

One tube of competent TOP10F' *E.coli* cells (50 µl) was thawed on ice for each transformation. 2 µl of the ligation reaction was pipetted into the cell suspension and mixed by gently stirring with the pipette tip. Once mixed, the tubes were incubated on ice for 30 minutes before being heat shocked at 42°C for exactly 30 seconds in a water bath (Julabo SW-20c; Germany). The tubes were then incubated for a further 2 minutes on ice after which 250 µl of LB broth (appendix I) was added and the tubes shaken horizontally at 37°C for 1 hour at 225 revolutions per minute (rpm) in a rotary shaking incubator (Gallenkamp, Loughbourgh, UK). Once complete 125 µl of the reaction was spread onto one LB/X-gal/IPTG/ampicillin agarose plate (appendix I) and the remaining 175 µl onto a second plate. The plates were inverted and placed in a 37°C incubator overnight (Gallenkamp, Loughborough, U.K).

2.2.4.2. TOPO-TA Cloning® vector

When using the TOPO-TA Cloning® kit, the PCR product concentration was calculated as before, but the ligation set up varied from the previous kit protocol. The ligation reaction constituted 0.5 - 4 µl fresh PCR product, 1 µl salt solution and 1 µl TOPO® vector, both supplied with the kit, and the mixture brought to 10 µl with sterile H_2O. The reagents were mixed gently in a 1.5 ml microcentrifuge tube before being incubated for 30 minutes at room temperature (~15°C). Once complete, one tube of Invitrogen™ One Shot *E. coli* per ligation was thawed on ice and 2 µl of the TOPO® cloning reaction added. This reaction mixture was incubated on ice for 5 minutes and subsequently heat-shocked for 30 seconds at 42°C in a waterbath (Julabo SW-20c; Germany). Afterwards, the tubes were transferred back to the ice and incubated for a further 2 minutes on ice before 250 µl of LB broth was pipetted

into each tube of *E. coli* cells. The tubes were capped and shaken at 37°C in a rotary shaking incubator at 225 rpm for 1 hour then plated out as above.

2.2.4.3. Preparation of pBluescript® II KS TA vector (Non-kit method)

The pBluescript® II KS (+/-) vector was first linearised at the polycloning site by digesting 2 μg of vector with 1 μl (20 units) of Eco RV enzyme (NewEngland Biolabs., MA, U.S.A.), 1 μl of No. 3 buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) (NewEngland Biolabs., MA, U.S.A.), 1 μl of 10X bovine serum albumin (BSA; NewEngland Biolabs., MA, U.S.A.) and the final volume brought to 10 μl with sterile H_2O. This was incubated at 37°C for 1 hour before purification though a GFX™ column (Amersham Pharmacia Biotech, Uppsala, Sweden) (appendix II). TA overhangs were added by incubating 2 μl of cut vector with 0.5 μl Thermoprime plus polymerase (Abgene, Surrey, U.K.), 1 μl of the corresponding buffer (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20), 0.6 μl 25 mM of $MgCl_2$, 0.5 μl of dTTP's (10 pmol) and 5.4 μl of H_2O at 72°C for 1 hour. The vector was then ready for use in a ligation reaction. Afterwards the PCR concentration was calculated as above and each ligation was performed as follows: 1.25 μl TA-vector, 1μl of T4 DNA ligase (Promega, Madison, WI, U.S.A.), 1 μl of corresponding buffer (10 mM HCl (pH 7.0), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA and 50% glycerol), the calculated concentration of fresh PCR product and brought to a final volume of 10 μl with sterile H_2O. The subsequent transformation was conducted as with the Clonetech pT-Adv kit (2.2.4.1.).

2.2.5. Colony screening

Briefly, recombinant (white) colonies were picked from the plates and screened for the presence of inserts by means of PCR. The positive colonies were then grown

overnight in selective LB/ampicillin media (appendix I) at 37°C in a shaking incubator with the plasmid being harvested the following day using the GFX™ microplasmid preparation kit (Pharmacia Biotech, Uppsala, Sweden) (appendix II).

Each white colony was picked using a sterile 20 μl pipette tip then stabbed twice into 5 μl of sterile H_2O in a 0.2 ml PCR tube and then into 20 μl of sterile selective LB/ampicillin broth (appendix I). To the 5 μl of H_2O was added 5 μl of PCR reaction mix which constituted 0.05 μl Thermoprime plus polymerase (Abgene, Surrey, U.K.), 1 μl of the corresponding buffer (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20), 0.6 μl of 25 mM $MgCl_2$, 0.5 μl (5 mM) of dNTP's, 0.1 μl (0.5 pmols) of M13 forward primer, 0.1 μl (0.5 pmols) of M13 reverse primer (appendix III) and brought to a final volume of 10 μl with H_2O. This reaction mixture was placed into a thermal cycler, (PTC-100) and run under the following conditions.

1 cycle: 95°C, 60 seconds,

25 cycles: 95°C, 20 seconds,

56°C, 30 seconds,

72°C, 1 minute and 30 seconds,

Once complete, 2 μl of loading dye (appendix I) was added to each tube and the PCR reactions run alongside 1 μl (500 ng) of 1kb (New England Biolabs, MA, U.S.A.) marker on a 1% agaroase gel containing 10 ng of ethidium bromide to stain the PCR products and 1x TBE buffer (appendix I). Electrophoresis was routinely done 80 volts at room temperature (~15°C). The resultant gels were visualised under UV light supplied by a transilluminator (UVP, San Gabriel, CA, U.S.A.) allowing the positive colonies to be identified. Consequently, the media containing the positive clones was used to inoculate 3 ml of selective LB/ampicillin medium (appendix I) and allowed to grow overnight before being harvested and the plasmids extracted from the cells

using the GFX™ microplasmid preparation kit (appendix II). The plasmids were then ready for sequencing as below.

2.3. DNA Sequencing

Sequencing of recombinant plasmids was accomplished using terminator cycle sequencing kits, DYEnamic ET and BigDye, in combination with an ABI Prism™ 377 DNA sequencer (CA, U.S.A.). The kits were based on a modification of traditional dideoxynucleotide chain termination chemistry in which terminators are labelled with fluorescent dyes for automated detection on the ABI Prism™ 377 (CA, U.S.A).

2.3.1. Big Dye™ V3.0 cycle sequencing kit

The cycling conditions and the reaction components for Big Dye (Applied Biosystems) sequencing are described below. The following reagents were added to a 0.2 ml PCR tube; 2.5 μl of purified plasmid, 2 μl of Big Dye terminator kit and 0.5 μl of primer (5 pmols). The tubes were then placed into the PTC-100 thermal cycler and run on the following cycling program for 25 cycles:

96°C, 10 seconds,
50 °C, 10 seconds,
60 °C, 4 minutes,

Once complete the products were purified by ethanol precipitation as follows. 15 μl of wash mixture (500 μl of 100% ethanol and 15 μl of 3 M sodium acetate) was added to each PCR tube, vortexed and incubated at –20°C for 15 minutes. The tubes were then centrifuged at room temperature for 15 minutes at ~15,000 rpm in a microcentrifuge (IEC micromax, Needham Heights, MA, U.S.A.) to pellet the DNA. Afterwards the pellets were washed with 150 μl of 70% ethanol which was slowly

and carefully pipetted in and then directly out again, and then the pellet air dryed. Once dry, the pellets were resuspended in 2 μl of formamide loading dye, supplied with the kit, and run on the sequencer.

2.3.2. DYEnamic ET terminator cycle sequencing kit

2 μl of DYEnamic reaction premix (Amersham Pharmacia, Uppsala, Sweden), 0.5 μl of primer (5 pmol), 6 μl (~600 ng) of plasmid preparation, 6 μl of ABI dilutent and 5.5 μl of H_2O was added to a 0.2 ml PCR tube and placed into PTC-100 thermal cycler and run on the following cycling program for 25 cycles.

95 °C, 20 seconds,
50 °C, 15 seconds,
60 °C, 60 seconds,

Once complete, the products were precipitated, by ethanol precipitation as follows. 2 μl of sodium acetate/EDTA buffer, supplied with the kit, and 80 μl of 95% ethanol was added to each tube, vortexed and centrifuged at room temperature for 15 minutes at ~12,000 rpm in a microcentrifuge. The supernatant was removed by aspiration and the pellets washed with 150 μl of 70% ethanol by pipetting the ethanol into the tube and removing it immediately. After washing, the pellets were dried and resuspended as before.

2.3.3. Operation of the ABI Prism™ 377 DNA sequencer

Operation of the ABI Prism™ 377 (CA, U.S.A) remained constant throughout the study using the following protocol compiled by the machines manufacturers.

2.3.3.1. Production of acrylamide gels

In order to run the sequencer a polyacrylamide gel must first be made. This was achieved by pouring the following mix between two custom made glass plates. 18 g urea (BioRad lab. Hercules, CA, U.S.A.), 5.2 ml of Long Ranger® gel solution (BioWhittaker Molecular Applications, Rcokland, ME, U.S.A.), 27 ml double distilled H_2O and 0.5 g mixed bed resin (Sigma) were added to a 50ml glass beaker and stirred slowly at room temperature until the urea dissolved and the solution had warmed to ~15°C. The resulting mixture was filtered, 5ml of 10X TBE (appendix I) added and degassed for 4 minutes under vacuum. After this time, 250 µl of 10% ammonium persulphate (APS; Amresco, Solon, Ohio, U.S.A.) and 35 µl of N,N,N',-tetramethylethylenediamine (TEMED; Sigma, Dorset, UK) were added. The resulting reaction mixture was stirred slowly, drawn into a 50 ml sterile syringe and injected between the glass plates. The gel was allowed to set for two hours before the samples could be run on the sequencer in 1X TBE buffer (appendix I)

2.3.3.2. Operation of the Sequencer

When the gel had set, the comb was added, the read region on the glass plates cleaned and the whole apparatus inserted into the main body of the sequencer. The buffer tanks were attached, filled with 1X TBE buffer and the heat block placed onto the gel. The sequencer was then ready for the initial pre-run. The pre-run heats the gel to 50°C at which point the machine was paused and the odd number lanes loaded with 1.5 µl of each sample. The machine was run on for a further 2 minutes before the even sample lanes were loaded, again 1.5 µl being loaded into each lane. The sequencer was then ready for the sequencing run.

2.4. Yeast Expression

The vector chosen to functionally characterise the putative fish genes was the yeast expression vector pYES2 from Invitrogen™ (CA, U.S.A.). The rationale behind this choice of vector was that yeast *Saccharomyces cerevisae*, does not possess the fatty acyl desaturating enzymes required to produce long chain highly unsaturated fatty acids whereas other expression systems, such as mammalian cell lines, possess these activities and so it would be difficult to distinguish activity of the putative fish gene from the activity of endogenous mammalian genes, present in the cell line. The yeast vector had also been used successfully by other workers functionally characterising putative desaturase genes. The vector contains an URA3 gene for selection of transformants in yeast host strains with a URA3 genotype, and has the yeast *GAL1* promoter for high level inducible protein expression in yeast by galactose and repression by glucose.

2.4.1. Cloning into pYES2 (Invitrogen™, CA, U.S.A.)

In order to functionally characterise the putative genes from the various fish species, the sequences had to be first restriction mapped to allow primers to be designed with appropriate restriction sites to ensure correct orientation of insertion into the pYES2 vector. The primers designed for each gene are given in Chapter 4 along with the appropriate cycling conditions. The PCR products were purified through a GFX™ column (appendix II), restricted with the appropriate enzymes, re-purified and ligated into the pYES2 vector, previously restricted with the corresponding enzymes and ligated as described in section 2.2.4.3.

2.4.2. Transformation into *Saccharomyces cerevisiae* INVSc1

Before transformants containing the insert/vector constructs could be produced, competent *S. cerevisae* cells had to be prepared. The production of the competent yeast cells and subsequent transformation was accomplished by using the S. c. EasyComp™ transformation kit (Invitrogen™, CA, U.S.A.).

2.4.2.1. Preparation of Competent yeast cells

The yeast was first streaked from a stab onto a YPD plate (appendix I) and incubated at 30°C for 2 days. A single colony was picked and used to inoculate 10ml of YPD media (appendix I). The culture was shaken overnight at 30°C in a rotary shaking incubator (Gallenkamp, Loughborough, UK). The following day the OD_{600} was determined using the Jenway 6405UV/Vis. Spectrophotometer (Essex, U.K.). The OD_{600} routinely varied between 3.0 and 5.0. The cells were diluted to an OD_{600} of 0.2 to 0.4 in a total volume of 10 ml of YPD media and grown on until the OD_{600} reaches 0.6 to 1.0 which usually took about 4 to 5 hours. After further growth the cells were pelleted by centrifugation at 500 x g (1500rpm) for 5 minutes at room temperature and the supernatant discarded. The pellet was then resuspended in 10ml of solution I, supplied with the kit and re-centrifuged, once again, at 500 x g (1500rpm) for 5 minutes to pellet the cells (Eppendorf, Hamburg, Germany). Once the cells had been pelleted the supernatant was disgarded and the pellet resuspended in 1 ml of solution II. The cells were then ready for transformation or freezing until required.

2.4.2.2. Transformation of yeast cells

50 μl of competent yeast cells were used for each transformation. To each 50 μl, 1 μg of vector DNA and 500 μl of solution III (kit) were added and the mixture vortexed. The reaction mix was incubated at 30°C for 1 hour and resuspended every 15

minutes to ensure good transformation efficiency. After 1 hour the cells were ready to be spread onto –uracil selection plates (appendix I). 100 μl from each transformation was spread onto two plates and incubated at 30°C for 2 to 4 days.

2.4.3. Induction with desaturase fatty acid substrates

2.4.3.1. Preparation of Na salts of PUFA

The assays for characterising desaturases routinely used substrate fatty acids in the concentration range of 0.5 – 1.25 mM due to the fact that as the chain length increases the ability of the yeast to take up the fatty acids decreased. Therefore, a concentration gradient of increasing concentration with increasing chain length was used in order to partially compensate for the decreased uptake concentration at longer chain lengths. The stock solutions were made at 100, 150, 200 and 300 mM so that a 1 in 200 dilution gave assay concentrations of 0.5 mM, 0.75 mM, 1.0 mM and 2 mM for C_{18}, C_{20}, C_{22} and C_{24} substrate fatty acids, respectively (appendix I). The substrates used in the reported experiments were in the form of free fatty acids but other delivery systems were tried including fatty in BSA.

2.4.3.2. Induction of the Gal1 promoter

Once the colonies had grown for 3 to 4 days they were ready for the next stage in the protocol. A single colony was used to inoculate 5 ml –uracil selective medium (appendix I) contained in a 50 ml centrifuge tube. This was grown overnight in a 30°C shaking incubator, as before, at 225 rpm. The following day the OD_{600} was measured as before and the amount of culture needed to inoculate 15 ml of –uracil selective medium was calculated using the following formula.

$$\frac{(0.4\ \text{OD/ml}) \times (15\ \text{ml})}{?\ \text{OD/ml}}$$

? = the OD_{600} of the overnight culture.

The calculated amount of culture was diluted with the appropriate amount of selective medium in a 250 ml glass conical flask. The flask was placed into the shaking incubator at 30°C and growth allowed to continue for a further 4 hours. After this time the flasks were removed and either 0.5 mM of C_{18} substrate, 0.75 mM C_{20} substrate, 1.0 mM of C_{22} or 2 mM of C_{24} substrate was added (appendix I) along with 1.2 ml of filtered 25% galactose (appendix I) which caused the induction of the *Gal1* promoter.

2.4.4. Fatty acid analysis

After 48 hours of growth the yeast cells were ready to be harvested and total lipid extracted and derivatised using one of two methods, the first for shorter chain substrates, C_{18}'s and C_{20}'s, and the second for longer chain substrates, C_{22}'s and C_{24}'s. Once extracted, fatty acid methyl esters (FAME) were prepared and analysed by gas chromatography (GC). Possible desaturase product peaks were confirmed by GC-MS (gas chromatography-mass spectrometry) of the methyl esters, and double bond position confirmed by GC-MS of the picolinyl esters. Unless otherwise stated all solvents were HPLC grade and obtained from Fisher Chemicals (Fisher Scientific, Leicestershire, U.K.).

2.4.4.1. Preparation of Fatty Acid Methyl Esters (FAME)

Method 1

Approximately equal amounts of yeast cells were transferred into glass test tubes after determination of culture densities at OD_{600}. The cells were collected by

centrifugation at 500 x g for 2 min, the pellets washed twice with 5 ml of ice-cold Hanks balance salt solution and dried under a stream of oxygen - free nitrogen (OFN) using an N-EVAP^{TM}III (Organomation Associates, Berlin, MA, U.S.A.). Fatty acid methyl esters (FAME) were prepared by incubating the dried yeast cells directly with 1 ml of methylation reagent containing 10 % (v/v) concentrated HCl, 5 % (v/v) 2,2-dimethoxypropane and 85% (v/v) dry methanol for 1 hour at 85^{0}C in a Hot Block (Stuart Scientific, Redhill, U.K.). After incubation, FAME were extracted by the addition of 1 ml of 1% NaCl solution and 0.5 ml of hexane containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. The mixture was vigorously mixed and centrifuged at 600 x g (Jouan C312, Saint Herblain, France) for 5 min to promote phase separation. The top phase was carefully removed and filtered through Whatman No. 1 filter paper into a clean glass test tube, and the solvent evaporated under a stream of OFN. The esters were then purified by thin layer chromatography (TLC). The methyl esters were applied onto 20 x 20 cm TLC plates (MERCK, Germany) as 2 cm streaks 1.5 cm from the bottom of the plate and separated by 2 cm and with a 2 cm margin at the edge of the plate. The plate was developed in isohexane/diethyl ether/acetic acid (90:10:1, by volume) to 1-1.5cm from the top of the plate. The plate was removed from the chromatography tank and the solvent evaporated in a fume cupboard. The outer edge of the plate was then sprayed lightly with 1% (w/v) iodine in $CHCl_3$ to visualise the FAMEs. The rest of the plate was masked with a blank glass plate so that only the very edge of the sample was exposed. The position of the FAME bands was marked with a pencil. The FAMEs chromatograph as a doublet, saturated and monounsaturated fatty acids form the upper band and polyunsaturated fatty acids the lower band. Both bands were marked and scraped together from the TLC plate into a test tube using a straight edge scalpel

or razor blade. FAME were eluted from the silica with 10 ml isohexane/diethyl ether (1:1, v:v), the elutant mixed by vortexing and centrifuged as described above to precipitate the silica. The solvent was removed to a clean 15 ml tube and evaporated under oxygen free nitrogen (OFN), the samples transferred to 2 ml sample vials in 1 ml isohexane, evaporated to dryness and redissolved in isohexane.

FAME were stored under argon at –20°C until GC analysis. FAME were separated using a Fisons GC8160 gas chromatograph equipped with a chemically bonded CP Wax 52CB fused silica wall coated capillary column (30 m x 0.32 mm i. d., Chrompack U.K. Ltd., London) with an on-column injection system and flame ionization detection. Hydrogen was used as carrier gas with an oven thermal gradient from an initial 50°C to 180°C at 40°C/min, and then to a final temperature of 235°C at 2°C/min. Individual FAME were identified by comparison with known standards, with a well-characterized fish oil, and by reference to published data, as described previously (Tocher and Harvie, 1988). FAME were quantified using a directly linked PC operating Chrom-Card Software (Thermo-Quest Italia S.P.A., Milan, Italy). All solvents contained 0.01% butylated hydroxytoluene as an antioxidant.

Method 2

The yeast samples were harvested and washed as above but after the supernatant had been removed the yeast pellets were extracted by the addition of 6 ml of chloroform/methanol (2:1, v/v) and the cells disrupted by homogenisation using the Ultramax™. The tubes were then stoppered and left on ice for 1 hour. After 1 hour, 2.5 ml of 0.88% KCl was added to the homogenised sample and mixed thoroughly by vortexing. After standing on ice for 5 minutes the samples were centrifuged at 400 g (1500 rpm) (C312; Juoan, Saint Herblain, France) for 3 minutes. The lower organic layer was transferred using a Pasteur pipette through pre-washed

(chloroform/methanol. 2:1, v/v) 9 cm Whatman no. 1 filter paper into clean glass test tubes and the solvent evaporated under a stream of OFN. FAME were prepared from the total lipid extract by the addition of 1 ml toluene and 2 ml methylating reagent (methanolic sulphuric acid; 1% H_2SO_4 in methanol) and the tubes mixed. The tubes were flushed with OFN and stoppered with a glass stopper and a piece of paper to prevent the stopper blowing out when the tube was heated. The tubes were incubated overnight (16hrs) at 50°C in a hot-block (Stuart Scientific, Redhill, U.K.). The following day the tubes were removed from the hot-block and allowed to cool. FAME were extracted by the addition of 2 ml 2% $KHCO_3$, followed by 5 ml of isohexane/diethyl ether (1:1, v/v) containing 0.01% (w/v) BHT and the tubes vortexed and centrifuged at 400 g to separate the two phases. The upper organic layer was transferred to a clean test-tube and the lower layer extracted again with a further 5 ml isohexane/diethyl ether (1:1 v/v) without BHT. The combined organic solvent layers were then evaporated under a stream of OFN and the resulting FAME redissolved in 100 μl isohexane. The methyl esters were then ready to be purified by thin layer chromatography and analysed by GC as described previously.

2.4.4.2. Gas chromatography-Mass Spectrometry.

The identities of fatty acids and positions of their double bonds were confirmed by subjecting the picolinyl esters to electron ionization (EI) gas chromatography/mass spectrometry (GC-MS). Picolinyl esters were prepared by the method of Balazy and Nies (1989) which involves activating the free fatty acid by reaction with 1,1'-carbonyldiimidazole to form the imidazolide which then reacts with 3-(hydroxymethyl) pyridine under basic conditions to form the picolinyl ester (see below). GC-MS of both fatty acid methyl esters and the picolinyl esters was performed using a Fisons GC8000 gas chromatograph coupled to an MD800 mass

spectrometer (Fisons Instruments, Crawley, U.K.). The gas chromatograph was equipped with a fused silica capillary column (60 m x 0.32 mm i.d, 0.25 mm internal film thickness) coated with Zebron ZB-Wax (Phenomenex, Macclesfield, U.K.) and used helium as carrier gas. Samples were applied using on-column injection with the oven temperature programmed to rise from 80°C to 250°C at 40°C per minute.

Preparation of Picolinyl Esters

The fatty acid methyl ester samples were transferred from the 2ml sample vial into a test tube, dried down under OFN and 2 ml of 0.1M KOH in 95% ethanol added. The sample were then hydrolysed for 1 hour at 78°C in a hot block under OFN (Stuart Scientific, Redhill, U.K.). Once incubation was complete, 5 ml of H_2O and 300 μl of 6M hydrochloric acid was added to acidify the sample to ~pH<3. The fatty acids were extracted by adding 4.5 ml of diethyl ether, mixed by shaking, centrifuged and the layer transferred to a clean test tube. This extraction procedure was repeated once more. To the combined upper layers, 3 ml of sterile H_2O was added, the tube stoppered, and mixed thoroughly to wash the sample, and centrifuged in the Jouan C312 centrifuge at 1500rpm (Saint Herblain, France). The top layer was transferred into another clean test tube, the sample dried down under OFN and resuspended in 100 μl of 1,1'-carbonyldiimidazole solution and 200μl of picolinyl reagent (appendix I). After vortexing, the samples were placed into a 37°C hot-block for 10 minutes (Stuart Scientific, Redhill, U.K.). After incubation, samples were evaporated to dryness under OFN, 5ml of isohexane and 2 ml distilled H_2O were added, the tubes stoppered, shaken and vortexed. The upper isohexane layer was filtered through a short (4 cm) column of anhydrous sodium sulphate prepared in a pasteur pipette and plugged with cotton wool, pre-washed with isohexane, and overlaid with a thin layer of sand. The aqueous layer was re-extracted with fresh isohexane (5 ml) and passed

through the column. The column was finally washed through with 1 ml isohexane and the combined eluents evaporated under OFN. The sample was resuspended in 100 μl of isohexane and purified further by TLC as before. The picolinyl esters were visualised by spraying the side of the plate with iodine as above, the lanes were marked, scraped and the silica eluted with 10 ml isohexane:diethyl ether (1:1, v/v) as above. The purified picolinyl esters were evaporated to dryness under OFN and resuspended in the appropriate amount of isohexane for GC/MS analysis.

Chapter 3

Putative Desaturase Gene Structure

Chapter 3 – Putative Desaturase Gene Structure

3.1. Introduction

3.1.1. Aims:

To clone and sequence the desaturase genes of a variety of fish species, including one freshwater, one marine and one anadromous fish species.

There are several ways to isolate a gene of interest, some of the techniques are described below together with the chosen technique for this study.

3.1.2. Gene hunting

3.1.2.1. DNA libraries

DNA libraries are useful for gene isolation when either there is no sequence information available for the gene of interest or where only a partial sequence is available. Two types of clone library are commonly used, genomic libraries and cDNA libraries. Genomic libraries are prepared from the chromosomal DNA of the organism under study and consist of a sufficiently large number of clones for there to be a statistically-high chance of the gene of interest being present. cDNA libraries are derived from the mRNA present in an individual tissue or cell type, and therefore contain copies of only those genes that are actively expressed in the tissue or cell type. Once the libraries have been produced they can be screened by hybridisation to a homologous radiolabelled polynucleotide probe and the positive clones selected and sequenced for complete identification (Brown, 1995).

3.1.2.2. Genome sequencing projects

There has been considerable effort to sequence the complete genomes of a variety of organisms including human and mice but also for model fish species such as fugu, the puffer fish (*Fugu rubripes*) and the zebrafish (*Danio rerio)*. This sequence data is accessible online through several websites and can be BLAST searched against a homologous gene from another species allowing the identification of orthologous genes.

3.1.2.3. EST's and PCR based isolation

Expressed sequence tags (EST's) are unique sequences which define a population of mRNA molecules. The procedure for acquiring EST's commences with establishing cDNA libraries from polyA-containing mRNA. cDNAs are then sequenced and a non-degenerate population of unique ESTs are then collated. These sequences can be used to identify cDNAs with putative functions of interest by comparison with the information held on DNA databases and the scientific literature. Once ESTs of interest have been identified they can be used to predict PCR primer sites unique to that message or used as hybridisation probes. The genes from which ESTs are derived can also be positioned and chromosomally mapped by screening radiation hybrids and YAC contigs. This is a very powerful approach as over half the genes expressed in humans are now codified as cDNA molecules. The overall aim is to define EST's for all regions of the genome so that whenever the gene for an inherited disorder or a particular trait is mapped to a chromosomal location a series of EST's will already be known for the region. These can then be assessed to find the gene responsible for the disorder or trait. EST collections are published for many organisms, including zebrafish *Danio rerio,* on the NCBI (National Centre for

Biotechnology Information) and EMBL (European Molecular Biology Laboratory) websites.

PCR-based cDNA isolation was the technique of choice for this study for several reasons. Firstly, the sequence of the human Δ5 and Δ6 desaturase genes, both of which had been functionally characterised were known and available, and secondly, zebrafish ESTs were also available that have high homology to the mammalian delta 6 desaturase genes. The zebrafish EST (AI497337) allowed primers to be designed for the first PCR-based isolation of a fish desaturase gene. This information, along with the desaturase sequences of other organisms that have periodically appeared in the GenBank database, enabled alignments to be made between the protein sequences. These alignments could then be analysed and the functionally important regions identified. This allowed degenerate primers to be designed and used in the isolation of the desaturase genes of other fish species. The structural characteristics of the Δ5 and Δ6 fatty acyl desaturase genes along with other desturase genes are outlined below.

3.1.3. The desaturase genes

3.1.3.1.Structural characteristics of the desaturase genes

The desaturase genes discussed below contain certain common structural characteristics, domains and motifs. Examination of the deduced amino acid sequences of the membrane fatty acid desaturases from mammals, fungi, insects, higher plants and cyanobacteria revealed three regions of conserved primary sequence, termed histidine boxes, containing $HX_{(3\text{ or }4)}H$, $HX_{(2\text{ or }3)}HH$, and $HX_{(2\text{ or }3)}HH$ (Shanklin *et al.*, 1994). It had been noted that these regions have a consistent positioning with respect to three potential membrane spanning domains. The histidine rich regions are always in the hydrophilic regions of the primary sequence

rather than in the membrane spanning domains and the functional importance of the histidine residues has been revealed through site-directed mutagenesis of the Δ6 desaturase gene from borage, a plant species. Mutation of any of the histidine residues or the glutamine to alanine resulted in loss or reduction of desaturase activity, thus suggesting these residues may provide some essential catalytic functionality (Shanklin *et al.*, 1994). Shanklin proposes that these His residues serve to bind iron as His residues are typical metal binding motifs in proteins, for example HH (Shapleigh *et al.*, 1992); HXH in β strands; HX2H in reverse turns; and HX3H in α helices (Regan, 1993). Shanklin and co-workers propose that the presence of the His residues allows the chelation of transition metals, and that data from other His-containing metalloproteins strongly support the assignment of the conserved histidine boxes specifically as binding residues (Shanklin *et al.*, 1994). Shanklin reports that there are enough His residues in these conserved regions in desaturases to potentially form binding sites for two iron atoms, termed diiron clusters (Figure 3). In other metalloproteins, these diiron clusters catalyse diverse reactions requiring O_2-activation chemistry (Sander-Loehr, 1988) such as oxidation of unactivated C-H bonds (Lipsomb, 1994).

Some desaturase genes also contain N or C-terminal cytochrome b_5 like domains. The cytochrome b_5 domains contain a characteristic motif His-Pro-Gly-Gly-Xaa$_8$-Gly-Xaa$_6$-Phe-Xaa $_{3\text{-}6}$-His where His-Pro-Gly-Gly corresponds to a haem-binding domain (Sayanonva *et al.*, 1999). Desaturases introduce double bonds into preformed acyl chains by oxygen-and electron-donor-dependent desaturation (Shanklin and Cohoon, 1998).

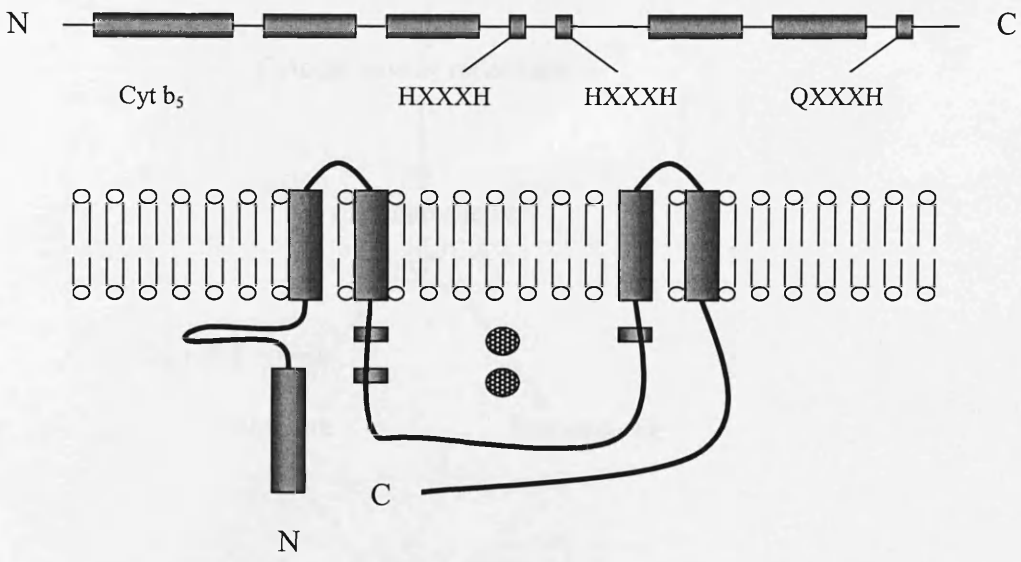


Figure 3. Structural characteristics of the mammalian Δ5 and Δ6 fatty acid desaturases containing the functionally essential histidine rich sequences (his boxes) with general motifs HXXXH, HXXHH, and QXXHH, which stabilise the two iron atoms (spotted circles) essential to the oxidation/reduction reaction, and an N-terminal cytochrome b_5 domain (Cyt b_5).

The immediate electron donor for many microsomal desaturases without an integral cyt b_5 domain is the cytochrome b_5 (Lederer, 1994). Cytochrome b_5 is a small hemoprotein that functions in a number of oxidation/reduction reactions, including NADH-dependent acyl-group desaturation and takes part in electron transport in the desaturation of fatty acids as shown in figure 4. The electrons are transferred from NADPH to microsomal cytochrome b_5 via a cytochrome b_5 reductase (Lehninger, 1978). It has been shown that that disruption of histidine residue within the haem-binding domain of the borage Δ6 desaturase results in complete loss of desaturase activity (Sayanova *et al.* 1999).

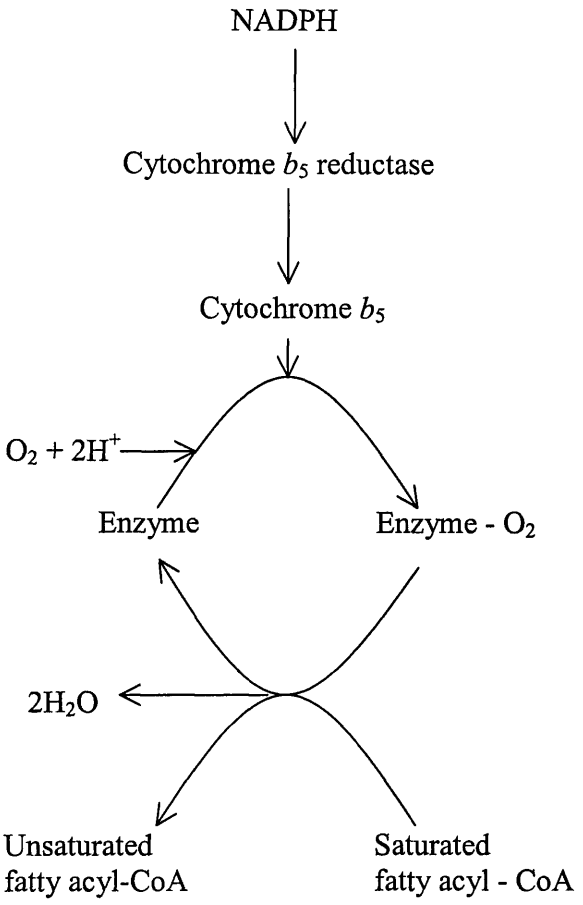


Figure 4. Pathway of electron transport in the desaturation of fatty acids.

3.1.3.2. The Δ9 desaturase genes

The Δ9 stearoyl desaturases are probably the best studied and most understood of all fatty acyl desaturases, particularly in molecular biological terms and certainly in animals, if not in general. There are two types of Δ9 stearoyl desaturase, one which is found in plants, the Δ9 stearoyl-ACP desaturase, and the other which is found in animals, the stearoyl-CoA desaturase (Tocher *et al.*, 1998). The Δ9 desaturases are responsible for converting 18:0 (stearic acid) to 18:1*n*-9 (oleic acid). The structural characteristics of these genes depends on which organism it has been isolated from. They contain the three conserved histidine boxes. However, in some instances they also contain a cytochrome b_5 domain (Mitchell and Martin, 1995). The Δ9 desaturase

has been isolated from many organisms including the fish species common carp (Tiku *et al.*, 1996) and grass carp (*Ctenopharyngodon idella*) (Chang *et al.*, 2001).

3.1.3.3. The Δ12 and Δ15 desaturase genes

Delta 12 desaturase enzymes are membrane bound enzymes that desaturate palmitoleic acid, 16:1*n*-7, and oleic acid, 18:1*n*-9, to 16:2*n*-4 and 18:2*n*-6, respectively, at the Δ12 position. The Δ15 desaturase enzymes are membrane associated enzymes that desaturate 18:2*n*-6 to 18:3*n*-3 at the Δ15 position. Most eukaryotic organisms, including mammals can introduce a double bond into an 18-carbon fatty acid at the Δ9 position, however mammals are incapable of inserting double bonds at the Δ12 and Δ15 positions. However, certain lower order animals, including some insects and nematodes can synthesise *de novo* all their component fatty acids including linoleate and linolenate meaning that they possess genes for the Δ12 and Δ15 desaturase enzymes. Genes for Δ12 desaturase were first isolated and characterised from cyanobacteria, plants and fungi *Helianthus annus* (Martinez-Rivas *et al.*, 2001), *Mortierella alpina* (Huang et al., 1999), Arabidopsis (Okuley *et al.*, 1994) and *Mucor Rouxii* (Passorn et al., 1999) but a number of homologous genes have also been discovered in invertebrate animals including the and the nematode *C. elegans* (Peyou-Ndi *et al.*, 2000).

The Δ12 desaturase genes sequenced to date contain several features in common with other desaturases. They possess the three histidine cluster motifs that are conserved in other membrane-bound desaturases and which are known to be essential for enzyme function (see above) (Jackson, *et al.*, 1998). However, unlike many other desaturase genes, they do not contain cytochrome b_5 domains, although cyt b_5 is required as an electron donor for desaturation in microsomal membranes of fungi (Jackson *et al.*, 1998). Therefore, Δ12 desaturases must interact with a separate

cytochrome b_5 to achieve activity. Similarly, the Δ15 (ω-3) desaturases also contain the three histidine boxes as other desaturases but do not contain cytochrome b_5 domains (Tocher *et al.*, 1998).

3.1.3.4. The Δ5 and Δ6 desaturase genes

The conventional pathway for the production of essential fatty acids involves the Δ5 and Δ6 desaturase genes (see chapter 1). At the time of writing, approximately 6 non-fish Δ5 desaturase sequences and approximately 10 non-fish Δ6 desaturase sequences were in the GenBank database, several of which have been functionally characterised. The human Δ5 and Δ6 desaturase genes are aligned in Figure 5. They both contain the three conserved histidine boxes and hydrophobic membrane spanning domains mentioned in section 3.1.3. The protein sequences of the Δ5 desaturases of *C. elegans* (Michaelson *et al.*, 1998; Wallis and Browse, 1999), *Mortierella alpina* (Michaelson *et al.*, 1998), human (Leonard, 2000, Cho *et al.*, 1999a), and the Δ6 desaturases of *Borago officinalis* (Sayanova *et al.*, 1997), *C. elegans* (Napier *et al.*, 1998), *Mortierella alpina* (Huang *et al.*, 1999), human and mouse (Cho *et al.*, 1999b), rat (Aki *et al.*, 1999) and *Ceratodon purpureus* (Sperling *et al.*, 2000) all contain N-terminal cytochrome b_5 domains. This information was important to the present project for two reasons. Firstly, it allowed the functional enzymatic characteristics of the desaturase genes to be described enabling any cloned sequence to be assessed for its potential as a desaturase gene. Secondly, it allowed degenerate primers to be designed for the isolation of the desaturase genes from fish cDNA (see below).

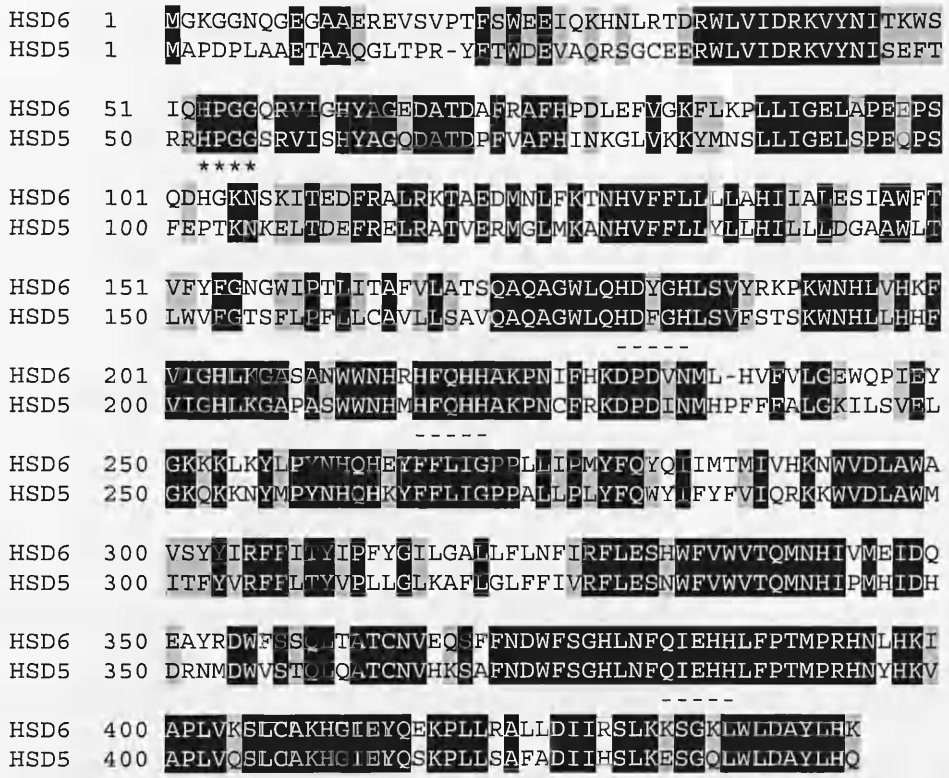


Figure 5. Alignment of the human Δ5 and Δ6 desaturase genes. The haem-binding domain contained within the N-terminal cytochrome b_5 is underlined with asterisks and the three conserved histidine boxes are underlined with dashes. The matrix used was BLOSUM 62 and the cut off for shading was 75%. Identical residues are shaded black, synonymous residues are shaded grey and non-synonymous residues are white.

3.1.3.5. The Δ8 desaturase genes

The Δ8 desaturase enzymes potentially provide an alternative pathway for the biosynthesis of 20-carbon polyunsaturated fatty acids. Instead of desaturation, the first step in the alternative pathway is elongation of the essential 18-carbon fatty acids to 20-carbon chain lengths producing 20:2 Δ11, 14 (20:2*n*-6) and 20:3 Δ11, 14, 17 (20:3*n*-3). Subsequent desaturation occurs via a Δ8-desaturase (Figure 6). The products of this pathway are the same as the more usual desaturation pathway, 20:3 Δ8, 11, 14 (20:3*n*-6) and 20:4 Δ8, 11, 14, 17 (20:4*n*-3). The alternative Δ8 pathway is present in the soil amoebae *Acanthamoebae* sp. (Ulsamer *et al.*, 1969), in the protozoan *Tetrahymena pyriformis* (Lees and Korn, 1966) and in euglenoid species.

However, the Δ8 desaturation pathway was also apparently demonstrated in mammalian rat testis (Albert and Coniglio, 1977) and in human testis (Albert *et al.*, 1979). While Δ8 activity has been detected in breast cancer cell lines (Grammatikos *et al.*, 1994) and in glioma (Cook *et al.*, 1991), no Δ8 activity was observed in a corresponding non-cancerous breast cell line (Grammatikos *et al.*, 1994) or in the brain (Dhopeshwarkar and Subramanian 1976). The significance of Δ8 desaturation in normal mammalian or cancer cells is unclear, since analysis of desaturase activities is frequently complicated by the presence of competing Δ6 reactions and chain shortening retroconversion of fatty acid substrates in tissue (Sprecher and Lee, 1975, Geiger *et al.*, 1993). However, there is no significant Δ8 activity in mammalian liver where the bulk of mammalian fatty acid metabolism occurs (Sprecher and Lee, 1975). There is no evidence for a Δ8 desaturase activity in fish (Sargent *et al.*, 2002). However, Euglena do appear to possess a true Δ 8 desaturase. The Euglenid Δ8 desaturase protein sequence is highly homologous with both mammalian and nematode Δ6 and Δ5 desaturase genes in that they contain an N-terminal cytochrome b_5 domain. The Euglenids cytochrome b_5 possesses 6 of the 8 most highly conserved amino acids characteristic of this domain and responsible for haem binding (Lederer, 1994). The essential three conserved histidine boxes are also present (Wallis and Browse, 1999). The Euglena protein also exhibits similarities with known type II multiple membrane spanning proteins. Hydropathy analysis indicated that the predicted protein has at least three significant hydrophobic regions long enough to span the membrane layer twice, again similar in structure to the Δ5 and Δ6 desaturase genes in higher animals.

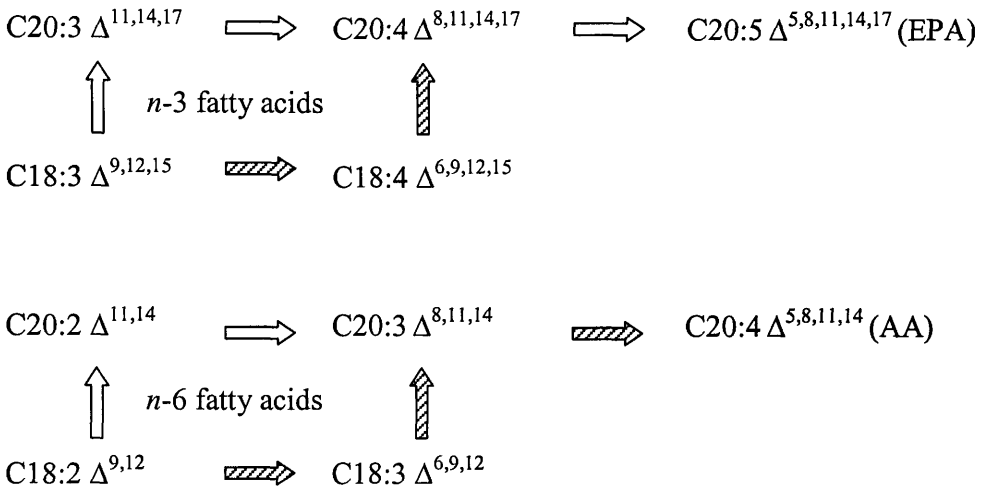


Figure 6. The alternative Δ8 desaturation pathway reproduced from Wallis and Browse (1999). The common pathway begins with a Δ6 desaturation of 18-carbon fatty acid followed by a 2-carbon elongation and then further desaturation and elongation (hatched arrows). The alternative pathway begins with an elongation to 20-carbon fatty acids, followed by Δ8 desaturation and a second desaturation at the Δ5-position (white arrows).

3.1.3.6. The Δ4 desaturase genes

A Δ4 desaturase is required in the traditional alternating desaturase-elongase, pathway for the biosynthesis of DHA from EPA. To date only one Δ4 desaturase gene has been cloned and functionally characterized, *Thraustochytrium sp.* (Qui *et al.*, 2001). Thraustochytrids are a common type of marine microheterotroph and are taxonomically aligned with the heterokont algae. They are unusual in that they produce substantial amounts of long chain polyunsaturated fatty acids such as DHA and DPA, and as such are an interesting organism in which to study mechanisms for the biosynthesis of HUFAs. The structure of the Thraustochytrid predicted Δ4 desaturase protein sequence is highly homologous to the Δ5 and Δ6 desaturase sequences in that it has an N-terminal extension in the form of a cytochrome b_5-like domain. It also contains the three conserved histidine domains as with all other desaturase genes (Qui *et al.*, 2001). A Δ4 desaturase is not required in the alternative

pathway, or "sprecher shunt", for the biosynthesis of DHA and EPA as proposed by Sprecher (Sprecher *et al.*, 1991).

3.1.3.7. PUFA synthesis by polyketide synthases

However, there is another system for producing HUFA without the need for desaturases and elongases by a specialised polyketide synthase (PKS). This anaerobic system uses the same small protein, acyl carrier protein as a covalent attachment for the growing carbon chain and also uses the same four reactions as the fatty acid synthesis (Wallis *et al.*, 2002). The difference between the systems is due to the cycle of reduction, dehydration and reduction being abbreviated in the PKS so that a wide variety of compounds containing keto and hydroxy groups, as well as methylene interrupted cis double bond systems can be produced (Metz *et al.*, 2001). However, the exact sequence of reactions involved in PUFA synthesis by the PKS system remains to be determined.

The PKS system is found in both prokaryotic and eukaryotic marine microbes (Metz, 2001). These PKS protein complexes are found in organisms such as the thraustochytrid marine protist *Schizochytrium*, this organism accumulates large quantities of triacylglycerols rich in docosahexaenoic acid (22:6*n*-3) and docosapentaenoic acid (22:5*n*-3). However, as discussed previously this is not the rule for thraustochytrids as some species possess a desaturation/elongation pathway. But it is present in many marine bacteria such as *Moritella marina* (Wallis *et al.*, 2002) and *Photobacterium profundum* (Allen *et al.*, 1999).

3.2. Materials and Methods

3.2.1. Zebrafish (*Danio rerio*) desaturase gene

3.2.1.1. Primer design

The first fish desaturase gene to be cloned in this project was that of the zebrafish (*Danio rerio*). Primers were designed using a zebrafish EST sequence (Acc: AI497337) entered into GenBank in March 1999. When the sequence was translated and BLAST searched against the GenBank database it was found to have high homology to the human Δ6 desaturase (AF126799; submitted February 1999). The translation was used to design a forward primer around the putative methionine start codon. This primer, ZF2A (appendix III), was used in conjunction with Not1PolyT for PCR based gene isolation. The Not1PolyT primer contained a run of dTTPs which, when used in a PCR reaction, should hybridise with the poly A tail in the cDNA enabling the 3' UTR end of the cDNA to be isolated.

3.2.1.2. Tissue and DNA

cDNA was produced from total RNA, as in sections 2.2.1. and 2.2.2., from the liver of a single fish from the AB line that was kindly provided by Dr. Pete Currie of the Western General Hospital, Edinburgh.

3.2.1.3. PCR conditions

The following constituents were mixed in a 0.2 ml thin walled PCR tube; 1 μl cDNA, 1 μl (10 pmol) ZF2A primer (appendix III), 1 μl (10pmol) Not1PolyT primer (appendix III), 0.4 μl (4 mM) of dNTPs, 0.5 μl Thermoprime plus polymerase (ABgene, Surrey, U.K.), 2 μl of the corresponding buffer (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20), 1.2 μl of 25 mM of $MgCl_2$ and

brought to a total volume of 20 μl with H_2O. The tubes were placed into a thermal cycler (PTC-100 MJ Research, MA, U.S.A.) and run under the following conditions:

1 cycle: 95°C, 60 seconds,

25 cycles: 95°C, 30 seconds,

58°C, 30 seconds,

72°C, 2 minutes,

Once complete, the reaction mixture was run on an agarose gel (section 2.2.5.) and the largest band selected, excised and purified (appendix II). This was then cloned (section 2.2.4), colony screened (section 2.2.5) and the positive clones sequenced (section 2.3.2.). The resulting sequence was used to design primers for the production of the yeast expression construct ZfishD6 (chapter 4).

3.2.2. Carp (*Cyprinus carpio*) desaturase gene

3.2.2.1. Primer design

The same primers were used for the carp as were used for the zebrafish (section 3.2.1.1.) to gain the initial putative sequence using the protocol in section 3.2.1.3. This information was then used to design forward and reverse primers at the start and stop codons which contain restriction sites and the Kozak sequence required for yeast expression (chapter 4). These primers were used in conjunction with *Pfu* DNA polymerase to clone and sequence clones CppYesA5, CppYesA7 and CppYesA9 (see chapter 4).

3.2.2.2. Tissue and RNA

cDNA was produced from total RNA, as above, from the liver of a single fish from a stock of common carp held within the Institute of Aquaculture. The fish was not a

recognised breeding line and therefore would be "wild type", with an origin in a British population.

3.2.2.3. PCR conditions

PCR and cycling conditions were the same as in section 3.2.1.3.

- 3'RACE

Once several PCR products (CppYesA5, CppYesA7 and CppYesA9) were cloned and sequenced, the data were used to design primers to gain further 3' ends in order to investigate the number of loci present in the carp genome. The following PCR components were added to a 0.2 ml PCR tube vortexed and split between 6 x 0.2ml thin walled PCR tubes: 2 μl cDNA, 2 μl (20 pmol) CpYesFor1 primer (appendix III), 2 μl (20pmol) Not1PolyT primer, 1.8 μl (18 mM) of dNTPs, 1 μl Thermoprime plus polymerase (ABgene, Surrey, U.K.), 9 μl of the corresponding buffer (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20), 5.4 μl of 25 mM of $MgCl_2$ and brought to a total volume of 90 μl with H_2O. The tubes were placed into a TGradient thermal cycler (Whatman Biometra GmbH, Göttingen, Germany) and run under the following conditions:

1 cycle:	95°C, 60 seconds,
25 cycles:	95°C, 30 seconds,
	50 to 60°C, 30 seconds,
	72°C, 2 minutes

The products of the 60°C PCR reaction were cloned (section 2.2.4.), colony screened (section 2.2.5) and the positive clones sequenced (section 2.3.2.).

3.2.3. Atlantic Salmon (*Salmo salar*) desaturase gene

3.2.3.1. Primer design

Isolating the carp and zebrafish desaturase genes was relatively straightforward by comparison with isolation of the genes of the other fish species. This was due to two reasons. Firstly, there was existing zebrafish sequence information and secondly, the primers designed for zebrafish coincidentally isolated the carp gene. However, neither of the aforementioned applied for the salmon gene. Instead, the carp and the zebrafish sequences were aligned to enable the design of the degenerate primers FishD6for and FishD6rev (appendix III). These primers were used for PCR isolation of the salmon desaturase genes from cDNA.

3.2.3.2. Tissue and RNA

cDNA was produced from total RNA, as in section 2.2.1. and 2.2.2. from two sources, RNA from the liver of an Atlantic salmon and an Atlantic salmon cell line (AS) (Tocher and Dick, 1990). The cell line has epithelial morphology and was derived originally from the posterior part of whole salmon fry. The liver was taken from a mature fish and the cell line is currently maintained at the Institute.

3.2.3.3. PCR conditions

- Initial PCR

The PCR reaction mix was the same as for carp and zebrafish only using FishD6for FishD6rev and primers. The thermocycling conditions were as follows:

1 cycle:	95°C, 60 seconds,
25 cycles:	95°C, 30 seconds,
	58°C, 30 seconds,
	72°C, 2 minutes,

Touchdown PCR was not required. Once the PCR reaction was complete the products were run on an agarose gel and the largest band excised, cloned and sequenced as above. This gave product SalA10 which was used to design a forward and reverse primers at the start and stop codons which contain restriction sites and the Kozak sequence required for yeast expression (chapter 4). These primers were used in conjunction with *Pfu* DNA polymersase to clone and sequence clones SalA9 and SalpYesB1 (see chapter 4).

- 3'RACE

Once several PCR products were cloned and sequenced the data were used to design primers to gain further 3' ends in order to investigate the number of loci present in the salmon genome. The following PCR components were added to a 0.2 ml thin walled PCR tube vortexed and then divided equally between 6 x 0.2 ml PCR tubes: 2 μl cDNA, 2 μl (20 pmol) SalFor5 primer (appendix III), 2 μl (20pmol) Not1PolyT primer, 1.8 μl (18 mM) of dNTPs, 1 μl Thermoprime plus polymerase (ABgene, Surrey, U.K.), 9 μl of the corresponding buffer (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20), 5.4 μl of 25 mM $MgCl_2$ and brought to a total volume of 90 μl with H_2O. The tubes were placed into a TGradient thermal cycler and run under the following conditions:

1 cycle:	95°C, 60 seconds,
25 cycles:	95°C, 30 seconds,
	50 to 60°C, 30 seconds,
	72°C, 2 minutes

Once the PCR reaction was complete the products were run on an agarose gel as above and the products from the tube containing the lowest annealing temperature were cloned and sequenced also as above.

3.2.4. Cod (*Gadus morhua*) desaturase gene

3.2.4.1. Primer design

Isolating the desaturase genes from marine species required the design of further degenerate primers around conserved regions of the desaturase gene as a number of degenerate primers designed around the methionine start codon of the known open reading frames were unsuccessful. At the time when an attempt was being made to isolate the salmon gene there were a number of mammalian desaturase genes in the GenBank database. The desaturase genes, as mentioned in the introductions to this chapter and thesis, contain a number of conserved regions including the three functionally essential histidine boxes. These regions are highly conserved, enabling the design of degenerate primers, UniD56-2A, Uni5/63A and Uni5/63B for PCR across these areas. The primer UniD56-2A was designed and used in the first round of nested PCR with the primer Not1PolyT (appendix III). The second round in the nested PCR used Uni5/63A and Uni5/63B (appendix III).

3.2.4.2.Tissue and RNA

cDNA was prepared from the liver RNA of a cod liver fed on a vegetable oil-based diet as part of a dietary trial carried out by colleagues in the Nutrition Group, Institiute of Aquaculture at the SOAEFD Marine Research Station, Aultbea, Scotland. The cDNA was prepared as in above.

3.2.4.3. PCR conditions

- Initial PCR

The first round of PCR contained the following constituents in a 0.2 ml thin walled PCR tube: 1 μl (10 pmol) of Uni5/62A, 1 μl (10 pmol) of Not1PolyT, 1 μl of cDNA, 0.4 μl (4 mM) dNTP's, 2 μl of corresponding buffer (750 mM Tris-HCl (pH 8.8),

200 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20), 1.2 μl of 25 mM $MgCl_2$, 0.25 μl of Thermoprime Taq DNA polymerase and brought to a total of 20 μl with H_2O. This was mixed and run on the thermal cycler (MJ Research, MA, U.S.A.) under the following touchdown PCR conditions:

1 cycle:	95°C, 60 seconds,
10 cycles:	95°C, 15 seconds,
	62°C, 30 seconds, -1.5°C/cycle
	72°C, 1 minutes and 30 seconds,
6 cycles:	95°C, 15 seconds,
	52°C, 30 seconds,
	72°C, 1 minute and 30 seconds,
24 cycles:	95°C, 15 seconds,
	51°C, 30 seconds,
	72°C, 1 minute and 30 seconds.

The second round of the nested PCR contained the following constituents in a 0.2 ml thin walled PCR tube: 1 μl (10 pmol) of Uni5/63A, 1 μl (10 pmol) of Uni5/63B, 1 μl of 1:10 dilution of the first round PCR product, 0.4 μl (4 mM) dNTP's, 2 μl of corresponding buffer (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20), 1.2 μl of 25 mM $MgCl_2$, 0.25 μl of Thermoprime Taq DNA polymerase and 13.15 μl of H_2O. This was mixed and run on the thermal cycler (PTC-100 MJ Research, MA, U.S.A.) under the touchdown PCR cycling conditions above. The PCR products were cloned and the positive colonies picked and sequenced as before.

- 3' RACE

Once the section between the second and third histidine boxes of the gene was cloned and sequenced it was used to design primers for 3' and 5' RACE (rapid

amplification of cDNA ends). Cod3Race1 (appendix III) was designed and used in conjunction with Not1PolyT on cod cDNA in the following PCR reaction. 1 μl cDNA, 2 μl (20 pmol) Cod3Race1, 2 μl (20 pmol) Not1PolyT, with the same amounts of buffer, Taq, $MgCl_2$ and dNTPs as before and brought to a final volume of 20 μl with H_2O. The cycling conditions were as follows:

1 cycle:	95°C, 60 seconds,
25 cycles:	95°C, 20 seconds,
	58°C, 30 seconds,
	72°C, 2 minutes,

A 1:10 dilution was made of the resulting product and used in the following nested PCR.

Nested PCR

The following constituents were added to a single 0.2 ml PCR tube before vortexing and subsequent alliquoting to a further 5 tubes. 6 μl (60 pmol) of Not1PolyT, 6 μl (60 pmol) of CodRace3 (appendix III), 6 μl of cDNA, 2 μl (20 mM) dNTP's, 12 μl of corresponding buffer (750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20), 7.2 μl of 25 mM $MgCl_2$, 1.5 μl of Thermoprime Taq DNA polymerase and 79.3 μl of H_2O. The six tubes were then run in a gradient thermocycler under the following conditions:

1 cycle:	95°C, 60 seconds,
25 cycles:	95°C, 20 seconds,
	50-60°C, 30 seconds,
	72°C, 2 minutes,

The resulting products of the lowest annealing temperature were cloned and sequenced as the lowest annealing temperature gave bands of within the expected size range.

- 5' RACE

Attempts were made to gain the 5' end of the cod desaturase cDNA using a variety of gene specific and non-specific primers however the full sequence was never attained.

3.2.5. Turbot (*Scophthalmus maximus*) desaturase gene

3.2.5.1. Primer design

The putative turbot desaturase gene was also isolated using PCR. The primers designed for the cod 5'RACE experiment were used in a nested touchdown PCR.

3.2.5.2. Tissue and RNA

The RNA was extracted from the liver of a single turbot held at the Institute of Aquaculture and provided by Prof. Steven George. The turbot had been maintained for many months on a standard trout pellet diet formulated with fish oil. The RNA was extracted from the tissue as in section 2.2.1., and the subsequent cDNA synthesised as in section 2.2.2. and 2.2.3.

3.2.5.3. PCR conditions

- 5'RACE

The following reagents were pipetted into a 0.2 ml thin walled PCR tube: 4.5 μl (1X) of UPM (appendix III), 1 μl (10 pmol) CodRev2 (appendix III), 1μl dNTP's (10 mM) 4.5 μl of Advantage 2 buffer (400 mM Tricine-KOH, 150 mM KOAc, 35 mM $Mg(OAc)_2$, 37.5 μg/ml BSA, 0.05% TWEEN-20, 0.05% nonidet-P40), 3 μl of

5'RACE cDNA (section 2.2.3.), 1 μl (1X) of Advantage 2 cDNA polymerase (Clontech, Palo Alto, CA, U.S.A.), and brought to a total volume of 45 μl with H_2O. The reaction mix was then divided between three 0.2 ml thin walled PCR tubes and run on the PTC-100 thermo cycler (MJ Research) under the following cycling parameters:

1 cycle:	95°C, 60 seconds,
10 cycles:	95°C, 15 seconds,
	62°C, 30 seconds, -1.5°C/cycle
	72°C, 1 minutes and 30 seconds,
6 cycles:	95°C, 15 seconds,
	52°C, 30 seconds,
	72°C, 1 minute and 30 seconds,
24 cycles:	95°C, 15 seconds,
	51°C, 30 seconds,
	72°C, 1 minute and 30 seconds.

Nested PCR

A 1:10 dilution was then made from one of the PCR reaction tubes, as all reactions produced the same results when viewed on an agarose gel, and used in a nested PCR reaction. The reagents were combined in the following amounts in a 0.2 ml thin walled PCR tube: 1 μl of (10 μm) NUP (appendix III), 1 μl (10pmol) of CodRev3, 1 μl (10 mM) dNTPs, 4.5 μl Advantage 2 buffer, 3 μl of template, 1 μl of Advantage 2 and 33.5 μl of sterile H_2O. The constituents were mixed, split into three separate 0.2 ml thin walled PCR tubes, loaded into the MJ Research thermocycler (MA, U.S.A.) and run under the same cycling parameters as the initial PCR.

The products were cloned and the colonies screened for the presence of inserts as per section 2.2.5. The positive clones were fully sequenced (section 2.3.1.).

- 3'RACE

The sequence results of the 5'RACE experiment enabled primers to be designed for 3' end RACE. The following PCR reaction mix was set up in a 0.2 ml thin walled PCR tube: 2 μl (20 pmol) of Turb3RaceA (appendix III), 2 μl (20 pmol) of Not1polyT, 1.8 μl (18 mM) dNTPs, 9 μl of Advantage 2 buffer, 2 μl Advantage 2 enzyme, 3 μl of turbot cDNA and brought to a final volume of 90 μl with H_2O. The reagents were mixed and split into 6, 0.2 ml, thin walled PCR tubes and run on the TGradient PCR thermo cycler and run under the following cycles:

1 cycle:	95°C, 60 seconds,
25 cycles:	95°C, 20 seconds,
	50-60°C, 30 seconds,
	72°C, 2 minutes,

Nested PCR

The lowest annealing temperature PCR was diluted 1:10 and used in nested PCR to amplify the 3' end of the turbot gene. This was done as follows: 1 μl (1:10) of the above PCR product, 2 μl (20 pmol) of Turb3RaceB (appendix III), 2 μl (20 pmol) of Not1polyT, 1.8 μl (18 mM) dNTPs, 9 μl of Advantage 2 buffer, 2 μl Advantage 2 enzyme, 3 μl of turbot cDNA (section 2.2.2.) and 69.2 μl of sterile H_2O. The constituents were mixed, divided into 6, 0.2 ml thin walled PCR tubes and run on the gradient thermocycler as above. 5 μl of PCR reaction from each tube were viewed on an agarose gel. The tube containing the PCR products that were subjected to the lowest annealing temperature were cloned and screened as above. The positives were

then sequenced and the results aligned with the turbot 5' end for positive identification.

3.3 Results

3.3.1. Zebrafish (*Danio rerio*)

3.3.1.1. Open reading frame

Only one clone derived from zebrafish was sequenced. The nucleotide sequence is presented in appendix IV and includes the 3' UTR, and the translated ORF sequence is outlined in Figure 7. However, when the nucleotide sequence was BLAST searched against the GenBank database many ESTs were found containing sequences similar to the ZfishD6 nucleotide sequence. Recently another full coding DNA sequence was entered into the database, (Acc. No. BC049438; submitted 31st of March 2003). This new clone was very similar to the sequence in appendix IV. There are 11 nucleotide changes of which only one causes a non-synonymous residue change.

```
ZfishD6   1   MGGGGQQTDRITDTNGRFSSYTWEEVQKHTKHGDQWVVVERKVYNVSQWV
ZfishD6  51   KRHPGGLRILGHYAGEDATEAFTAFHPNLQLVRKYLKPLLIGELEASEPS
ZfishD6 101   QDRQKNAALVEDFRALRERLEAEGCFKTQPLFFALHLGHILLLEAIAFMM
ZfishD6 151   VWYFGTGWINTLIVAVILATAQSQAGWLQHDFGHLSVFKTSGMNHLVHKF
ZfishD6 201   VIGHLKGASAGWWNHRHFQHHAKPNIFKKDPDVNMLNAFVVGNVQPVEYG
ZfishD6 251   VKKIKHLPYNHQHKYFFFIGPPLLIPVYFQFQIFHNMISHGMWVDLLWCI
ZfishD6 301   SYYVRYFLCYTQFYGVFWAIILFNFVRFMESHWFVWVTQMSHIPMNIDYE
ZfishD6 351   KNQDWLSMQLVATCNIEQSAFNDWFSGHLNFQIEHHLFPTVPRHNYWRAA
ZfishD6 401   PRVRALCEKYGVKYQEKTLYGAFADIIRSLEKSGELWLDAYLNK
```

Figure 7. Translation of the putative zebrafish desaturase nucleotide sequence.

Table 1 lists the accession number of the cds clone and the EST clones and their corresponding percentage identity to ZfishD6. The EST numbers listed are a chosen few which separately only span a section of the ZfishD6 sequence but together span the length of the ZfishD6 sequence.

Clone Type	*GenBank Acc. No.*	*Identity (%)*
EST	BF717772	98
EST	BQ481262	99
EST	BI473077	100
EST	BM156220	100
EST	BI877233	98
EST	BF717785	99
EST	BM264661	97
EST	AW281238	97
EST	BM156732	98
EST	AW018506	98
EST	BM264927	97
EST	BM156430	99
Complete cds	BC049438	99

Table 1. List of ESTs and cds sequences similar to ZfishD6.

3.3.2. Carp (*Cyprinus carpio*)

3.3.2.1. Open reading frame

Using the protocol described in section 3.2.2.3. three clones were sequenced. The nucleic acid sequence is presented in appendix V. Using BLASTN (NCBI) a comparison of the percentage identity was calculated between each of the three putative desaturase clones the results of which are given in Table 2.

Clones *(CPpYes)*	Identities	Identities (%)
A5 – A9	1231/1332	92.42
A7 – A9	1229/1332	92.27
A7 – A5	1326/1332	99.54

Table 2. A comparison of the carp clones nucleotide sequence, identities shown as raw number and percentage data.

The results in table 2 indicate that clones CPpYesA5 and CPpYesA7 are very similar to each other, 99.54%, and vary by only 6 nucleotides. However, both of these clones differ more from CPpYesA9, varying by approximately 8%. When compared to CPpYesA9 98 nucleotides were different with CPpYesA7 and 96 nucleotides with CPpYesA5. The nucleotide sequence of the three clones was translated allowing the identification of the base pairs which caused non-synonymous changes in the amino acid sequence. Figure 8 plots the resulting translation and alignment. From the alignment the percentage identities and percentage positives were calculated and are presented in Table 2.

```
CPpYesA5    1   MGGGGQQTDRITGTNARFSTYTWEEVQKHTKSGDQWIVVERKVYNVSQWV
CPpYesA7    1   MGGGGQQTDRITGTNARFSTYTWEEVQKHTKSGDQWIVVERKVYNVSQWV
CPpYesA9    1   MGGGGQQTDRIAGTNGRFGTYTWEEVQKHTKSGDQWIVVERKVYNVSQWV

CPpYesA5   51   KRHPGGLRIIGHYAGEDATEAFTAFRPDLPLVRKYMKPLLIGELEASEPS
CPpYesA7   51   KRHPGGLRIIGHYAGEDATEAFTAFHPDLPLVRKYMKLLLIGELEASEPS
CPpYesA9   51   KRHPGGLRIIGHYAGEDATDAFHAFHPNIQLVRKYMKPLLIGELEASEPS

CPpYesA5  101   QDRQKNAALVEDFRALRERLEAEGCFKTQPLFFLLHLGHILLLEVIALML
CPpYesA7  101   QDRQKNAALVEDFRALRERLEAEGCFKTQPLFFLLHLGHILLLEVIALML
CPpYesA9  101   QDRQKNGALVEDFRALRERLEAEGCFKTQPLFFILHLGHILLLEAIALML

CPpYesA5  151   VWYFGTGWINTAIVAVLMATAQSQAGWLQHDFGHLSVCKSSRWNHLVHKF
CPpYesA7  151   VWYFGTGWINTAIVAVLMATAQSQAGWLQHDFGHLSVCKSSRWNHLVHKF
CPpYesA9  151   LWYFGTGWINTAIVSVILATAQSQAGWLQHDFGHLSVFKNSRWDHLLHKF
```

Figure 8. Alignment of the translated three putative desaturase open reading frames cloned from the common carp. Identical amino residues are shaded black, synonymous residues are shaded grey and non-synonymous white. The matrix used was BLOSUM 62 and the cut off for shading was 75%.

Table 3 compares pairwise the translated sequences of the 3 carp cDNAs and presents data on the identities, the percentage of amino acids with similar properties, and on the number of positives, the number of amino acid residues which have similar properties.

Table 3. Comparison of the three translated putative desaturase open reading frames from common carp. Identities and positives shown as raw and percentage data.

CPpYes Clones	*Identities*	*Identities (%)*	*Positives*	*Positives (%)*
A5 – A9	397/444	83.36	421/444	94.82
A7 – A9	395/444	88.96	420/444	94.60
A7 – A5	440/444	99.10	441/444	99.32

Once again it is obvious that CPpYesA5 and CPpYesA7 are highly homologous to each other having over 99% positives, whereas CPpYesA9 is clearly distinct by comparison with both of these clones differing by over 5% (positives).

3.3.2.2. 3' ends

The sequence for the 3' untranslated regions were gained as in section 3.2.2.4. Five clones in total were fully sequenced and aligned (appendix V). Studying appendix V it is noticeable that there are 5 positions where base changes occur, 25, 67, 437, 621, 1068, all of which are typed in white. However, the most notable difference is at the extreme 3'end of the UTR, just before the poly T tail where clones CP3'B2 and CP3'C6 have an extra 13 nucleotides consisting of cytosine, guanine, and thymidine bases, also typed in white.

3.3.2.3. Matching 3'UTR with open reading frames

The following three Tables, Table 4 a,b and c, present the results of alignments between the clones containing the putative desaturase gene ORF and the 3'UTR sequences. The Tables present data of identity numbers and the corresponding calculated percentages. Upon comparison with the five 3'UTR sequences CPpYesA5 and CPpYesA7 both have very low homology only between approximately 90 and 93% similarity. However, when CPpYesA9 was aligned with the 3'UTRs and percentage homology calculated, it was much greater and varied between 98.88 and 99.55%.

Table 4. Comparison of carp clones containing 3' UTR and 3' end of the open reading frame.

a.

Clones	CPpYes*A5*	
	Identities	*Identities (%)*
CP3'B2	410/440	93.18
CP3'B3	793/869	91.25
CP3'C6	385/414	93.00
CP3'C7	816/890	91.69
CP3'C9	745/818	91.08

b.

Clones	CPpYes*A7*	
	Identities	*Identities (%)*
CP3'B2	410/444	93.18
CP3'B3	791/869	91.02
CP3'C6	385/414	93.0
CP3'C7	814/890	91.46
CP3'C9	743/818	90.83

c.

Clones	CPpYes*A9*	
	Identities	*Identities (%)*
CP3'B2	440/442	99.55
CP3'B3	863/869	99.31
CP3'C6	413/415	99.52
CP3'C7	880/890	98.88
CP3'C9	812/818	99.27

3.3.3. Salmon (*Salmo salar*)

3.3.3.1. Open reading frame

Three clones were gained using the protocol stated in section 3.2.3.3. SalpYesB1 was from salmon liver and SalA9 and SalA10 from the AS salmon cell line. The untranslated nucleotide sequences for all of these clones are aligned together in

appendix VI and Table 5 gives the percentage identity comparison. The translated amino acid sequences are aligned in figure 9.

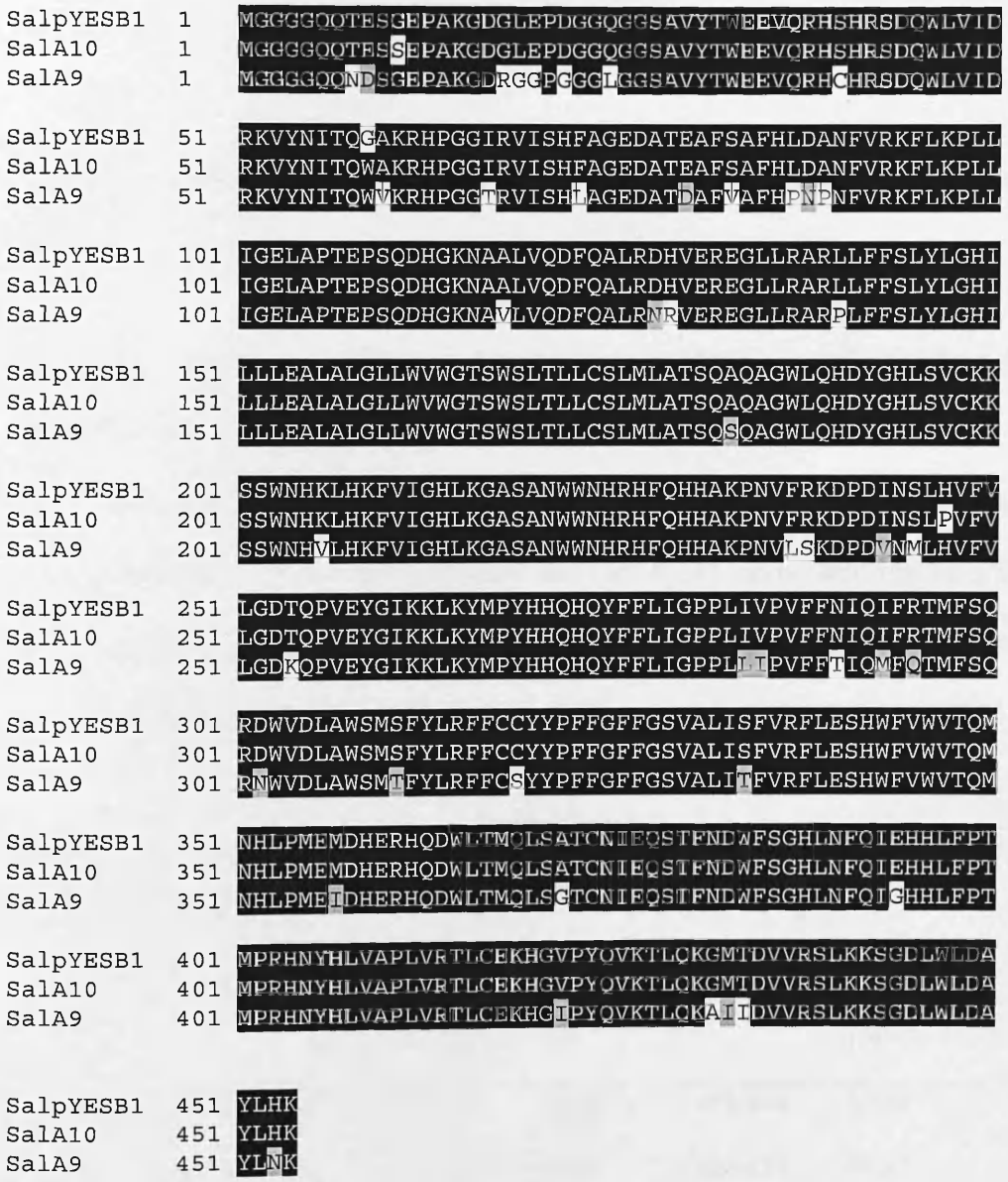


Figure 9. Alignment of the translated three putative desaturase open reading frames cloned from Atlantic salmon. Identical residues are shaded black, synonymous residues are shaded grey and non-synonymous white. The matrix used was BLOSUM 62, and the cut off for shading was 75%.

Clones	*Identities*	*Identities (%)*
SalpYesB6 – SalA10	1359/1365	99.56
SalA9 - SalA10	1289/1361	94.71
SalpYesB6 - SalA9	1291/1361	94.86

Table 5. A comparison of the salmon clones nucleotide sequence, identities shown as raw number and percentage data.

From the alignment in Figure 9 the percentage identities and positives were calculated (table 6). SalpYesB1 and SalA10 are highly homologous having only three amino acid changes all of which are non-synonymous. In comparison, the SalpYesB1 and SalA9 are quite different and vary by 45 amino acid residues, 28 of which cause non-synonymous changes. Similar percentage differences exist between SalA9 and SalA10.

Table 6. Comparison of the three translated putative desaturase open reading frames from Atlantic salmon. Identities and positives shown as raw and percentage data.

Clones	*Identities*	*Identities (%)*	*Positives*	*Positives (%)*
SalpYESB1 – SalA10	451/454	99.34	451/454	99.34
SalpYESB1 – SalA9	409/454	90.09	426/454	93.83
SalA10 – SalA9	408/454	89.88	425/454	93.61

3.3.3.2. 3' ends (including the 3'UTR)

Three clones were sequenced from the salmon liver cDNA which contained 3' UTRs and part of the open reading frame (appendix VI). Sal3'A1 and Sal3'A4 were very similar in the 3'UTR's but the third, Sal'A10, was entirely different showing almost no homology whatsoever (appendix VI).

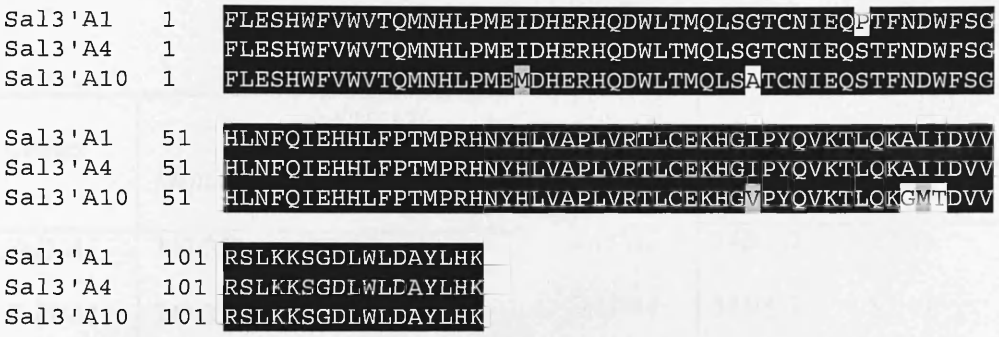


Figure 10. Alignment of the translated 3' end of the cDNA included in the 3'UTR clones from Atlantic salmon. Identical residues are shaded black, synonymous residues are shaded grey and non-synonymous white. The matrix used was BLOSUM 62, and the cut off for shading was 75%.

The areas that contained the open reading frame were translated and aligned in Figure 10. Figure 10 reveals that Sal3'A10 differs by 6 amino acid by comparison with the Sal3'A1 and Sal3'A4, 3 of which cause non-synonomous amino acid changes. Sal3'A1 differs by only one amino acid from Sal3'A4, caused by 1 base change.

3.3.3.3. Matching 3'UTRs with open reading frames.

The 3'UTR clones, nucleotide sequences were aligned with the open reading frame clones in order to evaluate which of the 3'UTRs, if any, could be associated with which open reading frames. The tables below, 7 a, b and c give the percentage identities between the 3' end of the open reading frame sequences and the 3'UTR sequences. It can be noted from the tables that Sal3'A10 has 100% similarity with SalpYESB1 and approximately 97% similarity to Sal3'A1 and Sal3'A4. Sal3'A1 and Sal3'A4 show highest homology, approximately 99%, with SalA9 and SalA10.

a.

Clones	*SalpYesB1*	
	Identities	*Identities (%)*
Sal3'A1	342/352	97.16
Sal3'A4	343/352	97.44
Sal3'A10	352/352	100

b.

Clones	*SalA10*	
	Identities	*Identities (%)*
Sal3'A1	342/352	97.16
Sal3'A4	343/352	97.44
Sal3'A10	352/352	100

c.

Clones	*SalA9*	
	Identities	*Identities (%)*
Sal3'A1	346/350	98.86
Sal3'A4	347/350	99.14
Sal3'A10	340/350	97.14

Table 7. Comparison of nucleotide sequences of salmon clones containing the open reading frame and those containing 3' UTR and three prime end of the open reading frame.

3.3.4 Cod (*Gadus morhua*)

3.3.4.1. Partial putative desaturase sequence (open reading frame)

An alignment of all known functionally characterised desaturase genes from a variety of organisms including mammals, fish and *C. elegans* was used to design degenerate primers around the second and third histidine boxes. These primers were used as outlined in section 3.2.4.3. to gain the following clones CodF4, F6, F7, F8, G6 and G11. The nucleotide sequences of these clones are presented in appendix VII. Figure 11 presents an alignment of the clones after translation into amino acid residues.

```
CodG11   1   VFSKDPDVNMLHVFVVGDIQPVEYGIKKIKYMPYHHQHQYFFLVGPPLLI
CodG6    1   VFSKDPDVNMLHVFVVGDIQPVEYGIKKIKYMPYHHQHQYFFLVGPPLLI
CodF4    1   VFSKDPDVNMLHVFVVGDIQPVEYGIKKIKYMPYHHQHQYFFLVGPPPLI
CodF7    1   VFSKDPDVNMLHVFVVGDIQPVEYGIKKIKYMPYHHQHQYFFLVGPPLLI
CodF6    1   VFSKDPDVNMLHVFVVGDIQPVEYGIKKIKYMPYHHQHQYFFLVGPPLLI
CodF8    1   VFSKDPDVNMLHVFVVGDIQPVEYGIKKIKYMPYHHQHQYFFLVGPPLLI

CodG11   51  PVYFHIQILRAMFSRRDWVDLAWSMSYYLRYFCCYAPFYGLLGSVALISF
CodG6    51  PVYFHIQILRAMFSRRDWVDLAWSMSYYLRYFCCYAPFYGLLGSVALISF
CodF4    51  PVYFHIQILRAMFSRRDWVDLAWSMSYYLRYFCCYAPFYGLLGSVALISF
CodF7    51  PVYFHIQILRAMFSRRDWVDLAWSMSYYLRYFCCYAPFYGLLGSVALISF
CodF6    51  PVYFHIQILRAMFSRRDWVDLAWSMSYYLRYFCCYAPFYGLLGSVALISF
CodF8    51  PVYFHIQILRAMFSRRDWVDLAWSMSYYLRYFCCYAPFYGLLGSVALISF

CodG11   101 V
CodG6    101 VRFLESHWFVWVTQMNHLPMNIDHEKQQDWLSMQLSATCNIEQSCFNDWF
CodF4    101 VRFLESHWFVWVTQMNHLPMNIDHEKQQDWLSMQLSATCNIEQSCFNDWF
CodF7    101 VRFLESHWFVWVTQMNHLPMNIDHEKQQDWLSMQLSATCNIEQSCFNDWF
CodF6    101 VRFLESHWFVWVTQMNHLPMNIDHEKQQDWLSMQLSATCNIEQSCFNDWF
CodF8    101 VRFLESHWFVWVTQMNHLPMNIDHEKQQDWLSMQLSATCNIEQSCFNDWF

CodG11   101
CodG6    151 SGH
CodF4    151 SGH
CodF7    151 SGH
CodF6    151 SGH
CodF8    151 SGH
```

Figure 11. Alignment of the translated section of the cod desautrase gene, between the second and third histidine boxes. Identical residues are shaded black and non-synonymous white. The matrix used was BLOSUM 62, and the cut off for shading was 75%.

3.3.4.2. 3'Ends (including the 3'UTR)

The partial ORF sequence above allowed the design of primers for gaining the end of the ORF and the 3' UTR as described in 3.2.4.3. 2 clones were gained using the above primers as outlined in section 3.2.4.3. The nucleotide sequences of these clones is presented in appendix VII. When translated these base changes confer two non-synonymous amino acid changes.

3.3.5. Turbot (Scophthalmus maximus)

3.3.5.1. Open reading frames

Figure 12 is the alignment of the translations of the nucleotide sequences from appendix VIII. The clones were gained using 5'RACE cDNA and using the protocol set out in section 3.2.5.3.

```
Turb5'B1   1   MGGGGQLTEQGETGSKRAGCVYTWEEVQSHSSRTDQWLVIDRKVYNTTQW
Turb5'B6   1   MGGGGQLTEQGETGSKRAGCVYTWEEVQSHSSRTDQWLVIDRKVYNTTQW
Turb5'C9   1   MGGGGQLTEQGETGSKRAGCVYTWEEVQSHSSRTDQWLVIDRKVYNSTQW

Turb5'B1   51  AKRHPGGFHVISHYAGQDATEAFTAFHPDLKFVQKFLKPLLIGELAATEP
Turb5'B6   51  AKRHPGGFHVISHYAGQDATEAFTAFHPDLKFVQKFLKPLLIGELAATEP
Turb5'C9   51  AKRHPGGFHVISHYAGQDATEAFTAFHPDLKFVQKFLKPLLIGELAATEP

Turb5'B1   101 SQDRNKNAALVQDFHTLRVKAESKGLFQARPLFFCLHLDHIVLLEALAWL
Turb5'B6   101 SQDRNKNAALVQDFHTLRVKAESKGLFQARPLFFCLHLGHIVLLEALAWL
Turb5'C9   101 SQDRNKNAALVQDFHTLRVKAESKGLFQARPLFFCLHLGHIVLLEALAWL

Turb5'B1   151 IIWVWGTNWILTFLCALLMTIAQSQAGWLQHDFGHLSVFKQSRWNHLLQK
Turb5'B6   151 IIWVWGTNWILTFLCALLMTIAQSQAGWLQHDFGHLSVFKQSRWNHLLQK
Turb5'C9   151 IIWVWGTNWILTFLCALLMTIAQSQAGWLQHDFGHLSVFKQSRWNHLLQK

Turb5'B1   201 FAIGHLKGASANWWNHRHFQHHAKTNIFRKDPDVNMLNIFVIGATQPVEY
Turb5'B6   201 FAIGHLKGASANWWNHRHFQHHAKTNIFRKDPDVNMLNIFVIGATQPVEY
Turb5'C9   201 FAIGHLKGASANWWNHRHFQHH

Turb5'B1   251 GVKKIKHMPYHHQH
Turb5'B6   251 GVKKIKHMPYHHQH
Turb5'C9   222
```

Figure 12. Alignment of the translated section of the turbot desaturase gene, between the second and third histidine boxes. Identical residues are shaded black, synonymous residues are shaded grey and non-synonymous white. The matrix used was BLOSUM 62, and the cut off for shading was 75%.

Three clones were sequenced using the above method turbot5'B1, B6 and B9. They differ by 2 amino acid residues, at positions 47 and 139. The change at position 47 is due to a A/T nucleotide substitution at position 316 in the clone turb5'C9 (appendix VIII). The amino acid change at position 139 is caused by A/G substitution at position 593 in the clone turb5'B1 (appendix VIII).

3.3.5.2. 3'end (including the 3' UTR)

Figure 13. Alignment of a section of the open reading frame (translated) of the turbot desaturase gene. Identical residues are shaded black, synonymous residues are shaded grey and non-synonymous white. The matrix used was BLOSUM 62, and the cut off for shading was 75%.

Once the 5' end clones of the turbot desaturase were fully sequenced they were used to design primers to gain the sequence of the 3' ends of the ORFs and the 3' UTR's. Five clones containing the 3' end of the gene were fully sequenced and appendix VIII presents the nucleotide sequence data of the clones. Figure 11 presents an alignment of the translated 3' end of the open reading frame. Clone turb3'4 contains the greatest number of amino acid differences, totalling 12, whereas clone turb3'8 contains 2.

3.3.6. Structural characteristics – A comparison with mammalian Δ5 and Δ6 fatty acyl desaturase genes

3.3.6.1. Percentage sequence similarity with mammalian Δ5 and Δ6 desaturases.

All the full length fish sequences were translated and the putative open reading frames BLAST searched against the GenBank database on the NCBI website. As expected, the sequences having the highest homology to the fish desaturase sequences above were the putative fatty acid desaturase genes from various fish species cloned by other researchers. However, the search also revealed the next highest homology to the fish putative desaturases was shown by the human Δ5 and Δ6 desaturases. Table 8a presents the results of that BLAST search and plots the percentage identities and positives of each of the fish species against the human Δ5 and Δ6 desaturases. It is obvious from Table 8b that all the fish putative desaturase genes are more homologous to the human Δ6 desaturase than to the human Δ5 desaturase gene, by approximately 2% (positives). CppYesA9 had the lowest percentage positives to the human Δ5 desaturase gene, 74.82%, whereas zfishD6 had the highest with 76.47%. As for the human Δ6 desaturase gene, the zfishD6 had the highest homology with 79.73% positives, whereas the carp, CppYesA7, had the lowest of 78.60%.

Table 8a. Human Δ6 desaturase

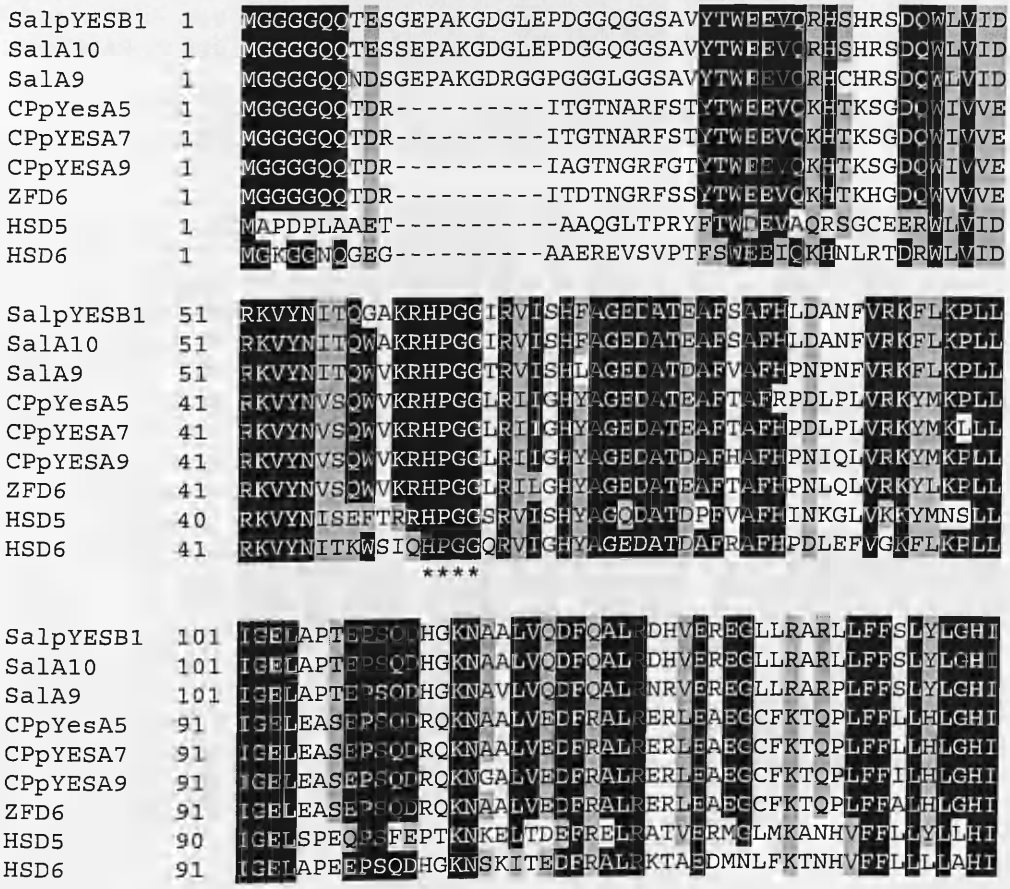
Clones	*Identities*	*Identities (%)*	*Positives*	*Positives (%)*
ZfishD6	288/444	64.86	354/444	79.73
CPpYesA5	284/444	63.96	350/444	78.83
CPpYesA7	284/444	63.96	349/444	78.60
CPpYesA9	284/444	63.96	354/444	79.73
SalpYesB1	290/454	63.88	359/454	79.07
SalA9	290/443	63.88	354/443	79.73

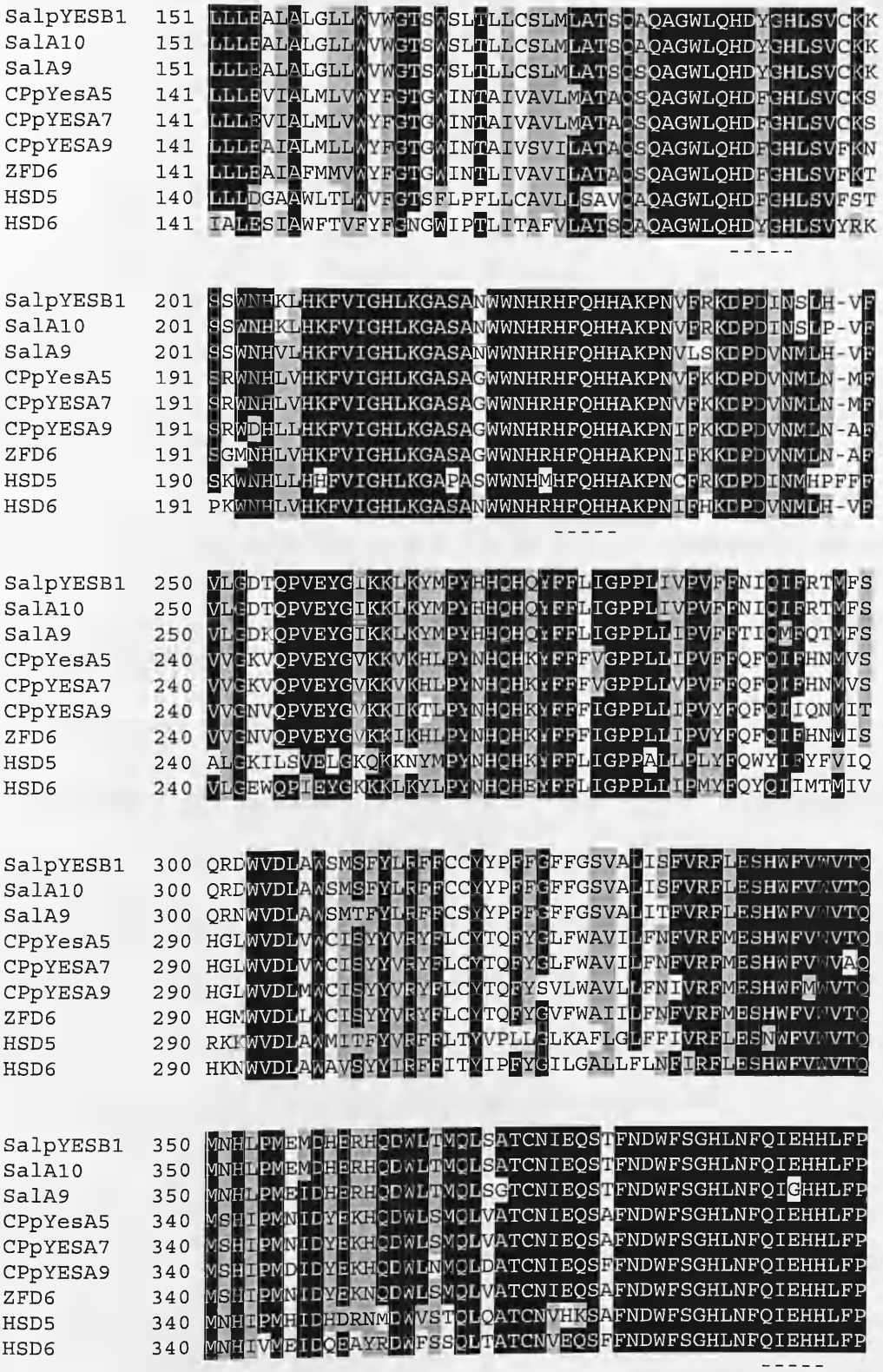
Table 8b. Human Δ5 desaturase

Clones	*Identities*	*Identities (%)*	*Positives*	*Positives (%)*
ZfishD6	249/425	58.59	325/425	76.47
CPpYesA5	246/425	57.88	324/425	76.24
CPpYesA7	246/425	57.88	324/425	76.24
CPpYesA9	244/425	57.41	318/425	74.82
SalpYesB1	258/426	60.56	322/426	75.59
SalA9	257/425	60.47	320/425	75.29

3.3.6.2. Cytochrome b_5-like domain

Analysis of the putative gene structures from all the fish species indicates several important similarities with the known, functionally characterised, human Δ5 and Δ6 fatty acyl desaturase genes. One of the key characteristics of the human Δ5 and Δ6 desaturases is the presence of a cytochrome b_5-like domain containing the haem binding region towards the N-terminus of the peptide. Figure 14 is an alignment of all of the putative fish desaturase genes aligned with the human Δ5 and Δ6 protein sequences, the cod and the turbot not shown in Figure 14. In Figure 14, the cytochrome b_5-like domain is highlighted for all the genes. It is immediately obvious that all the putative fish desaturase genes contain an N-terminal cytochrome b_5-like domain, as in the human Δ5 and Δ6 genes.




SalpYESB1 151 LLLEALALGLLWVWGTSWSLTLLCSLMLATSQAQAGWLQHDYGHLSVCKK
SalA10 151 LLLEALALGLLWVWGTSWSLTLLCSLMLATSQAQAGWLQHDYGHLSVCKK
SalA9 151 LLLEALALGLLWVWGTSWSLTLLCSLMLATSQSQAGWLQHDYGHLSVCKK
CPpYesA5 141 LLLEVIALMLVWYFGTGWINTAIVAVLMATACSQAGWLQHDFGHLSVCKS
CPpYESA7 141 LLLEVIALMLVWYFGTGWINTAIVAVLMATACSQAGWLQHDFGHLSVCKS
CPpYESA9 141 LLLEAIALMLLWYFGTGWINTAIVSVILATACSQAGWLQHDFGHLSVFKN
ZFD6 141 LLLEAIAFMMVWYFGTGWINTLIVAVILATACSQAGWLQHDFGHLSVFKT
HSD5 140 LLLDGAAWLTLWVFGTSFLPFLLCAVLLSAVCAQAGWLQHDFGHLSVFST
HSD6 141 IALESIAWFTVFYFGNGWIPTLITAFVLATSQAQAGWLQHDYGHLSVYRK
SalpYESB1 201 SSWNHKLHKFVIGHLKGASANWWNHRHFQHHAKPNVFRKDPDINSLH-VF
SalA10 201 SSWNHKLHKFVIGHLKGASANWWNHRHFQHHAKPNVFRKDPDINSLP-VF
SalA9 201 SSWNHVLHKFVIGHLKGASANWWNHRHFQHHAKPNVLSKDPDVNMLH-VF
CPpYesA5 191 SRWNHLVHKFVIGHLKGASAGWWNHRHFQHHAKPNVFKKDPDVNMLN-MF
CPpYESA7 191 SRWNHLVHKFVIGHLKGASAGWWNHRHFQHHAKPNVFKKDPDVNMLN-MF
CPpYESA9 191 SRWDHLLHKFVIGHLKGASAGWWNHRHFQHHAKPNIFKKDPDVNMLN-AF
ZFD6 191 SGMNHLVHKFVIGHLKGASAGWWNHRHFQHHAKPNIFKKDPDVNMLN-AF
HSD5 190 SKWNHLLHHFVIGHLKGAPASWWNHMHFQHHAKPNCFRKDPDINMHPFFF
HSD6 191 PKWNHLVHKFVIGHLKGASANWWNHRHFQHHAKPNIFHKDPDVNMLH-VF
SalpYESB1 250 VLGDTQPVEYGIKKLKYMPYHHQHQYFFLIGPPLIVPVFFNIQIFRTMFS
SalA10 250 VLGDTQPVEYGIKKLKYMPYHHQHQYFFLIGPPLIVPVFFNIQIFRTMFS
SalA9 250 VLGDKQPVEYGIKKLKYMPYHHQHQYFFLIGPPLLIPVFFTIQMFQIMFS
CPpYesA5 240 VVGKVQPVEYGVKKVKHLPYNHQHKYFFFVGPPLLIPVFFQFQIFHNMVS
CPpYESA7 240 VVGKVQPVEYGVKKVKHLPYNHQHKYFFFVGPPLLVPVFFQFQIFHNMVS
CPpYESA9 240 VVGNVQPVEYGVKKIKTLPYNHQHKYFFFIGPPLLIPVYFQFQIIQNMIT
ZFD6 240 VVGNVQPVEYGVKKIKHLPYNHQHKYFFFIGPPLLIPVYFQFQIFHNMIS
HSD5 240 ALGKILSVELGKQKKNYMPYNHQHKYFFLIGPPALLPLYFQWYIFYFVIQ
HSD6 240 VLGEWQPIEYGKKKLKYLPYNHQHEYFFLIGPPLLIPMYFQYQIIMTMIV
SalpYESB1 300 QRDWVDLAWSMSFYLRFFCCYYPFFGFFGSVALISFVRFLESHWFVWVTQ
SalA10 300 QRDWVDLAWSMSFYLRFFCCYYPFFGFFGSVALISFVRFLESHWFVWVTQ
SalA9 300 QRNWVDLAWSMTFYLRFFCSYYPFFGFFGSVALITFVRFLESHWFVWVTQ
CPpYesA5 290 HGLWVDLVWCISYYVRYFLCYTQFYGLFWAVILFNFVRFMESHWFVWVTQ
CPpYESA7 290 HGLWVDLVWCISYYVRYFLCYTQFYGLFWAVILFNFVRFMESHWFVWVAQ
CPpYESA9 290 HGLWVDLMWCISYYVRYFLCYTQFYSVLWAVLLFNIVRFMESHWFMWVTQ
ZFD6 290 HGMWVDLLWCISYYVRYFLCYTQFYGVFWAIILFNFVRFMESHWFVWVTQ
HSD5 290 RKIWVDLAWMITFYVRFFLTYVPLLGLKAFLGLFFIVRFLESNWFVWVTQ
HSD6 290 HKNWVDLAWAVSYYIRFFITYIPFYGILGALLFLNFIRFLESHWFVWVTQ
SalpYESB1 350 MNHLPMEMDHERHQDWLIMQLSATCNIEQSTFNDWFSGHLNFQIEHHLFP
SalA10 350 MNHLPMEMDHERHQDWLIMQLSATCNIEQSTFNDWFSGHLNFQIEHHLFP
SalA9 350 MNHLPMEIDHERHQDWLIMQLSGTCNIEQSTFNDWFSGHLNFQIGHHLFP
CPpYesA5 340 MSHIPMNIDYEKHQDWLSMQLVATCNIEQSAFNDWFSGHLNFQIEHHLFP
CPpYESA7 340 MSHIPMNIDYEKHQDWLSMQLVATCNIEQSAFNDWFSGHLNFQIEHHLFP
CPpYESA9 340 MSHIPMDIDYEKHQDWLNMQLDATCNIEQSFFNDWFSGHLNFQIEHHLFP
ZFD6 340 MSHIPMNIDYEKNQDWLSMQLVATCNIEQSAFNDWFSGHLNFQIEHHLFP
HSD5 340 MNHIPMHIDHDRNMDWVSTQLQATCNVHKSAFNDWFSGHLNFQIEHHLFP
HSD6 340 MNHIVMPIDQEAYRDWFSSQLTATCNVEQSFFNDWFSGHLNFQIEHHLFP

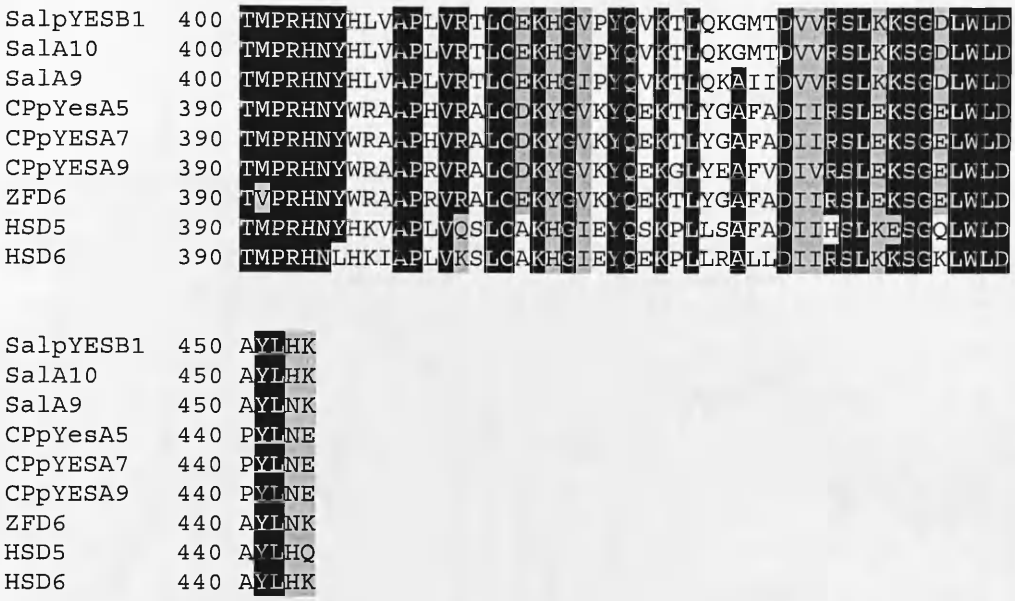


Figure 14. Alignment of all the fish species putative desaturase open reading and the, functionally characterised human Δ5 and Δ6 desaturase genes. The haem binding domain contained within the N-terminal cytochrome b_5 is underlined with asterisks and the three conserved histidine boxes are highlighted with dashed underlining.

3.3.6.3. Histidine boxes

Another important feature of the human Δ5 and Δ6 desaturases is the presence of the three functionally essential histidine regions. The three histidine boxes are highlighted in Figure 14. Regions I (HX_3H) and II (HX_2HH) are located between the two transmembrane domains (see below) and region III (QX_3H) is located near the carboxyl terminus of the peptide. Again it is clear that all the putative fish desaturase ORFs contain the characteristic histidine boxes.

3.3.6.4. Hydrophobicity plots

Analysis of the functionally characterised mammalian Δ5 and Δ6 desaturase genes through hydropathy profiles suggests that they contain at least two transmembrane domains. The putative desaturase nucleic acid sequences for zebrafish, carp, salmon and turbot were translated and entered into a hydropathy analysis programme in order to establish whether the sequences also contained hydrophobic membrane

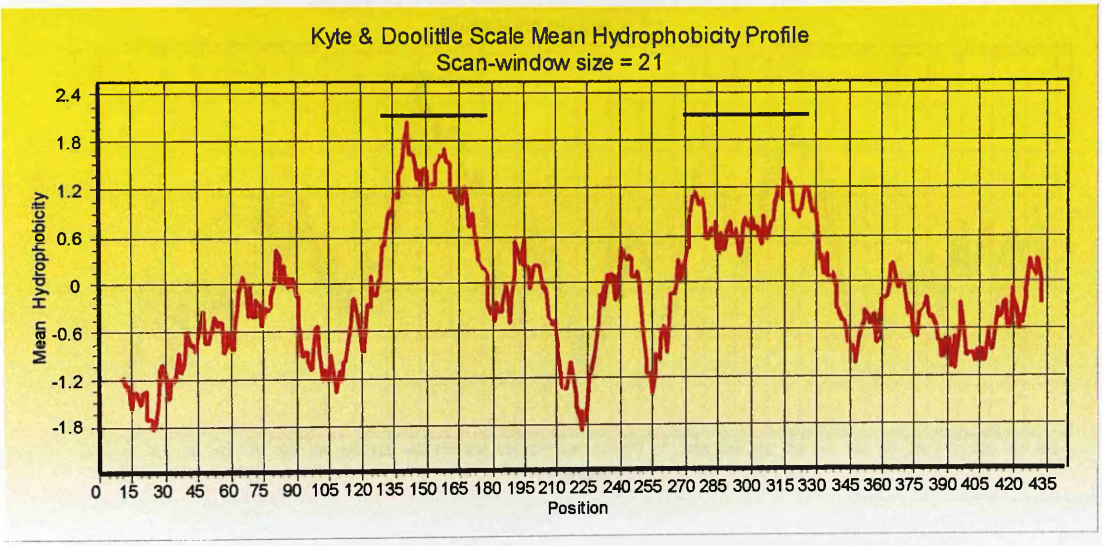


Figure 15a: Hydropathy profile of the putative zebrafish desaturase gene. The window size is 21. Black bars indicate possible transmembrane regions.

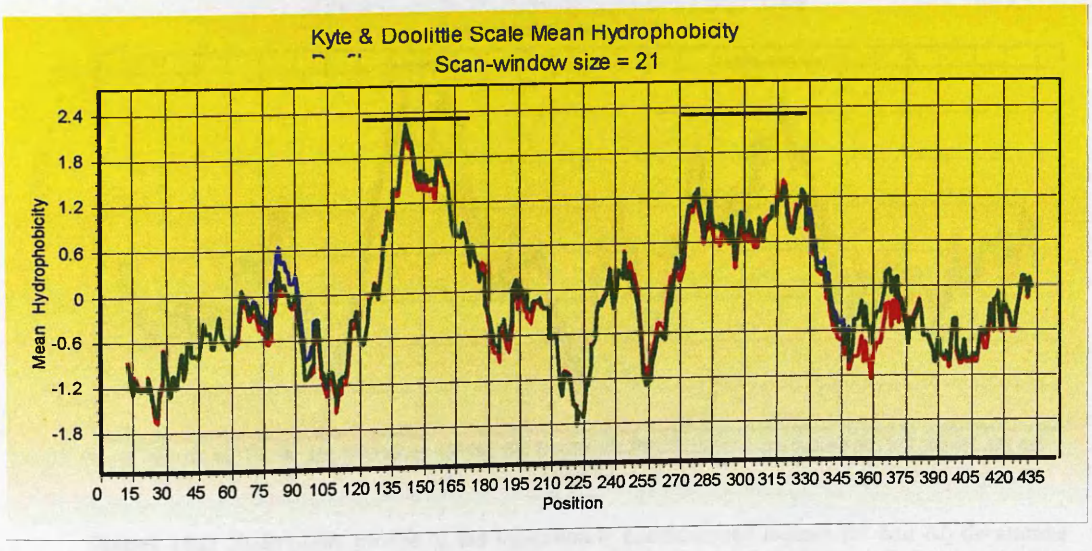


Figure 15b: Hydropathy profile of the putataive carp desaturase genes. The red line indicates CPpYesA9, the blue line, CPpYesA7 and green line, CPpYesA5. The window size is 21. Black bars indicate possible transmembrane regions.

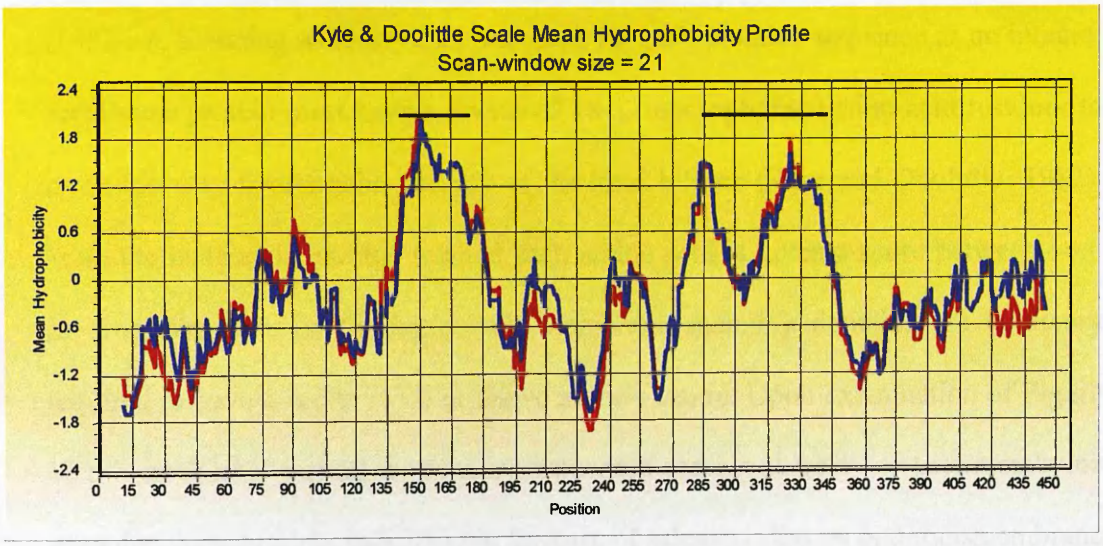


Figure 15c: Hydropathy profile of the putative salmon desaturase genes. The red line indicates SalpYesB1, and the blue SalA9. The window size is 21. Black bars indicate possible transmembrane regions.

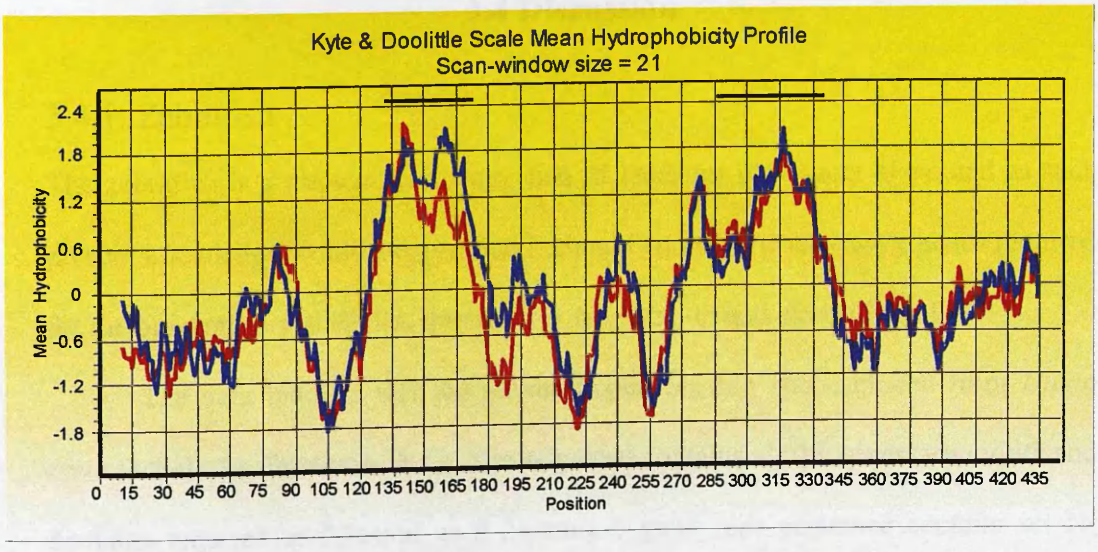


Figure 15d: Hydropathy profile of the functionally characterised human Δ5 and Δ6 desaturase genes. The red line indicates the human Δ6 desaturase and blue human Δ5 desaturase. The window size is 21. Black bars indicate possible transmembrane regions.

spanning domains. Figure 15 plots the hydrophobicity plots for all the fish putative desaturase open reading frames. The method used was that of Kyte and Doolittle (1982). A scanning window of 21 was used for each putative sequence as an integral membrane protein must have a stretch of 18-22 hydrophobic amino acid residues to cross the very hydrophobic interior of the lipid bilayer (Kyte and Doolittle, 1982). With the Kyte and Doolittle method each amino acid is given a score between –4.6 (hydrophilic) and 4.6 (hydrophobic). Membrane spanning domains are in theory required to have a score of 1.6 or above at their centre. Upon examination of Figure 15 it is noticeable that all the putative sequences contain at least one transmembrane spanning domain if not two. Only in the case of salmon is the second transmembrane segment convincing as the score is above 1.6. However, this is a good estimation of the overall number of transmembrane domains.

3.4 Discussion

3.4.1. Zebrafish

The zebrafish is a tropical freshwater fish. It feeds on freshwater algae and as such present knowledge would suggest that it should contain the necessary genes required for the biosynthesis of HUFA through the desaturation and elongation of PUFA.

The data indicate that the putative open reading frame cloned from *Danio rerio* is that of a desaturase gene. The sequence contains all the necessary motifs and domains required to function as a desaturase gene. The sequence contains an N-terminal cytochrome b_5, three essential histidine boxes, and the hydropathy plot suggest that the sequence may specify two transmembrane regions.

Amino acid sequence comparison of the zebrafish with the human Δ5 and Δ6 desaturase genes indicates that the zebrafish putative desaturase has a slightly greater

homology with the human Δ6 than the human Δ5. The percentage of amino acids in zebrafish that are similar (positives) to the human Δ6 desaturase is 79.73% compared to 76.47% to the human Δ5 desaturase. The percentage identities between human Δ6 and zebrafish is 64.86% whereas the percentage identities between human Δ5 and the zebrafish protein sequence is 58.59%. Thus, both the human Δ5 and Δ6 desaturase sequences are highly homologous to the zebrafish protein. It, therefore, remained to be established whether the zebrafish protein sequence confers Δ5 or Δ6 desaturase activity.

As for the number of alleles or genes that the zebrafish genome contains, the following is understood. In 1970 Ohno proposed that gnathostome vertebrates underwent two rounds of genome duplication close to the inception of this lineage however, the exact timing is unclear (Ohno, 1970). The first event separated the cephalochordates from the early agnathan (jawless) vertebrates. The second event apparently occurred within the subphylum vertebrata and coincided with the development of jaws from the second gill arch that took place in the Ordovician vertebrates (Ohno, 1999). The evidence for this is found in the Hox gene cluster. Jawless vertebrates carry two or three independent sets of Hox gene clusters while all jawed vertebrates , from fish to humans are endowed with at least four separate clusters of Hox genes (Garcia-Fernandez and Holland, 1994). However, this is not confined to Hox genes. Analysis of the results in section 3.2.1. indicates that there is at least one Δ6 like desaturase gene. Whether zebrafish contain one or several Δ6 like desaturase genes is open to debate and cannot be speculated at this point in time. However, it is clear that there is a Δ6 like desaturase sequence in the zebrafish genome and that multiple alleles at the locus almost certainly exist. However, zebrafish have a diploid complement of chromosomes with the diploid number

2n=50 (Daga *et al.*,1996) as humans and since both humans and zebrafish have undergone genome duplication it is conceivable that zebrafish have another gene and more alleles.

3.4.2. Carp

The common carp is also a freshwater fish preferring the colder temperate waters such as those found in UK lakes and lochs. They tend to feed off freshwater algae and, as with the zebrafish, current understanding is that they should possess all the necessary genes required for the biosynthesis of HUFA from C_{18} precursors. Their EFA requirements are well known (18:2*n*-6 mainly) so that is further evidence that they will have a complete pathway.

The chromosomal arrangement of the common carp (*Cyprinus carpio*) is very complex in comparison to the human chromosomal arrangement. Carp are considered to be tetraploid (Larhammar and Risinger, 1994). In evolution, tetraploidy has arisen through two different routes. Firstly, allotetraploidisation, tetraploidy through species hybridisation and secondly, autotetraploidy which occurs by genome doubling. In carp, tetraploidy is thought to have arisen through allotetraploidisation as no chromosomal quadrivalents have been observed during meiosis (Ohno *et al.*, 1967). In diploid species, there are two homologues of each chromosome and during 1st meiosis only bivalents are seen. In a recent autotetraploid, however, there are four homologues of each chromosome, and these four associate with each other and form one quadrivalent rather than two bivalents (Ohno, 1970). The exact number of chromosomes that carp have has been the subject of much discussion over the past 50 years. Ohno (1967) indicated the occurrence of 104 chromosomes. However, Ojima and Hitotsumachi (1967) claimed the diploid number was 100. The later research of Raicu *et al.* (1972) agreed with the chromosome number of Ojima and

Hitotsumachi. The DNA content per cell compared to that of 100% in human leucocytes is 52% for the common carp (Ritter *et al.*, 1969) as compared to diploid cyprinids which have between 20% and 38% DNA content (Wolf *et al.*, 1969). Regional duplications are thought to be the cause of diploid cyprinids which have greater than 28% DNA content. From the discussion above it is therefore conceivable that an individual common carp may have 2x2 alleles for any one gene.

For the common carp, three clones were identified in this study, each of which contained what appears to constitute the entire open reading frame of a desaturase gene. All three clones contain the necessary domains and motifs involved in Δ5 and Δ6 desaturase activity. However, two of the protein sequences are very similar to one another, while the third is distinctly different. When considering the percentage positives, CPpYesA5 and CPpYesA7 share 99.3% positives with each other and may constitute different alleles at the same locus, whereas CPpYesA9 is shares only 94.82% to CPpYesA5 and 94.6% to CPpYesA7. Thus, CPpYesA9 may constitute another gene or an allele from a different species and it may have been gained through the allotetraploid method of genome duplication. This is a very real possibility as it is known to have occurred in a variety of genes in the common carp. Hemoglobin alpha-I chain and hemoglobin alpha-II chain differ by 11.97% in amino acid bases. Somatotropin I and somatotropin II differ by 3.14% (6/191) and prolactin I and prolactin II differ by 5.91% (11/186) (Ohno, 1999). All are believed to have arisen by allotetraploidy. Ohno also estimated that in approximately 96% of the gene pairs of the common carp, redundancy did not result in gene silencing. Whether any of these carp desaturase cDNAs are functional is unclear at this point in time. However, it is possible that one of them could be non-functional or redundant. Being redundant, any extra copy genes can accumulate mutations, and may become

degenerate eventually joining the ranks of "junk DNA". But it is possible that mutations give rise to a new gene with a new or modified function, with different kinetic and physical properties that then come to occupy new metabolic niches of adaptive significance (Ferris and Whit, 1977).

Of the five 3'UTR clones gained from carp all match CPpYesA9 (98.88 – 99.55%) to a higher degree than either CPpYesA5 (91.08 – 93.18%) or CPpYesA7 (90.83 – 93.18%). However, all five 3'UTR clones are highly homologous to each other until the very 3' end of the UTR where there appears to be an insertion/deletion of 13 base pairs. As all the clones are highly homologous to each other to this point it would appear that there are two alleles within the 5 clones. As gene duplication occurred in carp probably by allotetraploidy and not autotetraploidy, the 3'UTRs of duplicate genes would have come from two different species, and 3'UTRs would therefore probably be very different from each other. However, since none of the 3'UTR sequences match either CPpYesA5 or CPpYesA7 it is possible that both these open reading frames are derived from the other species in the tetraploidisation event, and have very different 3'UTR sequences. CPpYesA5 and CPpYesA7 differ by 4 amino acids, of which one is a synonomous substitution. It is possible that these two clones are the sequences of two alleles as the sequence differences appear too small to cause vast differences in protein function. The exact number of genes and alleles is not clear from the information above but it probably indicates that there are 2 loci; CPpYesA5 and CPpYesA7 are two alleles for the first locus and that CPpYesA9 is one of the alleles for the second locus.

3.4.3. Salmon

Atlantic salmon are an anadromous species, and as such spend part of their life in freshwater and part in the ocean. They begin their life in the freshwater rivers and as

they mature they migrate downstream and into the oceans and seas, returning only to spawn. It is for this reason that they are an interesting species to study as they are neither a completely freshwater or completely saltwater species. They undergo many physiological changes upon migration to the sea, a process known as smoltification (Mills, 1971).

The salmonids have an even more complex chromosomal arrangement than carp and are thought to be partial tetraploids. They are believed to have undergone genome duplication by autotetraploidy. Three putative open reading frames from salmon were sequenced, and as with carp, two were very similar and the third was quite different. SalpYesB1 and SalA10 shared 99% positives, with only 3 amino acid non-synonomous substitutions. Whereas when the clones were compared to the SalA9 sequence, both differed by approximately 6.5%. As with the carp, it is impossible to know from the sequences alone whether these sequences confer functional or non-functional genes as they all contain the essential histidine boxes and N-terminal cytochrome b_5 domain. Since salmon are also tetraploid these sequences could represent redundant genes, genes with new functions, or they may be just further alleles at two loci with a common function.

Analysis of the 3' sequences reveals at least two alleles if not three. Again, as with the putative open reading frames, two are similar and the third is very different. SalpYesB1 and SalA10 nucleotide sequence is 100% identical to the Sal3'A10. Whereas SalA9 is most similar to Sal3'A1 and Sal3'A4. However, it is almost impossible to decide whether any of these differences are real and or just artifacts of the PCR method used to isolate these sequences.

3.4.4. Cod

Cod (*Gadus morhua*) are a diploid marine fish species and as such each specimen will only carry at most two alleles for each gene. The entire open reading frame was unable to be sequenced for the cod desaturase gene due to difficulties in 5'RACE amplification. The sequence information that is presented in section 3.2.4. indicates that two alleles for the desaturase gene were isolated. However, with so little information it is difficult to infer anything else.

3.4.5. Turbot

As mentioned in Chapter 1, there is some debate as to why marine or carnivorous fish have a limited ability to biosynthesise long chain fatty acids. Recent data on turbot cells are more consistent with a deficiency in the C_{18} to C_{20} elongase multi-enzyme complex (Ghioni *et al.*, 1999) whereas previous data suggest a deficiency in the Δ5 desaturase (Owen *et al.*, 1975, Tocher *et al.*, 1989). Turbot cells however, are known to have a very active Δ6 and also exhibit some EPA to DHA Δ4 desaturase activity (Bell *et al.*, 1995).

The results described in section 3.3.5. describe up to three desaturase genes with all the required features of a functioning Δ5 or Δ6 desaturase gene. As with carp and salmon there is one sequence containing the open reading frame that appears to have a considerable number of amino acid differences by comparison with the other two sequences. However, unlike carp and salmon, the turbot is a diploid species. The turbot contains 2n=44 chromosomes (Bouza *et al.*, 1994). Since one genome duplication probably occurred before the inception of the gnathostome vertebrates, and as the amino acid differences are numerous it is possible that the two more similar sequences represent two alleles from the same locus and the third less homologous sequence represents a gene at another locus.

It is concluded that turbot carry sequences which resemble those of the mammalian Δ5 or Δ6 desaturase genes, but whether these sequences code a functional enzyme and the specificity of that enzyme, remains to be determined. As to the number of alleles and number of loci, that can be only speculated upon at this time.

3.5. Summary

This chapter illustrates that both marine and freshwater teleosts possess putative gene sequences similar to those of the functionally characterised mammalian Δ5 and Δ6 desaturases. The coding regions of the teleosts genes contain all the elements of functional desaturases i.e., cytochrome b_5 domains, three conserved histidine regions, and up to two hydrophobic transmembrane regions. However, in order to distinguish if these putative sequences translate into functional proteins they must be functionally characterised. This is discussed in the following chapter.

Chapter 4

Functional Characterisation of the Putative Fatty Acyl Desaturase Sequences

Chapter 4 - The Functional Characterisation of the Putative Fatty Acyl Desaturase Sequences.

4.1. Introduction

4.1.1. Aims

To functionally characterise the teleost putative fatty acyl desaturase gene sequences.

Chapter 3 contains the putative fatty acyl desaturase gene sequences from a variety of marine and freshwater teleosts. However, the sequences require to be functionally expressed in order to characterise unequivocally the enzyme activities that the gene products possess. There are several systems available in which to functionally characterise desaturase genes; expression in yeast, expression in cell lines and expression in other organisms.

4.1.2. Functional characterisation in cell lines

The preferred method for the functional characterisation of the human Δ5 and the murine Δ6 desaturase genes was cellular expression. The murine Δ6 desaturase gene was expressed in primary rat hepatocytes and CHO (Chinese hamster ovary) cells by transfecting the cells with the cytomegalovirus promoter expression virus containing the putative mouse desaturase gene (Cho *et al.*, 1999a). Once transfected, the cells were incubated with the appropriate albumin-bound fatty acid substrates for a period of time before fatty acid extraction and analysis. CHO cells were also utilised for the functional characterisation of the human Δ5 desaturase (Cho *et al.*, 1999b). However, other workers used mouse L cells with the putative human Δ5 desaturase gene inserted into plasmid pMTK-bGH-C. The plasmid utilises the mouse metallothionein

I transcription regulatory element to direct human Δ5 desaturase transcription and the bovine growth hormone (bGH) polyadenylation signal for correct processing of the 3' terminus of the mRNA (Leonard *et al.*, 2000). But there are drawbacks to using mammalian cell cultures one of the most important being that they contain endogenous desaturases, Δ6 and Δ5, making it difficult to be definitive about the results.

4.1.3. Functional characterisation in plants

Transgenic plants can also be used to test the functionality of fatty acid desaturase genes. Spychalla and colleagues (1997) have utilised transgenic plants to prove the functionality of the *C. elegans* fat-1 gene. Desaturase deficient mutants of *Arabidopsis* are available which can then be transfected with the desaturase gene of interest. Spychalla took advantage of this and produced transgenic *Arabidopsis* plants containing the *C. elegans fat*-1 gene. The transgenic plants were then sprayed with the appropriate substrates and grown on. The plants were then harvested and fatty acids extracted and analysed. The transgenic plants desaturated 18:2*n*-6 into 18:3*n*-3 and 20:3*n*-6 and 20:4*n*-6 substrates to 20:4*n*-3 and 20:5*n*-3. Therefore the *C. elegans fat*-1 gene encodes a fatty acyl desaturase that recognises a range of 18 and 20 carbon *n*-6 substrates.

Tobacco transformant plants containing a Δ6 like desaturase cDNA from borage (*Borago officinalis*) have been used in the functional characterisation of the borage gene (Sayanova *et al.*, 1997).

4.1.4. Functional characterisation in yeast

By far the most popular expression system for the functional characterisation of a putative fatty acyl desaturase genes is the use of yeast transfected with the gene of

interest. Most yeast are incapable of Δ5 and Δ6 desaturation but contain all the cell components necessary for fatty acid desaturation. However, yeast have many other benefits, the cells are simple to harvest, easy to transfect and do not require sterile conditions in which to flourish, unlike animal cell line characterisation. The yeast are transformed with a vector containing the gene of interest, grown in media supplemented with the appropriate fatty acid substrates for a period of approximately 48 hours and then the cells harvested and the fatty acids extracted and analysed. The most commonly used yeast in this process is *Saccharomyces cerevisiae.*

Thus, the Δ6 fatty acyl desaturase genes of *C. elegans* (Napier *et al.*, 1998) and of the rat (Aki *et al.*, 1999) have been characterised in *Saccharomyces cerevisiae* as have the Δ5 desaturases of *Mortierella alpina*, an unusual filamentous fungi that is capable of *de novo* production of arachidonic acid (Michaelson *et al.*, 1998), and the nematode, *C. elegans* (Michaelson *et al.*, 1998, Watts and Browse 1999).

4.2 Material and Methods

Cloning into the pYes2 Vector

The fish putative fatty acid desaturase sequences were cloned into the pYes2 vector as described below. The primers used in the PCR for each putative desaturase clone functionally characterised are listed in Table 9 with the actual primer sequences listed in appendix III. The forward primer contains the Kozak nucleotide sequence, ANN (Kozak, 1990) required for the transcription of the gene and also contains an enzyme restriction site. The reverse primer contains a stop codon and another restriction site. The primers were used to isolate the gene of interest from the appropriate cDNA using *Pfu*, a proof reading enzyme, in order to reduce the number of base errors inserted through the PCR process. Using *Pfu* DNA polymersase (Promega Corp., Madison, U.S.A.) from *Pyrococcus furiosus* reduced the error rate

by 10% over *Taq* polymerase (Lundberg *et al.*, 2003; Andre, *et al.*, 1997). The following constituents were mixed in a 0.2ml thin walled PCR tube; 1 μl cDNA, (zebrafish, carp or salmon), 1 μl (10 pmol) appropriate forward primer (appendix III), 1 μl (10pmol) appropriate reverse primer (appendix III), 0.4 μl (4 mM) of dNTPs, 1 μl *Pfu* DNA polymerase, 2 μl of the corresponding buffer (200 mM Tris-HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 20 mM of $MgCl_2$, 1.0% Triton® X-100, 1mg/ml nuclease-free BSA) and 14 μl of H_2O, making a total volume of 20 μl. The tubes were placed into a thermal cycler (PTC-100 MJ Research, MA, U.S.A.) and run under the following conditions:

1 cycle: 95°C, 60 seconds,

25 cycles: 95°C, 30 seconds,

50 - 60°C, 30 seconds,

72°C, 2 minutes,

Clone	*Forward Primer (restriction enzyme)*	*Reverse Primer (restriction enzyme)*
ZfishD6	ZF2AHindIII (HindIII)	ZFRevXhoI (XhoI)
CppYesA5	ZF2AHindIII (HindIII)	CpRevNotI (NotI)
CppYesA7	ZF2AHindIII (HindIII)	CpRevNotI (NotI)
CppYesA9	ZF2AHindIII (HindIII)	CpRevNotI (NotI)
SaplA9	SalpYesFor(2) (HindIII)	SalpYesRev(2) (XhoI)
SalpYesB1	SalpYesFor(2) (HindIII)	SalpYesRev(2) (XhoI)

Table 9: List of primers used for each clone characterised and name of restriction site used in each.

Once complete the reaction mixture was run on an agarose gel (section 2.2.5.) and the band selected, excised and purified (appendix II). This was then cloned into pYes2 colony screened and the positive clones sequenced (Chapter 4). Once cloned

into the vector, the new plasmids were transformed into yeast *Saccharomyces cerevisiae* (section 2.4.2.2.). The modified yeast was then grown under optimal conditions, the expression of the vector induced with galactose and the appropriate desaturase fatty acid substrates added. The following substrates were added: 18:2*n*-6 and 18:3*n*-3 as substrates for Δ6 desaturase activity, 20:3*n*-6 and 20:4*n*-3 as substrates for Δ5 activity, 22:4*n*-6 and 22:5*n*-3 to test for Δ4 activity and 24:4*n*-6 and 24:5*n*-3 as substrates for Δ6* activity. Once grown for 48 hours under the above conditions, total lipids were extracted, fatty acid methyl esters prepared and then separated and analysed by GC and GC-MS analyses (section 2.4.4.).

4.3. Results

4.3.1. Zebrafish (*Danio rerio*)

4.3.1.1. Substrate specificity

Only one putative desaturase cDNA from zebrafish was functionally characterised, ZfishD6. The cDNA was characterised by determining the fatty acid profiles of transformed *S. cerevisae* containing either the pYES vector alone or the vector plus the zebrafish cDNA insert (ZfishD6). Both were tested for Δ6, Δ5, Δ4 or Δ6* desaturase activity as stated above. The fatty acid composition of the yeast transformed with the vector alone showed the four main fatty acids normally found in *S. cerevisae* namely 16:0, 16:1*n*-7, 18:0 and 18:1*n*-9 together with the four exogenously derived fatty acids (Figure 16). This result is consistent with the fact that *S. cerevisae* do not normally express Δ5 or Δ6 fatty acid desaturase activities. Additional peaks were obtained in the profiles of ZfishD6-transformed yeast grown in the presence of the Δ6 desaturase substrate fatty acids, 18:2*n*-6 and 18:3*n*-3 and also in the profiles of ZfishD6 transformed yeast grown in the presence of the Δ5

desaturase substrate fatty acids 20:3*n*-6 and 20:4*n*-3. (Figure 17 A-D). Based on the GC retention times, the additional peaks associated with the presence of the zebrafish cDNA, indicated in Figure 17 A-D, were identified as 18:3*n*-6, 18:4*n*-3, 20:4*n*-6 and 20:5*n*-3, respectively and confirmed by GC-MS of the methyl esters.

EI GC-MS of fatty acid picolinyl esters were used to unequivocally determine the structures represented by the additional PUFA peaks produced in yeast cultures containing ZfishD6. The samples all showed prominent ions at *m/z* = 92, 108, 151, and 164, which are characteristic of picolinyl esters representing fragments about the pyridine ring (Figure 18). The EI spectra of the additional fatty acid in yeast containing zebrafish Δ6 and cultured in the presence of 18:2*n*-6 showed a fragmentation pattern with a mass ion of 369 *m/z* and prominent peaks at 354, 340, 326, 312, 298, 272, 258, 232, 218 and 192 *m/z* (Figure 18A). The initial interval of 15 (369-354) represented the terminal methyl and was followed by four intervals of 14, indicating four methylene groups. The intervals of 26 (298-272, 258-232, and 218-192) denoted the positions of three double bonds, indicating that this fatty acid is $^{\Delta 12,9,6}$18:3 = 18:3*n*-6. The EI spectra of the additional fatty acid from cells incubated with 18:3*n*-3 showed a mass ion of 367 *m/z* and fragments at 338, 312, 298, 272, 258, 232, 218 and 192 m/z confirming this fatty acid as $^{\Delta 15,12,9,6}$18:4 = 18:4*n*-3 (Figure 18B). The EI spectra of the additional fatty acid produced in cells incubated with 20:3*n*-6 showed a fragmentation pattern with a mass ion of 395 *m/z* and prominent ions at intervals of 26 (324-298, 284-258, 244-218, and 204-178 *m/z*), denoting the position of four double bonds and indicating that this fatty acid is $^{\Delta 14,11,8,5}$20:4 = 20:4*n*-6 (Figure 18C). Similarly the EI spectra of the additional fatty acid in cells incubated with 20:4*n*-3 showed a fragmentation pattern with a mass ion of 393 m/z with prominent ions at intervals of 26 (364-338, 324-298, 284-258, 244-

218, and 204-178 *m/z*), confirming that this fatty acid is $^{\Delta 17,14,11,8,5}$20:5 = 20:5*n*-3 (Figure 18D). The GC-MS data of fatty acid picolinyl esters unequivocally confirmed that the zebrafish clone, ZfishD6, is a fatty acid desaturase that introduces double bonds into 18:2*n*-6 and 18:3*n*-3 at the Δ6 position and also into 20:3*n*-6 and 20:4*n*-3 at the Δ5 position.

The zebrafish cDNA was also tested for both Δ4 and Δ6* desaturase activities. The zebrafish cDNA did not desaturate either of the Δ4 desaturase substrate fatty acids, 22:4*n*-6 and 22:5*n*-3, as indicated in Figure 17 E and F. However, Figure 17 G and H shows small additional peaks with GC retention times corresponding to those for 24:6*n*-3 and 24:5*n*-6 where the fatty acid products of desaturation of 24:5*n*-3 and 24:4*n*-6 GC retention times should run. The additional peaks were too small to be regarded as conclusive evidence that the zebrafish cDNA expressed Δ6* activity and therefore the FAME were analysed by GC-MS. The results of the GC-MS of FAME gave a mass that was consistent with of the products of Δ6* activity, 24:6*n*-3 and 24:5*n*-6 (Figure 19). However, the peaks were too small to give sufficient material for GC-MS of picolinyl esters due to losses during the conversion of FAME to picolinyl esters.

4.3.1.2. Percentage conversion of substrate fatty acids

The amount of fatty acid substrate converted to desaturated product varied depending both on chain length and on position of the first double bond i.e. *n*-3 versus *n*-6. Figure 20 plots the percentage of total exogenously added substrate fatty acid that was recovered as a desaturated product by the transformed *S. cerevisae* containing the zebrafish cDNA.

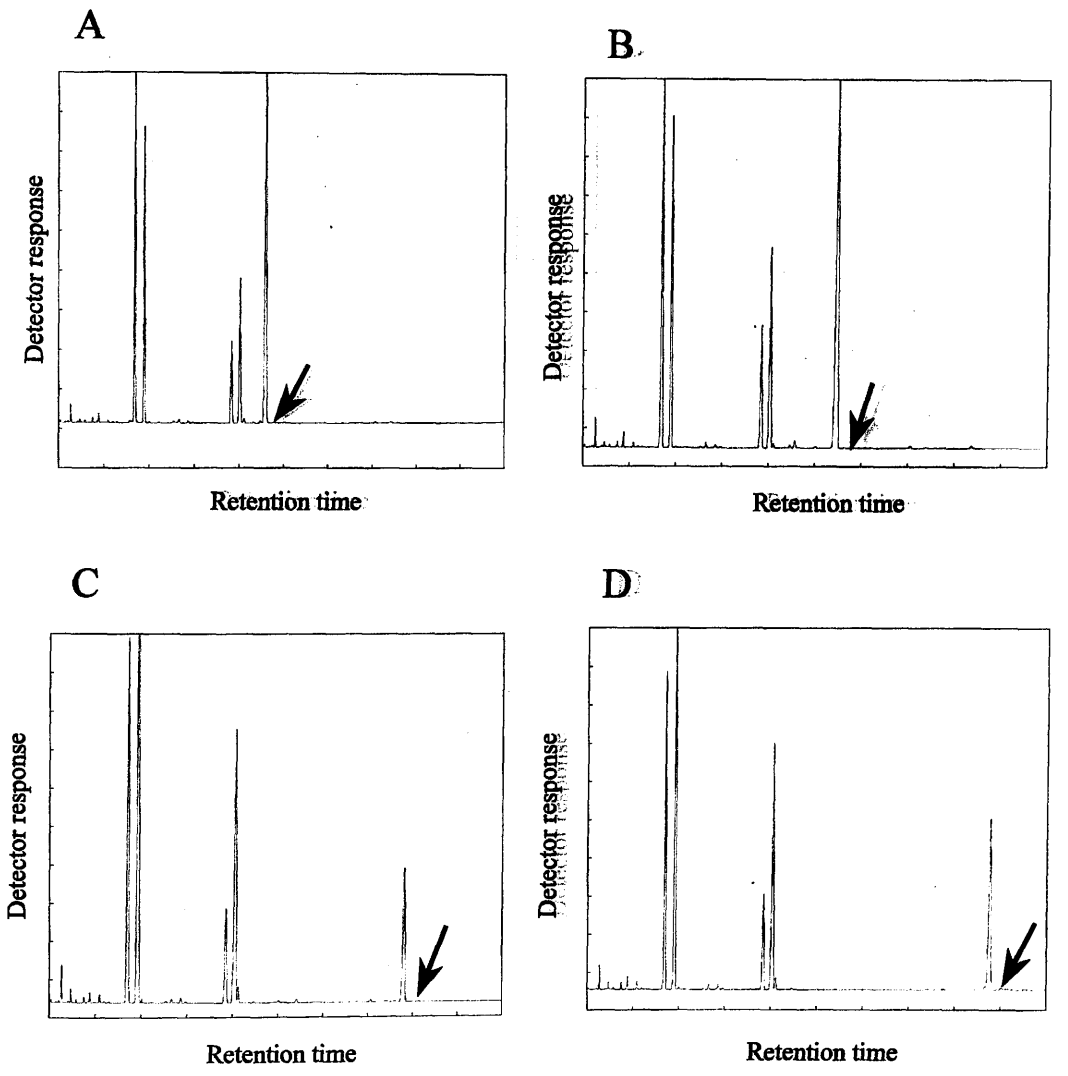


Figure 16: Identification of fatty acid desaturation products in transgenic yeast. FAMEs were extracted from yeast transformed with pYES2 vector (control) grown in the presence of 18:2*n*-6 (A), 18:3*n*-3 (B), 20:3*n*-6 (C) and 20:4*n*-3 (D). The black arrows in each panel point where peaks 18:3*n*-6 (A), 18:4*n*-3 (B), 20:4*n*-6 (C) and 20:5*n*-3 (D) should be but are missing.

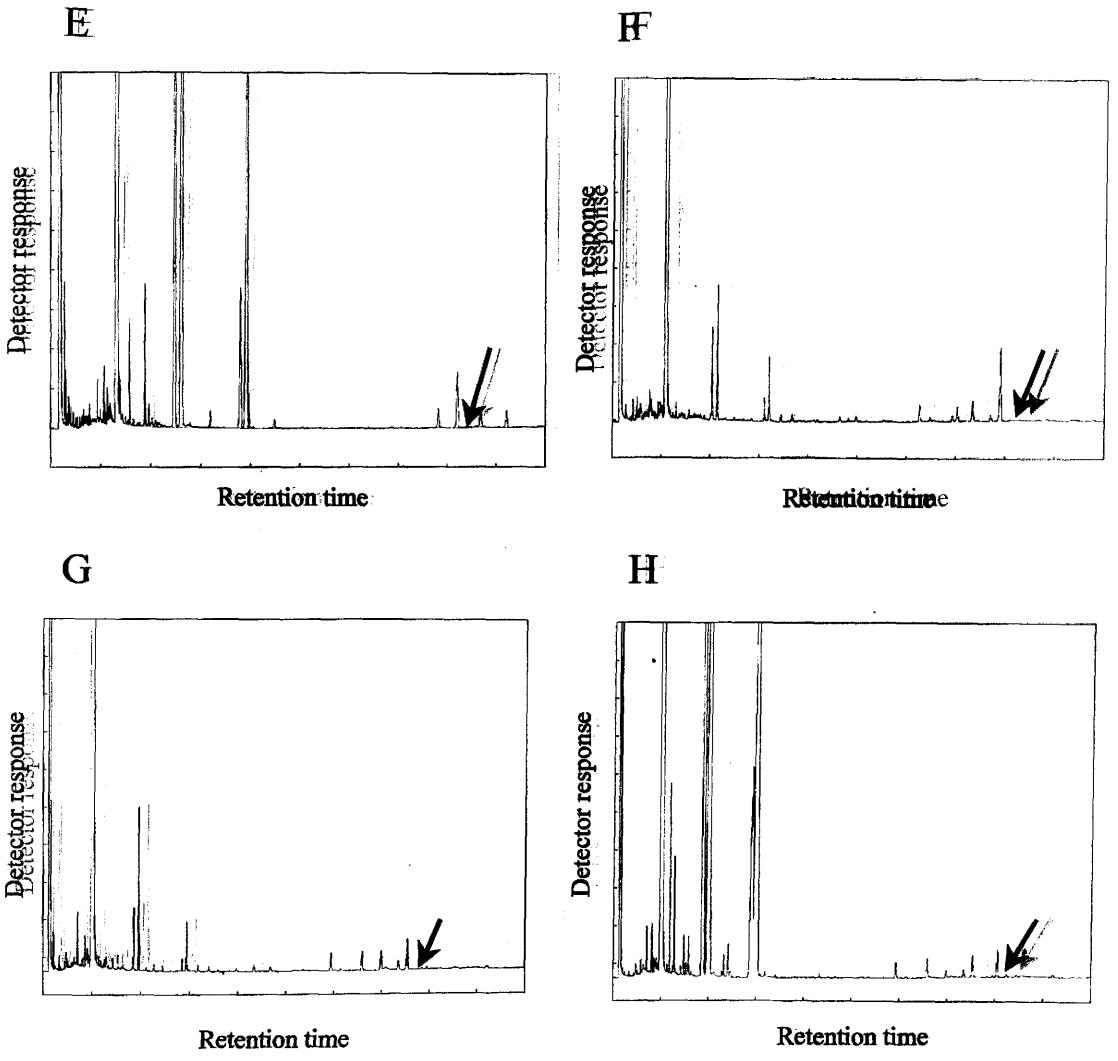


Figure 16: Identification of fatty acid desaturation products in transgenic yeast. FAMEs were extracted from yeast transformed with pYES2 (control) grown in the presence of 22:4*n*-6 (E), 22:5*n*-3 (F), 24:4*n*-6 (G) and 22:5*n*-3 (H). The black arrows in each panel point to possible peaks of 22:5*n*-6 (E), 22:6*n*-3 (F), 24:5*n*-6(G) and 24:6*n*-3 (H).

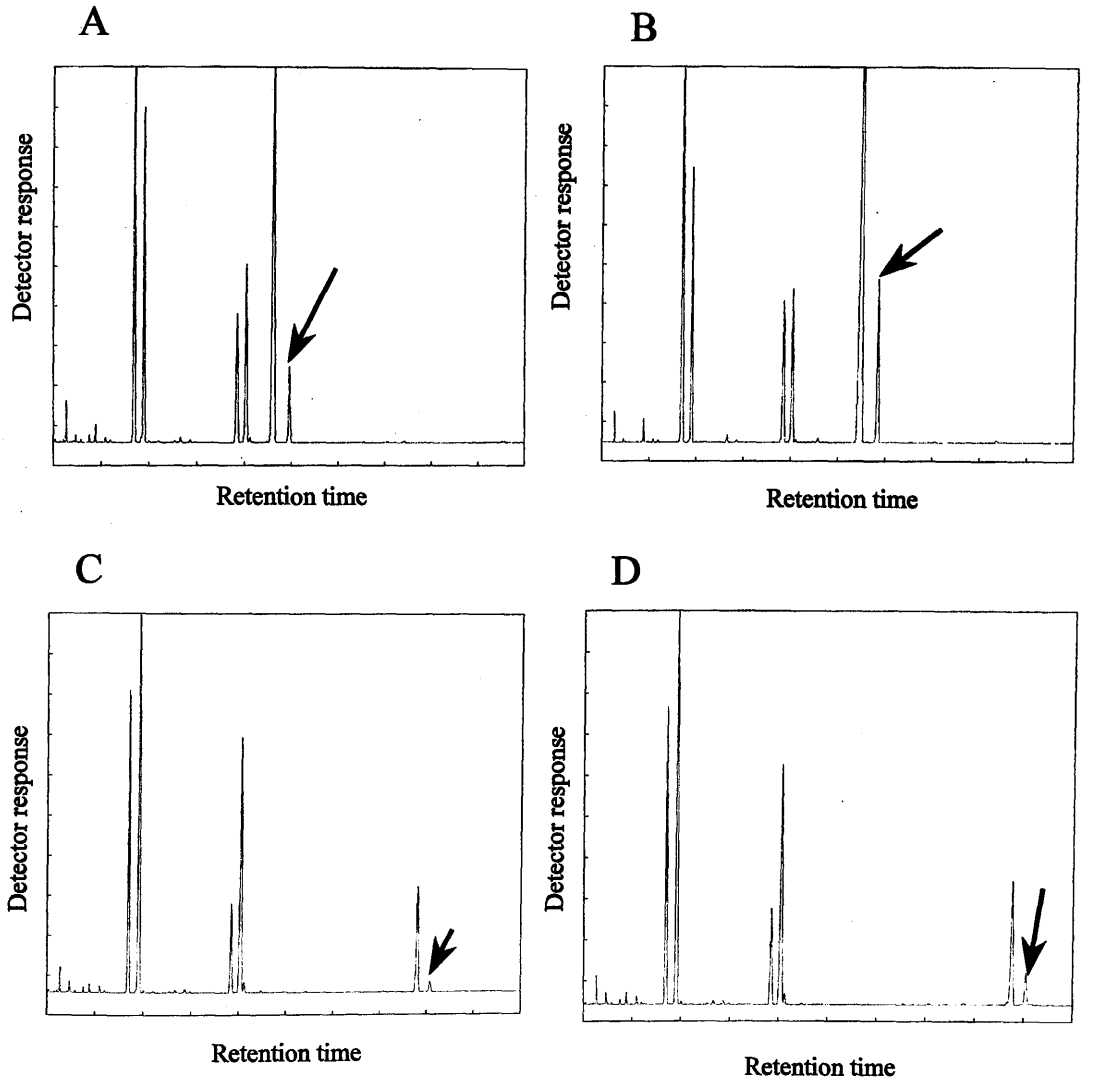


Figure 17: Identification of fatty acid desaturation products in transgenic yeast. FAMEs were extracted from yeast transformed with ZfishD6 grown in the presence of 18:2*n*-6 (A), 18:3*n*-3 (B), 20:3*n*-6 (C) and 20:4*n*-3 (D). The black arrows in each panel point to 18:3*n*-6 (A), 18:4*n*-3 (B), 20:4*n*-6 (C) and 20:5*n*-3 (D).

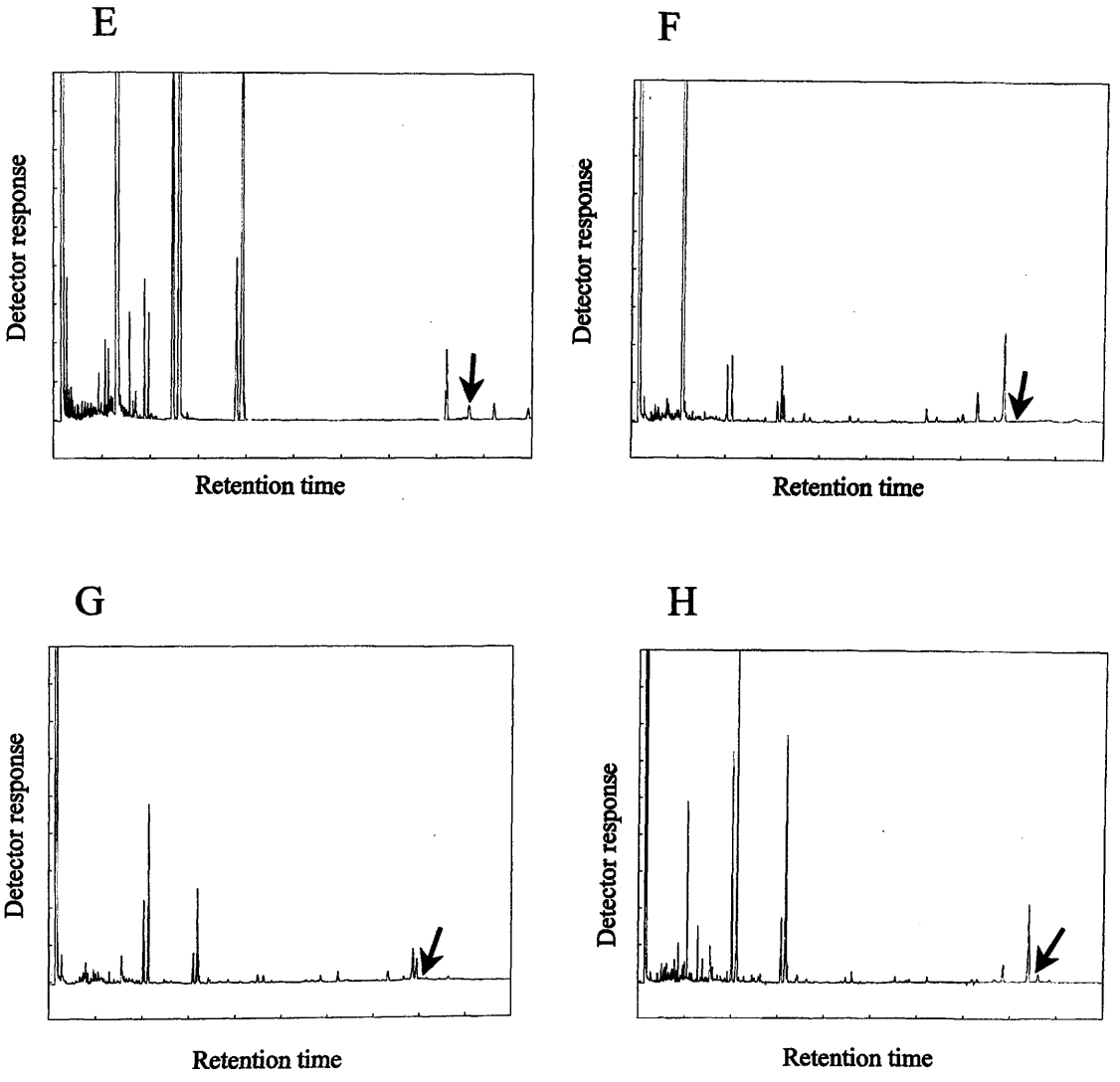
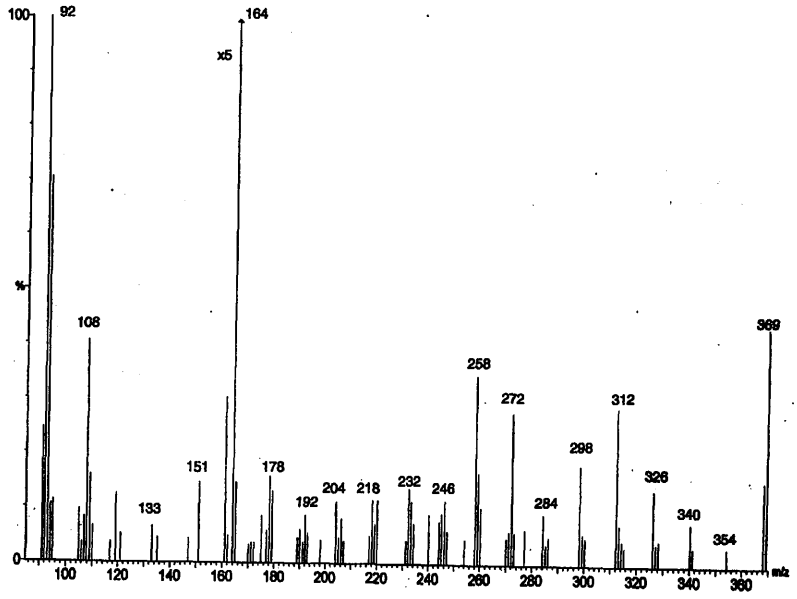


Figure 17: Identification of fatty acid desaturation products in transgenic yeast. FAMEs were extracted from yeast transformed with ZfishD6 grown in the presence of 22:4*n*-6 (E), 22:5*n*-3 (F), 24:4*n*-6 (G) and 24:5*n*-3 (H). The black arrows in each panel point to possible peaks of 22:5*n*-6 (E), 22:6*n*-3 (F), 24:5*n*-6(G) and 24:6*n*-3 (H).

A



B

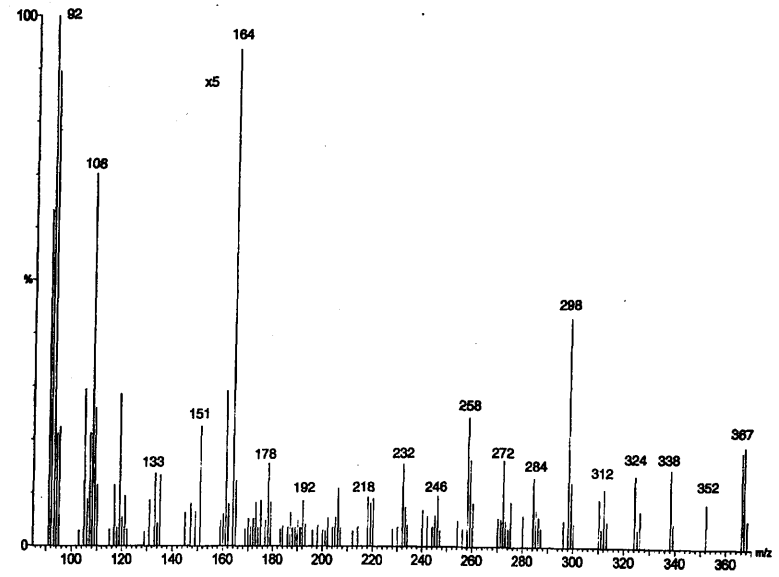


Figure 18: Mass spectra of the arrowed peaks in Fig. 17, 21 and 23 A-B. Picolinyl esters were prepared from FAMEs extracted from yeast transformed with ZfishD6, CppYesA9 and SalpYesB1 grown in the presence of 18:2*n*-6 (A) and 18:3*n*-3 (B) and analysed by GC-MS as described in chapter 2. The identities of the peaks were confirmed as 18:3*n*-6 (A) and 18:4*n*-3 (B).

Figure 18: Mass spectra of the arrowed peaks in Fig. 17, 21 and 23 D-E. Picolinyl esters were prepared from FAMEs extracted from yeast transformed with ZfishD6, CppYesA9 and SalpYesA10 grown in the presence of 20:3*n*-6 (C) and 20:4*n*-3 (D) and analysed by GC-MS as described in chapter 2. The identities of the peaks were confirmed as 20:4*n*-6(A) and 20:5*n*-3 (B).

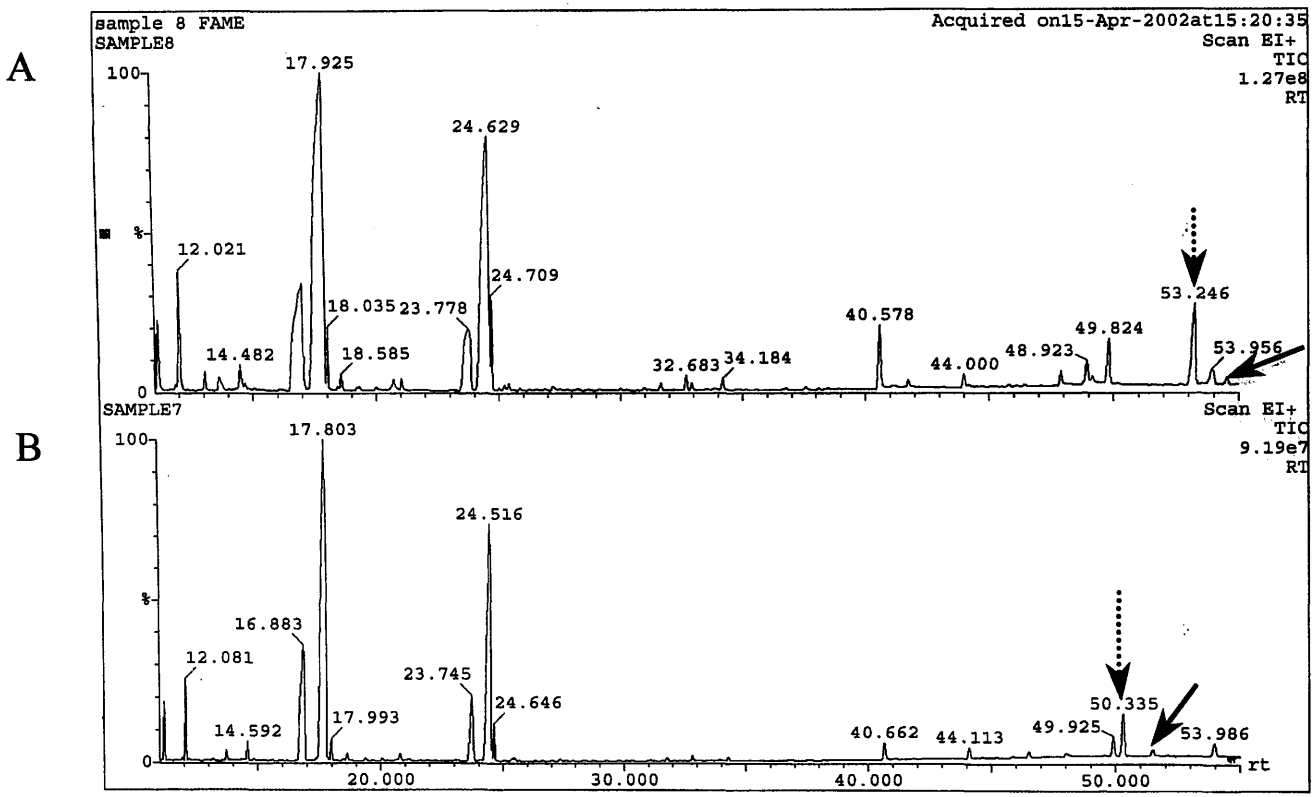


Figure 19: GC-MS of FAMEs extracted from transgenic yeast. The yeast were transformed with SalpYesA10 or ZfishD6 and grown in the presence of 24:5*n*-3 (A) and 24:4*n*-6(B) (dashed arrows). The identities of the peaks were confirmed by mass as that of 24:6*n*-3 and 24:5*n*-6 (solid arrows).

Figure 20: Percentage conversion of fatty acid substrate into desaturated product by yeast transformed with ZfishD6 vector.

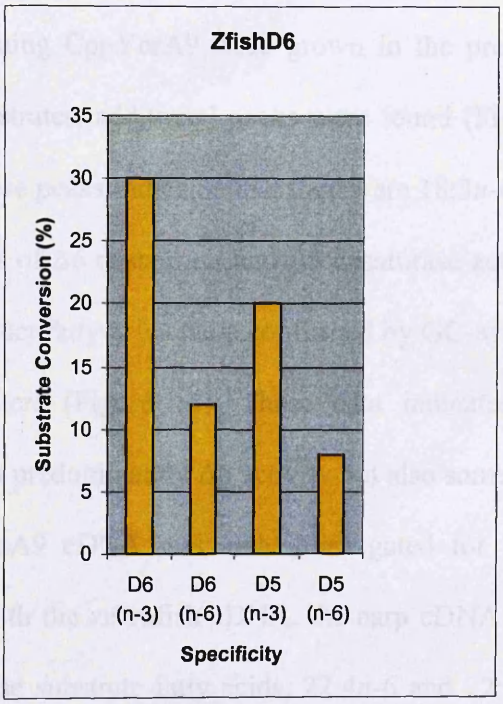


Figure 20 indicates that the zebrafish cDNA coded for a product which possesses both Δ5 and Δ6 activity, however, the enzyme has a greater ability to desaturate at the Δ6 position than at the Δ5 position. Figure 20 also shows that the resulting enzyme has a greater affinity and ability to desaturate *n*-3 fatty acids over *n*-6 fatty acids with approximately 30% of the *n*-3 Δ6 substrate and 20% of the *n*-3 Δ5 substrate being desaturated compared to only 12% of *n*-6 Δ6 and 8% of *n*-6 Δ5 substrates.

4.3.2. Common carp (*Cyprinus carpio*)

4.3.2.1. Substrate specificity

Three cDNAs were isolated from carp, CppYesA5, CppYesA7 and CppYesA9. Two, CppYesA5 and CppYesA7, when transformed into *S. cerevisae* and grown in the presence of Δ6, Δ5, Δ4, or Δ6* desaturase substrates conferred protein products that

were non-functional as desaturase enzymes, with no additional peaks present in the respective GC traces, irrespective of substrate fatty acid. However, when transformed *S. cerevisae* cells containing CppYesA9 were grown in the presence of the same desaturase fatty acid substrates, additional peaks were found (Figure 21 A-D). The GC retention times of these peaks indicated that they were 18:3*n*-6, 18:4*n*-3, 20:4*n*-6 and 20:5*n*-3, the products of Δ6 desaturase and Δ5 desaturase activity, respectively. The identities of the product fatty acids were confirmed by GC-MS of FAME and EI GC-MS of picolinyl esters (Figure 18). These data indicated that CppYesA9 conferred an enzyme with predominantly Δ6 activity but also some Δ5 activity.

The carp CPpYesA9 cDNA was also investigated for both Δ4 and Δ6* desaturase activity. As with the zebrafish cDNA, the carp cDNA did not desaturate either of the Δ4 desaturase substrate fatty acids, 22:4*n*-6 and 22:5*n*-3 (Figure 21 E and F). However, Figure 21 G and H show small additional peaks where the desaturated fatty acid products GC retention times should run. The additional peaks were too small, once again, to be regarded as conclusive evidence that the carp cDNA has Δ6* activity. As before with the zebrafish enzyme, it was impossible to generate picolinyl esters from such small peaks as the losses in the chemical reactions were too great. However, the GC-MS of the methyl esters gave mass ion results that indicated that the additional peaks were not desaturated products and they were therefore regarded as artefacts.

4.3.2.2. Percentage conversion of substrate fatty acids

The percentage conversion was calculated for each fatty acid substrate and the results plotted in Figure 22. It can be noted that the carp cDNA encoded an enzyme similar to that of the zebrafish, in as much as it was capable of both Δ6 and

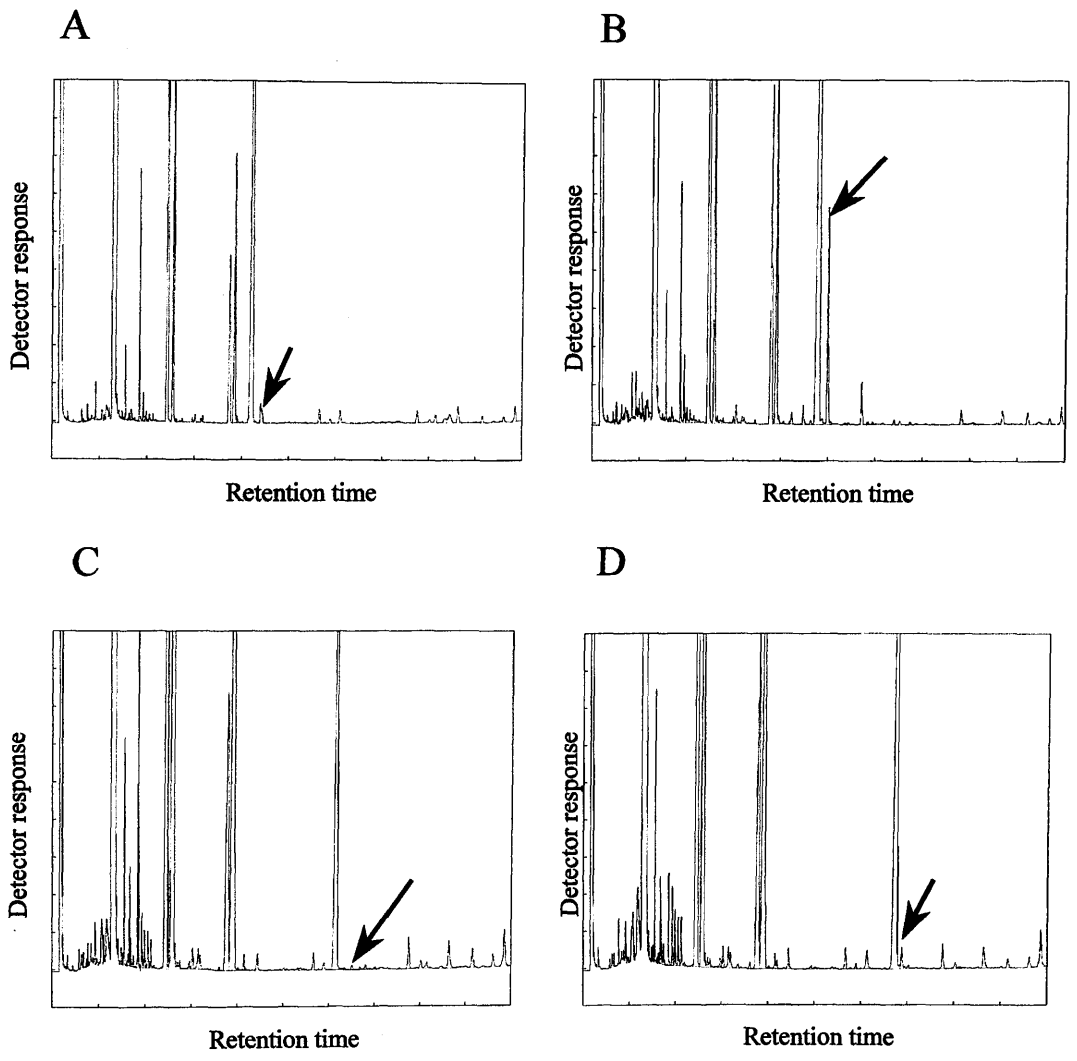


Figure 21: Identification of fatty acid desaturation products in transgenic yeast. FAMEs were extracted from yeast transformed with CPpYesA9 grown in the presence of 18:2*n*-6 (A), 18:3*n*-3 (B), 20:3*n*-6 (C) and 20:4*n*-3 (D). The black arrows in each panel point to 18:3*n*-6 (A), 18:4*n*-3 (B), 20:4*n*-6 (C) and 20:5*n*-3 (D).

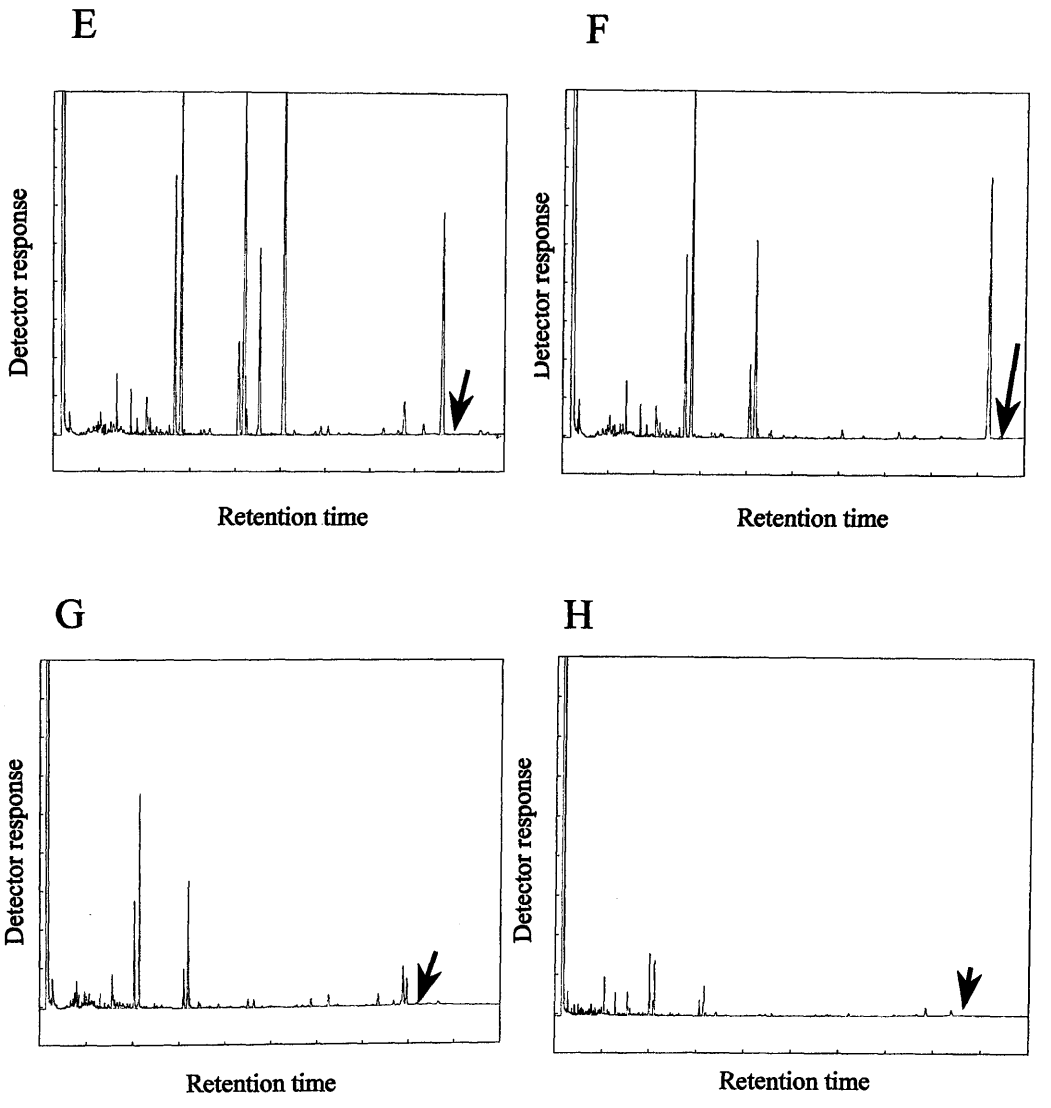


Figure 21: Identification of fatty acid desaturation products in transgenic yeast. FAMEs were extracted from yeast transformed with CppYesA9 grown in the presence of 22:4*n*-6 (E), 22:5*n*-3 (F), 24:4*n*-6 (G) and 24:5*n*-3 (H). The black arrows in each panel point to possible peaks of 22:5*n*-6 (E), 22:6*n*-3 (F), 24:5*n*-6 (G) and 24:6*n*-3 (H).

Δ5 activity. However, it's capacity to desaturate all substrates was considerably limited compared to the zebrafish cDNA product.

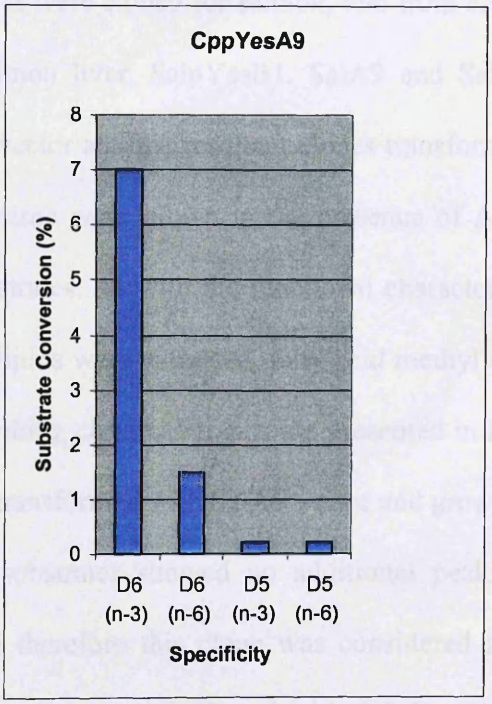


Figure 22: Percentage conversion of fatty acid substrate into desaturated product by yeast transformed with CppYesA9 vector.

Although the pattern of specificity was similar to the zebrafish, the much lower activity meant that the carp cDNA produced a product that only showed physiologically significant amounts of Δ6 activity and only towards the *n*-3 fatty acid substrate, 18:3*n*-3, with a percentage conversion of 7%. Only 1.5% of 18:2*n*-6 was desaturated to 18:3*n*-6 and less than 0.5% of both Δ5 substrates were converted into the appropriate desaturated products, 20:4*n*-6 and 20:5*n*-3, respectively. Thus, whereas the zebrafish cDNA product could be classed as a bifunctional enzyme the carp cDNA product was more a Δ6 with possibly just residual Δ5 activity.

4.3.3. Atlantic salmon (*Salmo salar*)

4.3.3.1. Substrate specificity

As with carp, three cDNAs were cloned for salmon, two from cell lines, SalA10 and SalA9 and one from salmon liver, SalpYesB1. SalA9 and SalpYesB1 were both inserted into the pYES2 vector and the resultant clones transformed into yeast cells. The transformed *S. cerevisae* were grown in the presence of Δ6, Δ5, Δ4 and Δ6* desaturase fatty acid substrates. As with the functional characterisation of the other fish species cDNAs, the lipids were extracted, fatty acid methyl esters produced and analysed by GC. The resulting chromatographs are presented in Figure 23 A-H. The GC traces for yeast cells transformed with SalA9 vector and grown in the presence of Δ5 and Δ6 fatty acids substrates showed no additional peaks corresponding to desaturated products and therefore this clone was considered non-functional as a desaturase gene. However, in yeast transfected with SalpYesB1, and grown in the presence of the Δ5 and Δ6 fatty acid substrates, additional peaks were observed in the GC traces (Figure 23 A-D). The retention times of these additional peaks were consistent with the fatty acids products of Δ5 and Δ6 desaturase activities. EI GC-MS of the picolinyl esters positively identified the structures represented by the additional PUFA peaks produced in cultures containing SalpYesB1 and incubated with Δ5 and Δ6 substrates as appropriately desaturated products. The SalpYesB1 clone was also tested for Δ4 activity but no actvitiy was found (Figure 23 E-F). As for Δ6* activity, when the methyl esters were subjected to GC-MS it was discovered that the additional peaks, as shown in Figure 23 G-H, had the correct mass ion to be the products of Δ6* activity, suggesting that the SalpYesB1 clone was capable of producing 24:5*n*-6 and 24:6*n*-3 from 24:4*n*-6 and 24:5*n*-3, respectively (Figure 19). However, there was insufficient desaturated product to allow the structures to be

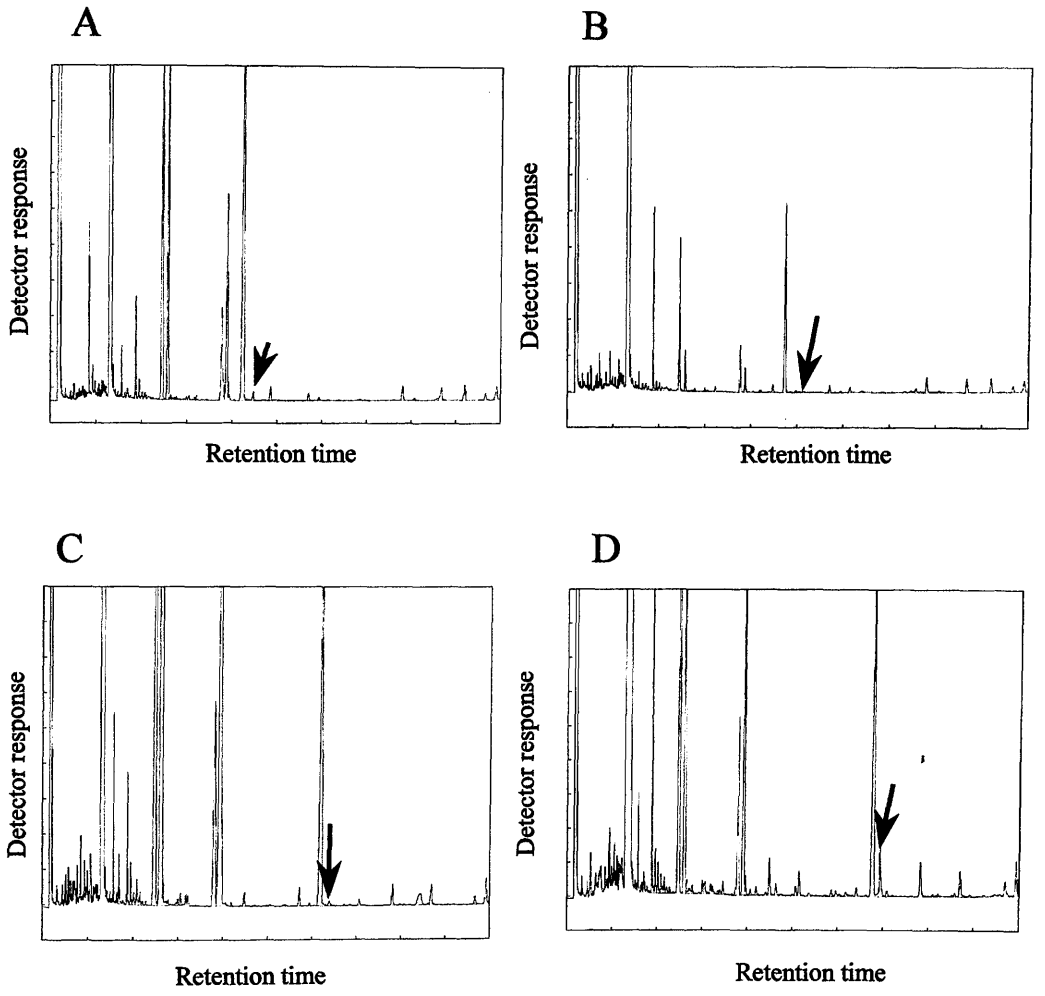


Figure 23: Identification of fatty acid desaturation products in transgenic yeast. FAMEs were extracted from yeast transformed with SalpYesB1 grown in the presence of 18:2*n*-6 (A), 18:3*n*-3 (B), 20:3*n*-6 (C) and 20:4*n*-3 (D). The black arrows in each panel point to 18:3*n*-6 (A), 18:4*n*-3 (B), 20:4*n*-6 (C) and 20:5*n*-3 (D).

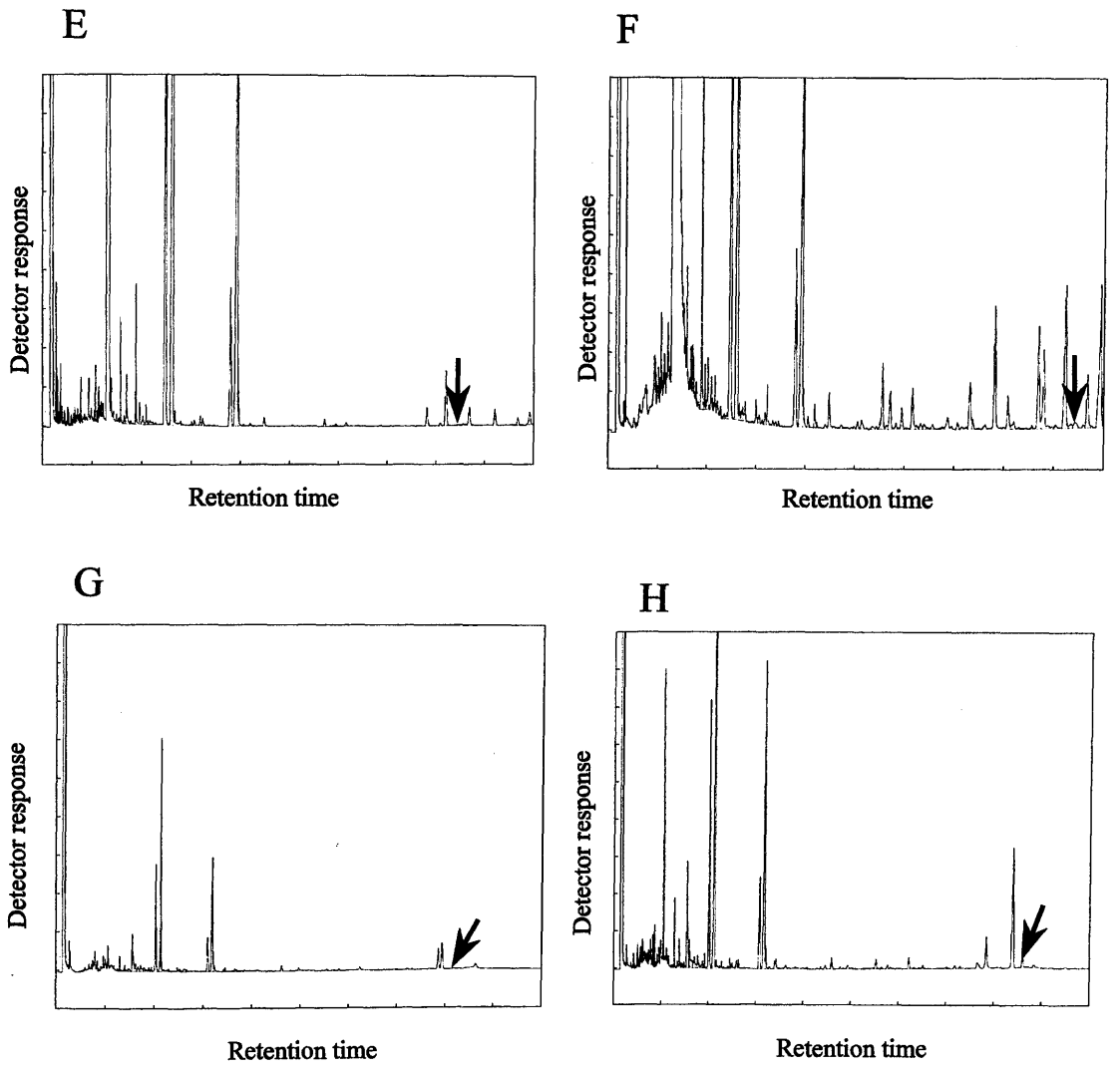


Figure 23: Identification of fatty acid desaturation products in transgenic yeast. FAMEs were extracted from yeast transformed with SalpYesB1 grown in the presence of 22:4*n*-6 (E), 22:5*n*-3 (F), 24:4*n*-6 (G) and 24:5*n*-3 (H). The black arrows in each panel point to possible peaks of 22:5*n*-6 (E), 22:6*n*-3 (F), 24:5*n*-6 (G) and 24:6*n*-3 (H).

further characterised by EI GC-MS of the picolinyl esters for absolute conformation of the double bond positions.

4.3.3.2. Percentage conversion of substrate fatty acids

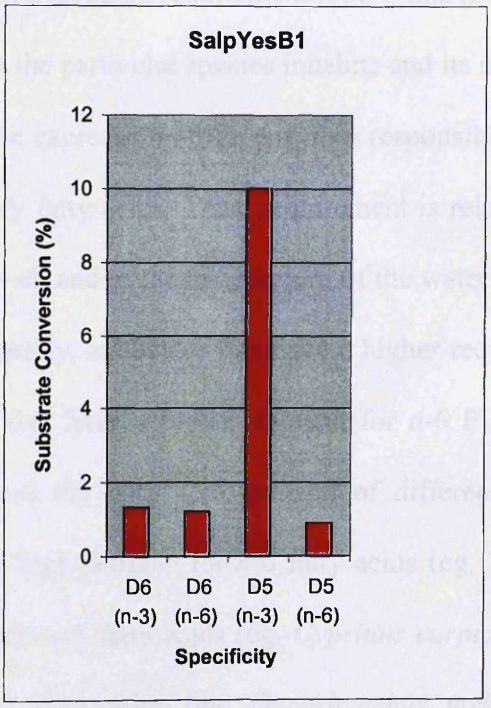


Figure 24: Percentage conversion of fatty acid substrate into desaturated product by yeast transformed with SalpYes1 vector.

The Atlantic salmon cDNA SalpYesB1 encoded an enzyme capable of desaturating fatty acids at the Δ5 and Δ6 positions including Δ6* activity (Δ6 activity towards C_{24} substrates). However, as with the carp cDNA, the salmon confers a desaturase with a narrower specificity compared to the zebrafish cDNA. The salmon clone is very specific in that it only desaturates 20:4*n*-3 to any real degree. This is shown in Figure 24 where the conversion ratio of 20:4*n*-3 to 20:5*n*-3 is approximately 10% compared to the other Δ5 and Δ6 substrates which have conversion ratios of less than 2%. To date, no cDNA has been isolated from any other fish species which has conferred a product with predominantly Δ5 desaturase activity.

4.4. Discussion

What type of desaturase activity is expected to be present in the different fish species?

There is a species dependent requirement for *n*-3 and *n*-6 PUFA that is related to both the environment the particular species inhabits and its dietary niche. It is also likely to be related to the expression of the enzymes responsible for the desaturation and elongation of dietary fatty acids. Thus, requirement is related to the position of the species in the food web and to the temperature of the water in which the fish live (Watanabe, 1982). Generally, coldwater fish have a higher requirement for *n*-3 fatty acids and warm water fish have a higher demand for n-6 PUFA (Meinelt, 2000). Takeuchi (1996) grouped the fatty acid demand of different fish species into 3 categories: species with high demand for *n*-6 fatty acids (eg. *Tilapia*); species with equal demand for *n*-3 and *n*-6 fatty acids (eg. *Cyprinus carpio*): and species with a higher demand for *n*-3 fatty acids (eg. *Oncorhynchus mykiss*). Carnivorous or piscivorous fish can obtain all their essential fatty acid requirements by feeding on other fish and are, therefore, characterised by a poor ability to biosynthesise HUFA. Omnivorous fish species obtain their dietary fatty acids from the range of aquatic insects and animal plankton they consume and can survive on vegetable/plant material alone as they appear able to biosynthesise HUFA in sufficient quantities to survive (Sargent *et al.*, 1989, Henderson and Tocher, 1987).

Zebrafish, a warm freshwater, omnivorous fish have been placed into Takeuchi's group 1, demanding a high level of *n*-6 PUFA. Studies with zebrafish hepatocytes have shown that they possess all the necessary enzymes to desaturate and elongate C_{18} PUFA into HUFA (Tocher *et al.*, 2002). Therefore, the zebrafish should contain the cDNAs which encode all the enzymes required.

The common carp, *Cyprinus carpio*, is a freshwater species found in the warmer waters of Southern European countries and in the cooler waters of Britain as an introduced species. The EFA requirements of common carp can be met by C_{18} PUFA alone (Watanabe, 1982) and are determined as 1% each of 18:2*n*-6 and 18:3*n*-3 (Takeuchi and Watanabe 1977). This means that carp are capable of desaturating and elongating the C_{18} PUFA to HUFA as demonstrated biochemically by experiments with established carp cell lines, EPC (Tocher and Dick, 1999).

Unlike the previous two species, the Atlantic salmon is an anadromous fish species living in the colder waters of the Northern hemisphere. As an anadromous fish species they spend part of their life in freshwater and part in saltwater and as such their dietary requirements may change throughout their life. Studies with an Atlantic salmon cell line (AS) have shown that salmon cells possess both Δ6 and Δ5 desaturase activities and therefore must express the enzymes which confer this activity (Tocher and Dick, 1990). However, dietary studies with live fish indicate that their ability to synthesise HUFA may depend on what stage they are in their life cycle. It has been shown that as parr they are capable of elongating and desaturating C_{18} PUFA to C_{20} and C_{22} HUFA (Bell *et al.*, 1997). It has been suggested that after they undergo smoltification, the post-smolt becomes unable to desaturate and elongate PUFA (Ackman and Takeuchi, 1986). This may not be wholly correct but it is likely that these activities are not highly expressed during the post-smolt seawater phase of its life cycle (Bell *et al.*, 1989).

Turbot, however, are a marine species which inhabit the warmer waters of the Mediterranean to colder waters of the Northern hemisphere. They are carnivorous/piscivorous by nature, and early studies suggested they were unable to produce HUFA readily (Owen *et al.*, 1975). However, as mentioned previously, there

is some debate as to where the block in the HUFA biosynthesis pathway occurs. Turbot have a very active Δ6 desaturase and also produce DHA from EPA whereas Δ5 desaturase appears to be deficient (Bell *et al.*, 1995). However, more recent data on turbot cells are more consistent with a deficiency in the C_{18} to C_{20} elongase multi-enzyme complex rather than a deficiency in Δ5 desaturase (Ghioni *et al.*, 1999).

Therefore, all the fish species choosen for this study are known to have some type of desaturase activity. However, until now the cDNAs for these genes have not been isolated and therefore the functions never tested.

What type of desaturase activity was observed in the cDNAs cloned?

Zebrafish

The most immediately outstanding result was that of the zebrafish, *Danio rerio*, as not only did this cDNA encode a desaturase with high levels of activity, but more importantly it had two activities, being capable of desaturating both *n*-3 and *n*-6 fatty acids at both Δ6 and Δ5 positions. This dual Δ6/5 desaturase activity had never been observed or reported before in any desaturase from any other organism. Human, Rat, mouse, the nematode, *C. elegans* and even the filamentous fungi, *M. alpina*, all have single function genes, one cDNA which encodes a Δ6 desaturase and another that encodes a Δ5 desaturase. However, single genes that encode an enzyme with multiple functions do exist. For example a bifunctional Δ6-fatty acyl acetylenase/desaturase has been isolated from the moss *Ceratodon purpureus* (Sperling *et al.*, 2000). This bifunctional enzyme can introduce a Δ6-cis-double bond into 9,12(15)-C18-polyenoic acids as well as converting a Δ6-cis-double bond to a Δ6-triple bond. Moreover, when *C. alpina* acetylenase (Lee *et al.*, 1998) and the *Lesquerella. fendleri* hydroxylase (Broun *et al.*, 1998a) were expressed in yeast they showed Δ12-desaturase activity in addition to their acetylenase and hydroxylase

activities. In has been proposed that only four amino acid substitutions are required to convert a Δ12-desaturase into a Δ12 hydroxylase but it is more difficult to convert a Δ12 desaturase into an acetylenase (Broun *et al*, 1998b). For example the Δ12-oleate desaturase from *Arabidopsis thaliana* which has lost its chain length specificity as it can also desaturate palmitoleic acid (16:1*n*-7) as well as 18:1*n*-9 (Broun *et al.*, 1998b). The hydroxylase and acetylenase enzymes are desaturase-like enzymes containing three equivalent histidine clusters that have been implicated in iron binding and shown to be essential for catalysis. As mentioned above only four amino acid changes around the active site histidines are required to convert a strict desaturase into an enzyme that retains some desaturase activity but is also an efficient hydroxylase (Broun *et al.*, 1998b). This may be due to changes in active site geometry, for example the relative positioning of the substrate with respect to the iron centre, the coordination geometry of the iron atoms, or the active site hydrogen bonding network. In a similar way, it may be that a small number of amino acid changes are needed around the active site to change either a strictly Δ6 desaturase or a strictly Δ5 desaturase into one capable of both types of activity.

With the zebrafish clone the substrate conversion rates were greater for Δ6 desaturation of 18:3*n*-3 to 18:4*n*-3 and Δ5 desaturation of 20:4*n*-3 to 20:5*n*-3 compared to the conversion rates of the equivalent *n*-6 substrates. This result is entirely consistent with the result of biochemical fatty acid desaturation assay data reported in zebrafish hepatocytes where the main products of fatty acid desaturation were 18:4*n*-3 and 20:5*n*-3 (Tocher *et al.*, 2002). However, zebrafish as a tropical fish, possibly have a greater requirement for *n*-6 fatty acids, as observed in feeding trials (Meinelt *et al.*, 2000). So why this enzyme is better at desaturating *n*-3 fatty acids makes little evolutionary sense, but it seems to be a general feature of

desaturases. One possible explanation is that this is not the only desaturase cDNA and that there may be other cDNAs coding for a further Δ5/Δ6 enzyme or even separate Δ5 and Δ6 enzymes in zebrafish. This is a real possibility as the human Δ5 and Δ6 desaturase genes are situated close to each other on chromosome 11 and are thought to have occurred through genome duplication (Marquardt *et al.*, 2000). Genome duplication has occurred at least twice in zebrafish, the first was before the separation of mammals from fish and the second was after this separation (Ohno, 1970). Some organisms are also known to contain two cDNAs coding the same enzyme for example the cellular slime mould *Dictyostelium discoideum* has two Δ5 fatty acid desaturase genes (Saito *et al.*, 2000). The two cDNAs, Dd des5-1 and Dd des5-2, however, produce products that have different levels of desaturase activity, one much greater than the other. Dd des5-1 is capable of desaturating 16:0, 16:1*n*-9, 18:1*n*-9 and 18:1*n*-11 while Dd des5-2 can only desaturate 18:1*n*-9 and 18:1*n*-11. Furthermore, Dd des5-1 has approximately a 7-fold and a 3-fold greater conversion rate of 18:1*n*-9 and 18:1*n*-11 respectively, over Dd des5-2 (Saito *et al.*, 2000). However, these are a rather different enzymes to the Δ5 desaturases in vertebrates which desaturates very different fatty acid substrates.

Carp

Three cDNAs were functionally characterised for the common carp, *Cyprinus carpio*, two of which were found to be non-functional as desaturase genes and a third which was functional and capable of Δ6 desaturation, with residual Δ5 desaturase activity. However, the substrate conversion rate and therefore the level of desaturation was considerably lower in the functional carp desaturase gene than in the functional zebrafish gene. Furthermore, although CppyesA9 was capable of both Δ6 and Δ5 desaturation, the substrate conversion of Δ5 substrates was almost

insignificant compared to the conversion rates of the Δ6 substrates. This may just be due to the temperature at which the cDNA was expressed. The yeast containing the functional conscript were grown in media at 30°C (Tocher and Dick, 1999). Carp require equal concentrations of *n*-3 to *n*-6 fatty acids (Takeuchi and Watanabe, 1977), and they are known to be able to thrive on a diet containing C_{18} PUFA alone and so if this was the only gene conferring a functional desaturase product then the fish would have great difficulty in surviving on C_{18} PUFAs alone. However, since genome duplication has occurred in this species since the separation of fish from mammals, it is possible that they contain further genes encoding functional Δ5/Δ6 or separate Δ5 and Δ6 desaturases, as discussed with zebrafish. Why there are non-functional cDNAs in the genome is not clear. It may be that these are redundant copies of the gene caused by genome duplication. Genome duplication causes an increase in the number of gene copies, these "extra" copies often incur mutations causing them to become non-functional (see chapter 3 and 6 for further discussion).

Salmon

Two cDNAs from salmon were functionally characterised. When translated, the predicted amino acid sequences were highly homologous to each other but had lower homology to the putative carp and zebrafish sequences. When functionally expressed, the results were very different to those of the zebrafish. Firstly, one of the salmon cDNAs was non-functional and secondly, the cDNA which produced a functional enzyme, had a substrate specificity very different from that of the zebrafish and carp genes. The cDNA which produced the functional desaturase encoded an enzyme that was specific for *n*-3 fatty acids and only desaturated at the Δ5 position to any substantial degree. However, Atlantic salmon are known to be capable of both Δ6 and Δ5 desaturation (Bell *et al.*, 1997) and are known to readily

convert Δ6 and Δ5 substrates with a preference for *n*-3 PUFA (Tocher and Dick 1990). Therefore, unless the cDNA confers an enzyme which has an optimal enzymatic temperature below that in which the transformed yeast were grown (30°C) then there is a possibility that there are further desaturases genes or alleles conferring higher desaturase activity. This is a real possibility as an Atlantic salmon would not survive at a water temperature of 30°C and it has been noted that the optimal temperature of the Atlantic salmon cell line (AS) was between 22°C and 24°C (Tocher & Dick, 1990). It is possible that the enzyme would have more activity at a lower temperature i.e. the specificity and activity of the enzyme would be different at lower temperatures. However, it is also very possible that there are other desaturase genes to be discovered. Again, as with the carp this is possible as there has also been genome duplication after the separation of mammals from fish (see chapter 3).

The homology of the predicted amino acid sequences between each of the fish species varies significantly. The carp and zebrafish are the most highly homologous pair in relation to each other in comparison with salmon which has more variability. However, no matter how homologous the sequences are, determining the substrate specificity is impossible from amino acid sequences alone. As the zebrafish and carp are so similar it would have been expected that the substrate specificity would be similar also. However this was not the case as the specificities of the functional zebrafish and carp cDNAs are different.

4.5. Conclusions

All of the fish species studied in this project contain sequences for functional desaturase genes. The fish desaturase genes are unusual in comparison to all other vertebrate desaturase genes functionally characterized to date as they are capable of introducing double bonds at the Δ6 and Δ5 positions although the level of activity for

each type of desaturation is species specific. It is not entirely clear why this should be but it is probably due to amino acid changes conferring structural changes within the enzymes active site. However, this is only speculation as little is known about the active sites of these enzymes since they are membrane-bound rendering them insoluble and therefore making it very difficult to study the protein's crystal structure.

The functional characterisation of the putative fish desaturase genes reported in this chapter, which showed that some cDNAs with very high homology to active desaturases but which do not produce a product capable of Δ5 or Δ6 desauration, has emphasized clearly that the putative desaturase genes require functional characterisation in an expression system before classifying them conclusively.

Very little is known about the active sites of the desaturase enzymes and this chapter has shown that sequences which are highly homologous at the amino acid level can confer products with very different of Δ5 and Δ6 activities, and in some instances non-functional desaturase products. Therefore, the next chapter will attempt to identify amino acids that are potential causes for these functional differences.

Chapter 5

Structure Function Relationships

Chapter 5 – Structure Function Relationships

5.1. Aims

The results described in the previous chapter, indicating that the fatty acid substrate specificity of the desaturase gene depended on the species from which the cDNAs was isolated, raises significant questions in the context of structure-function relationships. What causes the genes to have the specificities they have? Is it possible to predict what specificities each cDNA product will have, and why some cDNAs encode non-functional products? This chapter will attempt to relate protein structural characteristics of the cDNAs to the respective substrate specificities.

5.2. Introduction

The previous chapter described a number of fish desaturase genes, the enzyme products of which had different fatty acid substrate specificities. The carp and salmon enzymes were only able to desaturate one fatty acid substrate effectively. The carp was specific for *n*-3 fatty acids and introduced a double bond at the Δ6 position whereas the salmon enzyme introduced a double bond at the Δ5 position of predominantly *n*-3 fatty acids. In contrast, the zebrafish desaturase was capable of desaturating at both the Δ5 and Δ6 positions effectively, with both *n*-3 and *n*-6 substrates although there was a preference for *n*-3 fatty acids. Therefore, the carp and salmon desaturases seem to be specific for fatty acids of C_{18} and C_{20} chain length respectively, and possessing a double bond at the *n*-3 position, whereas the zebrafish enzyme was capable of desaturating both *n*-3 and *n*-6 fatty acids and with both C_{18} and C_{20} chain lengths. The structural basis for these substrate preferences is not obvious from the sequence comparisons, since all the enzymes share high levels of similarity, particularly carp and zebrafish. Moreover, some of the cDNAs appear to

encode products with no enzyme activity despite sharing high identities and similarities (i.e. no. of positives) with functional cDNAs from the same species. The overall protein conformation and structure should give answers and provide possible explanations as to why the above results were observed. To date, the crystalline structure of the insoluble membrane-bound desaturases has not been determined and therefore no information exists as to the nature or location in the primary structure of its active sites (Libisch, 2000). However, they are part of a super family of related proteins and enzymes with similar motifs and functions and some information is available on the active site and protein conformation of the other soluble desaturases and related enzymes. Using this information and the results of mutagenesis studies (site-directed and chimeras) it may be possible to develop theories of how these desaturase enzymes function, and how the related protein primary structure and folding determines the specificities described in chapter 4.

5.2.1. How do the fatty acids sit in relation to the active sites?

Just how the substrate is inserted in the protein for desaturation is unknown for the fused desaturases. However, according to the model of the crystalline stearoyl-ACP desaturase from plants, a soluble desaturase, the hydrophobic segment of the acyl group extending to and including the methyl end has to be released from the carrier protein to become available for insertion into the substrate channel of the enzyme, and penetrate deeply into the desaturase interior until the methyl end touches the bottom of the channel (Lindqvist *et al.*, 1996). This pulls the double bond insertion site close to the di-iron complex and initiates the reaction at a site completely shielded from the protein surface and the solvent. The question possed by Sperling and Heinz (2001) is whether membrane-bound desaturases form a similar extended substrate

channel, with only one opening for entrance and release into which acyl groups have to be inserted, with the methyl end entering first and accordingly most deeply.

The mammalian prostaglandin H_2 synthase-1 is another related membrane-bound enzyme which contains a haem group involved in catalysis, and also operates in the membrane transition zone mentioned above. The crystalline structure of the prostaglandin synthase displays a similarly extended hydrophobic channel which is accessible only from the membrane interior. Crystallisation of this enzyme in the presence of AA shows the bent conformation of the bound substrate which has to crawl into the enzyme with the methyl end in front and penetrating most deeply. The carboxyl end of the AA is fixed by the guanidinium group of an arginine residue located at the entrance of the substrate channel (Malkowski *et al.*, 2000).

As for the vertebrate membrane-bound desaturases, one of the first models proposed that an active centre was lying in an extended cleft or trench formed at the surface of the desaturase (Brett *et al.*, 1971). The first contact of the acyl group with this open trench could occur at any segment of the chain which could subsequently be put in place by specific interactions with the polar region surrounding and including C1 (the carboxyl end), with the hydrophobic methyl end and/or unsaturated bonds as reference centres for subsequent regioselective alterations. This would also depend on the regioselectivity of the actual desaturase as this model would not necessarily require the binding of an acyl group for its entire length compatible with the action of the ω-desaturases which measure the distance from the methyl end (Tocher *et al.*, 1998).

5.2.2. How do the desaturase enzymes function?

Little is known about how the fatty acid desaturases actually function in mechanistic terms as most of the available information is speculative, particularly with regard to

the insoluble, membrane-bound Δ5 and Δ6 desaturases. The crystalline structure for the Δ5 and Δ6 desaturases have not been determined and so, as discussed above, little is known about their active sites. Thus, most of the information is derived from studies on soluble enzymes such as the stearoyl Δ9 desaturases. Extrapolations and hypotheses can be drawn from studies on the structure of these similar enzymes and by undertaking mutagenesis studies, including site-directed mutagenesis and the use of chimeras.

In eukaryotes, fatty acid desaturases are all NAD(P)H and O_2-dependent multiprotein enzyme complexes (Fulco, 1974). Whether soluble or membrane-bound, the desaturases have several important similarities including the catalytic requirement for iron (Nagai & Bloch, 1968), the inhibition by metal chelators (Jaworski & Stumpf, 1974) and the kinetic isotope effects observed for C-H bond cleavage (Morris, 1970). These similarities indicate that there is a common mechanism for the desaturation reaction which may extend to include a structurally related catalytic active site. There have been studies on the structure of the soluble stearoyl-CoA Δ9 desaturase. Mössbauer spectroscopy has shown that the soluble Δ9 desaturase protein contains oxo or hydroxo-bridged di-iron clusters (diiron-oxo proteins) (Fox *et al.*, 1993). Other enzymes with similar reaction mechanisms include the alkane hydroxylase (ω-hydroxlyase) and xylene monooxygenase (Shanklin *et al.*, 1994). These data suggest that the membrane fatty acid desaturases and the hydrocarbon hydroxylases have a related protein fold, possibly arising from a common ancestral origin (Shanklin *et al.*, 1994). Further information from studies on the soluble desaturase enzymes, and the alkane hydroxylases and xylene monooxygenase may enable us to understand better how the membrane-bound desaturases function.

Fatty acid desaturation by membrane-bound desaturases requires electron transport from NADH via the component FAD of the NADH:cytochrome b_5 oxidoreductase and the heme-iron in the hydrophilic domain of cytochrome b_5 to the di-iron complex of the desaturase, which, in the μ-oxo-diferric state, is the final acceptor of the two electrons required per double bond formed (Broadwater *et al.*, 1998). Thus, fatty acid desaturases of mammals, fish, fungi, insects, higher plants and cyanobacteria are all iron-containing enzymes that catalyse the NAD(P)H and O_2-dependent introduction of double bonds into fatty acyl chains. Examination of the deduced amino acid sequences of the membrane desaturases has revealed three regions of conserved primary sequence containing $HX(_{3\text{ or }4})H$, $HX(_{2\text{ or }3})$ HH and $HX(_{2\text{ or }3})HH$ (Shanklin *et al.*, 1994). The His-containing regions are positioned in the hydrophilic regions but also have a consistent positioning with respect to the possible membrane spanning domains. The histidine boxes are thought to act as ligands for the iron atom(s) contained in these enzymes (Shanklin *et al.*, 1994) and thus form similar di-iron clusters as mentioned above for soluble desaturases (Shanklin *et al.*, 1997). The importance of these His-regions has been confirmed in mutation studies where mutation of any of the eight His residues results in a complete loss of desaturase activity. This has been reported both for the membrane-bound Δ12 acyl-lipid desaturase of cyanobacteria *Synechocystis* and the soluble rat stearoyl-CoA Δ9 desaturase gene (Avelange-Macheral, 1995, Shanklin *et al.*, 1994).

5.2.3. Active sites

The position and orientation of the active site will control the position of the fatty acids in relation to the desaturase enzyme and how the enzyme functions. The different desaturase proteins act at various positions along the fatty acid carbon chain which, in the membrane bilayer models, are close to or mix with the interfacial layers

covering the hydrophobic core of the bilayer. The interfacial layer is characterised by a steep gradient of polarity regarding solvation and electrical charge (Sperling and Heinz, 2001). It has been suggested that since the active sites of the desaturases may be situated in this environment at the membrane surface the di-iron complexes are surrounded by sequences of intermediate polarity/hydrophobicity. This position represents a compromise regarding the chances to interact with both the hydrophilic region of the electron donor and the hydrophobic substrate segments emerging from the interior (Sperling and Heinz, 2001). As previously mentioned, the membrane-bound desaturases belong to a superfamily of membrane iron proteins that possess the purported iron ligand. These His-rich motifs are also present in the bacterial membrane enzymes, AlkB1 and xylene monooxygenases, and share strikingly similar residue spacings, not-withstanding the different catalytic abilities, to the desaturase enzymes. Similar to the model for Δ5 and Δ6 desaturases, the predicted position of the His-rich residues of the bacterial enzymes is in the cytoplasmic face of the membrane rather than in the membrane-spanning domains. The substrates for all these associated enzymes are hydrophobic and will likely associate with the lipid bilayer. Overall these features suggest that an active site assembled from these His-containing sequences may occur at or near the membrane surface. The currently accepted structural model for the membrane-bound desaturases was proposed by Stukey *et al.* (1990). The model has two long hydrophobic regions of about 50 residues which are each predicted to span the membrane twice. Also, according to this model, most of the protein is located on the cytosolic side of the ER membrane. This model was supported by van Beilen (1992) working on the membrane-bound alkane hydroxylase of *Pseudomonas oleovorans*.

However, recent studies on the membrane topology of the acyl-lipid desaturase from *Bacillus subtilis* have challenged Stukey's model. The membrane topology of the Δ5-Des from *B. subtilis* was studied by constructing a series of fusion proteins with the reporter alkaline phosphatase PhoA (Diaz *et al.*, 2002), a similar method to that used previously to study the topology of the *P. oleovorans* membrane-bound alkane hydroxylase (van Beilen *et al.*, 1992). The results revealed that the *B. subtilis* Δ5-Des is a polytopic membrane protein with five to six transmembrane and one membrane-associated domain (i.e. does not enter the membrane) which likely represents a substrate-binding motif. The apparent topographic difference found among bacterial membrane-associated desaturases could also extend to mammalian desaturases involved in PUFA biosynthesis. The hydrophobicity plots for murine and human Δ6 desaturase (access. No. NP_062673, AAD20018) predict only four transmembrane segments (Cho *et al.*, 1999a) whereas the human Δ5 desaturase (GenBank access. No. AAF 29378) shows a similar profile to *B. subtilis* Δ5-Des (Cho *et al.*, 1999b). It has been speculated that the additional transmembrane regions may be essential for desaturation of the acyl chains of membrane glycerophospholipids by stabilising the structure and/or activity of bacterial acyl-lipid desaturases (Diaz *et al.*, 2002).

5.2.4. Mutageneis studies

Whittle and Shanklin (2001) recently engineered a Δ9-16:0-acyl carrier protein (ACP) desaturase by redesigning the castor Δ9-18:0-ACP desaturase. The crystal structure of the wild type Δ9-18:0-ACP desaturase revealed a pocket capable of accommodating an C_{18} substrate in the extended conformation adjacent to the di-iron active site (Lindqvist et al., 1996). Such a pocket capable of binding C_{18} substrates could also

structurally accommodate C_{16} substrates. The cost, however, is reduced turnover correlated with a loss of binding energy. It was realised that small changes in the positioning of the substrate relative to the active site was shown to exert large effects on catalytic rates (Cannon *et al.*, 1996, and Whitty *et a*l., 1995). Thus, Whittle and Shanklin replaced the threonine and glycine at positions 117 and 188 respectively, at the end of the binding pocket with bulkier arginine and leucine shortening the binding pocket and making it more effective for a C_{16} substrate but less effective for an C_{18} substrate. Therefore, a Δ9-18:0-ACP desaturase was converted into an enzyme with substrate preference for 16:0-ACP by replacement of two amino acid residues with larger residues which restricts the bottom of the active site enough to prevent the accommodation of the two additional carbon atoms at the methyl end of 18:0-ACP.

A previous study utilised mutatgenesis in order to alter the substrate specificity replaced specific amino acid residues in a Δ6-palmitoyl (16:0)-ACP desaturase with their equivalents from a Δ9-stearoyl (18:0)-ACP desaturase. Mutants were identified that had altered chain length specificities and inserted double bonds into either Δ6 or Δ9 positions of 16:0- and 18:0-ACP (Cahoon *et al.*, 1997). These studies outline the importance of the geometry of the lower portion of the binding pocket to the substrate chain length and, as such it is the residues at the bottom of the substrate channel or pocket which are likely to be most critical for determining preference for substrate fatty acid chain length (Cahoon *et al.*, 1997).

Alterations in the double bond positional specificities of the above mutants, are more difficult to understand. This class of desaturase is a classical "front-end" desaturase which inserts a double bond at a characteristic position determined from the carboxyl end of the acyl chains (Cahoon & Ohrogge, 1994). Therefore, the site of insertion is likely to be associated with interactions between the upper part of the

active site and the ACP portion of the fatty acid substrate. If the ACP binds in the same way as in acyl-ACP desaturases, then differences in the amino acid side chains at the enzyme surface and/or those lining the upper portion of the substrate binding channel would allow the enzyme to accommodate different lengths of the fatty acid chain between the site of double bond insertion and the thioester linkage to ACP. But this is speculation (Cahoon *et al.*, 1997). Residues that contribute to altered double bond positional activity include alanine-200 which is on the surface pointing away from the substrate and amino acids 205-207 that are located outside the active site. It is therefore possible that these residues can cause changes in specificity by means of the packing effects in the enzyme rather than through direct interactions with substrates (Cahoon *et al.*, 1997).

However, there has been some mutagenesis work on the borage Δ6 fatty acid desaturase. The borage Δ6 fatty acid desaturase (*Boof*d6) is similar to the vertebrate Δ6 fatty acid desaturases as it is also a "front-end" desaturase i.e. it introduces a double bond into the fatty acid between the carboxyl-end and a pre-existing double bond (Napier *et al.*, 1999). Recently the borage Δ8-sphingolipid desaturase (*Boof*d8) was isolated and found to have 58% identity and 77% similarity to *Boof*d6 (Sperling *et al.*, 2001). Both are thought to be evolutionarily related, either the Δ6 desaturase having evolved from the sphingolipid desaturase or they have both evolved from a common ancestor. So, in order to identify sequence differences which are responsible for different catalytic functions of the *Boof*d6 and *Boof*d8 proteins, Libisch and colleagues (2000) developed a set of chimeras combining sequences from the two enzymes. One chimera in particular indicated that the membrane helices 1 and/or 2 are involved in the formation of the active site (Libisch *et al.*, 2000). The chimera, C2, was a *Boof*d6 but which contained helices 1 and 2 with a linker region to the

cytochrome b_5 domain all which come from the *Boof*d8. This chimera was unable to desaturate 18:2*n*-6 and 18:3*n*-3, but was able to desaturate 16:1*n*-7 and 14:1, myristoleic acid. The results indicate that the C2 chimiera enzyme was not simply defective in its function but it had altered substrate-binding properties probably due to modifications in its substrate binding site (Libisch *et al.*, 2000). Chimeras have also been used to determine the regions or residues involved in substrate binding in soluble fatty acid desaturases, as above, and in hepatic and lipoprotein lipases (Davis *et al.*, 1992). In the acyl-ACP thioesterase, it was shown through protein engineering that the final two thirds of the C-terminal were critical in determining the substrate specificity (Yuan *et al.*, 1995). It is thought that small change in a tripeptide from MRR to RRH might have reduced the flexibility of the β-structure immediately following the tripeptide, which may have led to a reduction of flexibility in the substrate binding pocket and the active site (Yuan *et al.*, 1995).

5.2.5. Counting carbon atoms and double bonds

Fatty acid desaturase genes have obvious structural relationships, such as those observed between cyanobacterial and plant ω–3 and Δ12 desaturases. However, it has been observed that there are possible differences in the mechanism for determining positional specificity of double bond insertion into the acyl backbone (Tocher *et al.*, 1998). For all organisms double bonds are located at exact positions, for example in C_{18} fatty acids the first double bond is introduced into 18:0 at the Δ9 position and the subsequent double bonds are generally introduced at the Δ12 and Δ15 (or ω3) positions respectively. Furthermore, in some groups of cyanobacteria a double bond can be introduced at the Δ6 position. These data indicate that the desaturases can count the exact number of carbon atoms in the hydrocarbon chain and can introduce

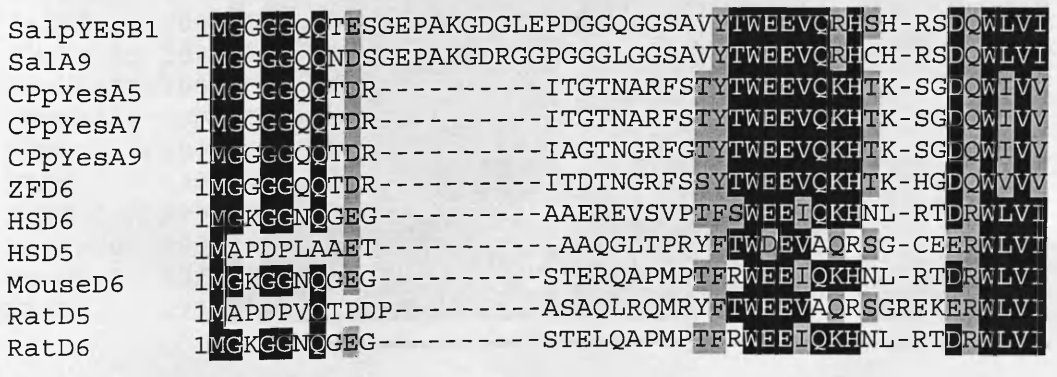
the double bond at specific positions. However, the mechanisms whereby they can exactly position the double bonds can vary. Higashi & Murata (1993) noted that in the cyanobacteria *Synechocystis* cultures grown on odd chain fatty acid precursors, the last double bond was always inserted three carbons from the methyl end of $17{:}2^{\Delta 9,12}$ and $19{:}2^{\Delta 9,12}$ to form $17{:}3^{\Delta 9,12,14}$ and $19{:}3^{\Delta 9,12,16}$. Thus, the *Synechocystis* "Δ15" desaturase actually counts carbons from the methyl end and so is now more commonly referred to as an "ω3 desaturase". However, *Synechocystis* Δ12 desaturase always inserted a double bond 12 carbons from the carboxyl end of $18{:}1^{\Delta 9}$ to form $18{:}2^{\Delta 9,12}$. Higashi & Murata's results also revealed that the Δ9 desaturase also counts carbons from the carboxyl end. As for the Δ6 and Δ12, it is clear that they do not count carbon atoms from the methyl end. However, it remains unclear as to whether either of these enzymes can count carbons from the carboxy terminus or from the double bond at the Δ9 position (Higashi & Murata, 1993).

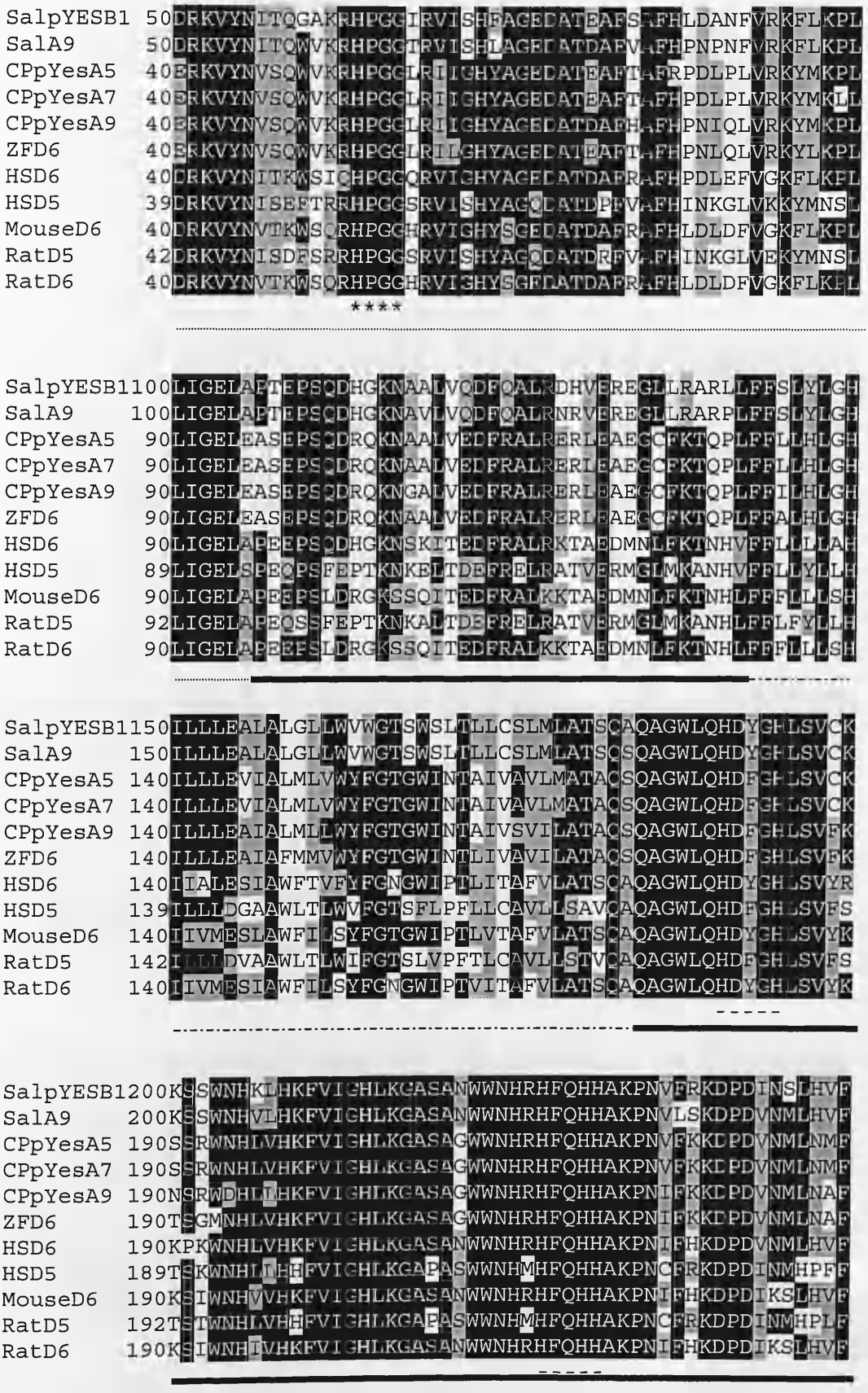
How the position of the double bond insertion is determined in vertebrate Δ5 and Δ6 desaturases is not understood. Given the results obtained with *Synechocystis,* and the observation that the products of the reactions are all methylene-interrupted, allied to the fact that the zebrafish enzyme is capable of both Δ5 and Δ6 desaturation, it would suggest that the animal enzymes recognise the carboxyl-proximal double bond (i.e. the Δ9, Δ6 or Δ5 position) and insert a double bond three carbons toward the carboxyl end. As it is possible to obtain Δ5 and Δ6 specificity in highly related enzymes, it is likely that just a few residues might determine the size and shape of a binding pocket for the carboxyl end carbons and thus determine the specificity. The way in which the substrate is inserted into the enzyme and the substrate/enzyme interaction, is not known and can only be speculated upon. From the above studies, it could be hypothesised that the active site is situated around the histidine boxes. A

second possible hypothesis using information from the chimeric studies on borage Δ6 and Δ8 sphingolipid desaturase above is that the first and second helices which form the first transmembrane region are involved in shaping a substrate binding pocket. If this is true, then amino acid residues in these areas will affect the substrate specificity and the efficiency of the desaturase reaction. Therefore, the remaining sections of this chapter will study amino acid residues within these areas of the primary sequences of the fish desaturases. Specifically they will attempt to determine if there are any obvious differences which could confer the different specificities of the enzymes and determine the resulting efficiency of the desaturase reaction.

5.3. Results and Discussion

Figure 25 is an alignment of the amino acid sequences from fish which code for proteins that are both functional and non-functional as desaturase enzymes. The transmembrane regions, conserved histidine boxes and cytochrome b_5 domains are highlighted. In the analysis, the second transmembrane region, the histidine boxes and the cyt b_5 have been considered the least likely to determine desaturase substrate specificity and enzyme catalytic efficiency for reasons that have been discussed and the other areas are focussed upon.

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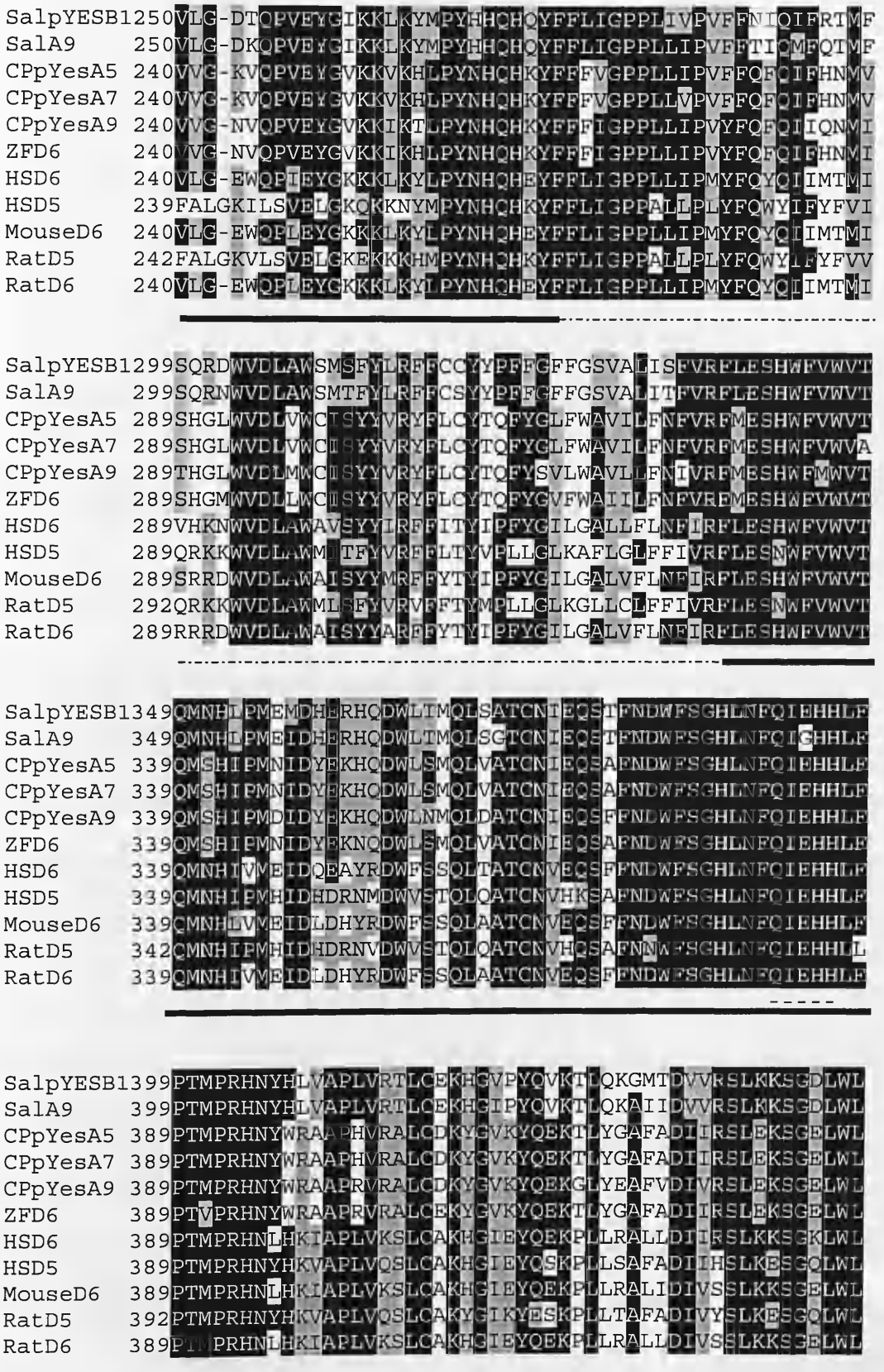
SalA9
CPpYesA5
CPpYesA7
CPpYesA9
ZFD6
HSD6
HSD5
MouseD6
RatD5
RatD6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SalA9 250VLG-DKQPVEYGIKKLKYMPYHHQHQYFFLIGPPLLIPVFFTIQMFQTMF
CPpYesA5 240VVG-KVQPVEYGVKKVKHLPYNHQHKYFFFVGPPLLIPVFFQFQIFHNMV
CPpYesA7 240VVG-KVQPVEYGVKKVKHLPYNHQHKYFFFVGPPLLVPVFFQFQIFHNMV
CPpYesA9 240VVG-NVQPVEYGVKKIKTLPYNHQHKYFFFIGPPLLIPVYFQFQIIQNMI
ZFD6 240VVG-NVQPVEYGVKKIKHLPYNHQHKYFFFIGPPLLIPVYFQFQIFHNMI
HSD6 240VLG-EWQPIEYGKKKLKYLPYNHQHEYFFLIGPPLLIPMYFQYQIIMTMI
HSD5 239FALGKILSVELGKQKKNYMPYNHQHKYFFLIGPPALLPLYFQWYIFYFVI
MouseD6 240VLG-EWQPLEYGKKKLKYLPYNHQHEYFFLIGPPLLIPMYFQYQIIMTMI
RatD5 242FALGKVLSVELGKEKKKHMPYNHQHKYFFLIGPPALLPLYFQWYLFYFVV
RatD6 240VLG-EWQPLEYGKKKLKYLPYNHQHEYFFLIGPPLLIPMYFQYQIIMTMI
SalpYESB1299SQRDWVDLAWSMSFYLRFFCCYYPFFGFFGSVALISFVRFLESHWFVWVT
SalA9 299SQRNWVDLAWSMTFYLRFFCSYYPFFGFFGSVALITFVRFLESHWFVWVT
CPpYesA5 289SHGLWVDLVWCTSYYVRYFLCYTQFYGLFWAVILFNFVRFMESHWFVWVT
CPpYesA7 289SHGLWVDLVWCISYYVRYFLCYTQFYGLFWAVILFNFVRFMESHWFVWVA
CPpYesA9 289THGLWVDLMWCISYYVRYFLCYTQFYSVLWAVLLFNIVRFMESHWFMWVT
ZFD6 289SHGMWVDLLWCISYYVRYFLCYTQFYGVFWAIILFNFVRFMESHWFVWVT
HSD6 289VHKNWVDLAWAVSYYIRFFITYIPFYGILGALLFLNFIRFLESHWFVWVT
HSD5 289QRKKWVDLAWMLTFYVRFFLIYVPLLGLKAFLGLFFIVRFLESNWFVWVT
MouseD6 289SRRDWVDLAWAISYYMRFFYTYIPFYGILGALVFLNFIRFLESHWFVWVT
RatD5 292QRKKWVDLAWMLSFYVRVFFTYMPLLGLKGLLCLFFIVRFLESNWFVWVT
RatD6 289RRRDWVDLAWAISYYARFFYTYIPFYGILGALVFLNFIRFLESHWFVWVT
SalpYESB1349QMNHLPMEMDHERHQDWLIMQLSATCNIEQSIFNDWFSGHLNFQIEHHLF
SalA9 349QMNHLPMEIDHERHQDWLTMQLSGTCNIEQSTFNDWFSGHLNFQIGHHLF
CPpYesA5 339QMSHIPMNIDYEKHQDWLSMQLVATCNIEQSAFNDWFSGHLNFQIEHHLF
CPpYesA7 339QMSHIPMNIDYEKHQDWLSMQLVATCNIEQSAFNDWFSGHLNFQIEHHLF
CPpYesA9 339QMSHIPMDIDYEKHQDWLNMQLDATCNIEQSFFNDWFSGHLNFQIEHHLF
ZFD6 339QMSHIPMNIDYEKNQDWLSMQLVATCNIEQSAFNDWFSGHLNFQIEHHLF
HSD6 339QMNHIVMEIDQEAYRDWFSSQLTATCNVEQSFFNDWFSGHLNFQIEHHLF
HSD5 339QMNHIPMHIDHDRNMDWVSTQLQATCNVHKSAFNDWFSGHLNFQIEHHLF
MouseD6 339QMNHLVMEIDLDHYRDWFSSQLAATCNVEQSFFNDWFSGHLNFQIEHHLF
RatD5 342QMNHIPMHIDHDRNVDWVSTQLQATCNVHQSAFNNWFSGHLNFQIEHHLL
RatD6 339QMNHIVMEIDLDHYRDWFSSQLAATCNVEQSFFNDWFSGHLNFQIEHHLF
SalpYESB1399PTMPRHNYHLVAPLVRTLCEKHGVPYQVKTLQKGMTDVVRSLKKSGDLWL
SalA9 399PTMPRHNYHLVAPLVRTLCEKHGIPYQVKTLQKAIIDVVRSLKKSGDLWL
CPpYesA5 389PTMPRHNYWRAAPHVRALCDKYGVKYQEKTLYGAFADIIRSLEKSGELWL
CPpYesA7 389PTMPRHNYWRAAPHVRALCDKYGVKYQEKTLYGAFADIIRSLEKSGELWL
CPpYesA9 389PTMPRHNYWRAAPRVRALCDKYGVKYQEKGLYEAFVDIVRSLEKSGELWL
ZFD6 389PTVPRHNYWRAAPRVRALCEKYGVKYQEKTLYGAFADIIRSLEKSGELWL
HSD6 389PTMPRHNLHKIAPLVKSLCAKHGIEYQEKPLLRALLDIIRSLKKSGKLWL
HSD5 389PTMPRHNYHKVAPLVQSLCAKHGIEYQSKPLLSAFADIIHSLKESGQLWL
MouseD6 389PTMPRHNLHKIAPLVKSLCAKHGIEYQEKPLLRALIDIVSSLKKSGELWL
RatD5 392PTMPRHNYHKVAPLVQSLCAKYGIKYESKPLLTAFADIVYSLKESGQLWL
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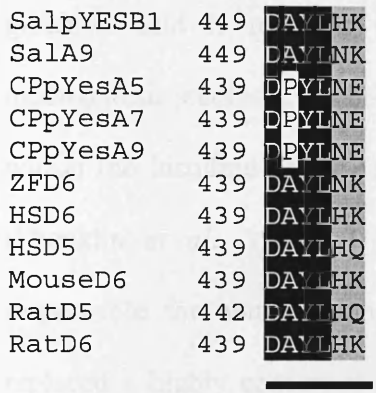


Figure 25. Alignment of the protein sequence of the functionally characterized cDNAs from fish and including the rat and human Δ5 and Δ6 desaturase genes and the mouse Δ6 desaturase gene which have also been functionally characterized. The cytochrome b_5 domain is highlighted by a dotted line and the haem-binding domain within is highlighted by stars. The three histidine boxes are marked by dashes, the transmembrane regions are underlined by a dot-dash line and all other areas are marked by a solid thick black line.

5.3.1. Non-functional cDNAs

The cDNAs producing the non-functional desaturases are highly homologous to the functional cDNAs from the same species (~94% positives). Therefore the number of amino acids potential causing or correlated with dis-functional enzymes is low. Thus it may be possible to determine which amino acids cause or potentially cause the cDNAs to produce products that are non-functional as desaturase enzymes.

5.3.1.1. Atlantic salmon

When functionally expressed in yeast, the Atlantic salmon desaturase cDNA SalA9 did not desaturate the *n*-3 or *n*-6 substrate fatty acids at the Δ5 or Δ6 position. The predicted sequence of the SalA9 clone contained a number of amino acid residues which are completely different from both mammalian Δ5 and Δ6 desaturase and other functionally characterised fish desaturase genes. The amino acid residues are positions N128, L237, S238, K254, G372, G394 and I433. Possibly the most obviously important difference is in the third histidine box where the conserved

glutamic acid is replaced with a glycine, G394, residue. From site directed mutatgenesis studies is has been demonstrated that replacement of any of the residues within the histidine boxes causes a reduction in the desaturase enzymatic activity (Shanklin *et al.*, 1994). This change is therefore a good candidate for being responsible for non-functionality. However, other residues such as G372 which replaced a highly conserved alanine, and L237 which replaced a highly conserved phenylalanine residue could also contribute to the lack of desaturase activity. The other four amino acid residues that were different were in less highly conserved areas.

5.3.1.2. Common carp

For common carp there were two cDNAs which produced products that did not desaturate fatty acids at the Δ5 or Δ6 positions, CPpYesA5 and CPpYesA7. None of the functionally characterised desaturases aligned in Figure 25 have the following amino acids S190, M238 and H402. Therefore, it is possible that one or all of these residues may cause the cDNA product to be non-functional with respect to Δ6 and Δ5 desaturation of fatty acids. It is also possible that it is none of these changes, and that rather it is a defect within the cytochrome b_5 domain that is producing a non-functional product in regards to desaturase activity. However, the *S. cereivisiae* yeast expression system contains an endogenous cytochrome b_5 (Vergeres *et al.*, 1993; Yoshida *et al.*, 1993) so it is possible that the desaturases inserted in transformed yeast would be able to utilise the endogenous cyt b_5 and successfully desaturate fatty acids. The above discussion is speculation and without further studies using site-directed mutatgenesis it is impossible at this stage to draw any further conclusions.

5.3.2. Functional cDNAs

Attempting to determine which residues may be responsible for the differences in substrate specificity between the functional desaturase genes appears more complex than determining residues responsible for catalytic activity. For one thing, there are many more changes in amino acid residues to be considered. Thus, studying the protein sequence of the functional enzymes it soon becomes apparent that it is very difficult to determine exactly which amino acid residues could be involved in crucial conformational changes leading to differences in substrate specificities.

5.3.2.1. Atlantic salmon

Transgenic yeast containing the Atlantic salmon gene construct, SalpYesB1, preferentially desaturated at the Δ5 position for *n*-3 fatty acid substrate, specifically 20:4*n*-3. It would be expected then, on comparison with the human Δ5 and Δ6 desaturase sequences, that the SalpYesB1 sequence would have higher homology to the Δ5 than for the human Δ6 desaturase. However, this is not so. As discussed in Chapter 3, the SalpYes1 sequence has greater homology with the human Δ6 desaturase enzyme. Upon studying the differences in what are believed to be the functionally important regions of the different genes, there are a great number of amino acid residues within these conserved areas that vary between the functional cDNAs. The changes in amino acids at the same position within the different functional cDNAs often result in amino acids with very different properties given scope for the changes having functional effects. For example, one amino acid out-with the cytochrome b_5 domain and the transmembrane regions, specifically position 138 in salmon, is a conserved threonine residue in protein sequences of products with specific or greater Δ6 activity, but is a conserved alanine in the protein sequence of

enzymes with a specific or higher Δ5 activity. There is another residue, in the first transmembrane region, which has similar conservation depending on whether the enzymes are either specific or have greater Δ5 or Δ6 desaturase efficiencies. This is position 167, which is conserved serine in Δ6 desaturases and glycine in Δ5 desaturases. The salmon has an alanine residue in position 138 and a glycine in position 167 as mammalian Δ5 desaturases. Therefore, there are one or two candidate amino acid residues that may be involved in determining the substrate specificity of the salmon desaturase described in the previous chapter.

5.3.2.2. Common carp

The carp, as with the salmon, preferentially desaturates one fatty acid. For carp the functional cDNA, CppYesA9, preferentially desaturates C_{18} *n*-3 fatty acids at the Δ6 position, specifically 18:3*n*-3. Therefore, upon comparison with the mammalian Δ5 and Δ6 desaturases one would expect the carp protein sequence to be more homologous to the human Δ6 than to the Δ5 desaturases, and this is the case. As for positions 128 and 157 (138 and 167 in salmon), the carp contains threonine and serine. These are the residues that are conserved in these positions in all other Δ6 desaturases. However, deciding which residues cause the enzymes substrate specificity is, once again, speculation, and as above there could be one or a number of amino acid residues which cause the observed difference.

5.3.2.3. Zebrafish

In contrast with both the salmon and carp functional desaturase enzymes, the zebrafish functional cDNA produced a protein which desaturated both *n*-3 and *n*-6 fatty acids and at both the Δ5 and the Δ6 positions. Upon comparison of conversion percentages between the *n*-3 and *n*-6 at the both Δ6 and Δ5 positions, the enzyme was

more efficient at desaturating at the Δ6 position. Thus, the zebrafish cDNA encodes an enzyme that firstly is more efficient at desaturating *n*-3 over *n*-6 fatty acids and secondly at the Δ6 over the Δ5 position. In agreement with the above results, the zebrafish shows higher homology to the mamammlian Δ6 desaturase than to the mammalian Δ5 desaturase. In agreement too to the above discussion, amino acid residues 128 and 157 (138 and 167 in salmon) correspond to the conserved residues of threonine and serine, respectively. However, once again it is impossible to decipher which amino acids are responsible for the enzymes varying desaturase abilities.

5.4. Conclusions

It is difficult to draw firm conclusions, in terms of primary sequence and specific amino acid residues, as to why the fish desaturase enzymes have the fatty acid specificities discussed above. Only in one case, SalA9, the non-functional salmon desaturase cDNA, may it be possible to identify real possibilities. However, in all other cases it was very difficult. In order to understand how these enzymes function and to establish which areas are important in determining activity and substrate specificity, chimeric studies must be undertaken, where short regions of each of the cDNAs are replaced by the equivalent regions of one of the other fish desaturases. Then, once the areas of importance are identified, site-directed mutagenesis could follow. Thus site-directed mutagenesis would enable the residues of importance with regards to substrate specificity to be identified and would facilitate the engineering of desaturase genes with chosen substrate specificity or with increased catalytic efficiency.

It is possible that the zebrafish enzyme is an enzyme in a prototypic vertebrate pathway and that the salmon and carp cDNAs, as suggested in Chapter 3, may be newly evolved enzymes produced by gene duplication. It is believed that low stability and poor catalytic rates are properties shared by many newly evolved enzymes that arise by gene duplication events in which selection for stability and/or turnover is released while mutations accumulate that finally result in an alteration of function (Govindarajan & Goldstein, 1998).

It would be interesting to test the zebrafish cDNA for further desaturase activities. For example, the protein sequences of the Δ5 and Δ6 desaturase enzymes share high homology to the sequences of Euglenid (Wallis *et al.*, 1999) and borage (Sperling *et al.*, 2001) Δ8 desaturases, containing N-terminal cytochrome b_5 domain and the three highly conserved histidine boxes. If the zebrafish desaturase enzyme was also capable of desaturating the fatty acids at the Δ8 position it would be of great importance, as Δ8 activity has been represented in mammals and has been proposed as an alternative pathway in the production of arachidonic acid in the animal HUFA biosynthesis pathway (Napier *et al.*, 2003). Obviously this depends on where the constraints of the zebrafish desaturase lie, if it is the number of double bonds, position of first double bond or the number of carbon atoms in the chain. Testing for Δ8 desaturase activity would test how the number of double bonds affects desaturase activity as the only difference between the Δ8 substrate eicosadienoic acid (20:2*n*-6) and di-homo γ-linolenic acid (20:3*n*-6) is one double bond.

The cDNAs which were non-functional when tested in yeast could be tested by site-direct muatagenesis for each amino acid residue that differed between the functional and non-functional cDNAs from the same species. This would also further

our understanding of the desaturase enzymes and give an indication as to which areas in the protein sequence are important.

Ultimately, the number of carbon atoms, the number of double bonds and position of the first double bond in the fatty acid substrates all play a role in the resulting specificity and efficiency of the fish desaturase genes. However, further studies are required to identify both the areas of the enzyme and the specific amino acid residues that are important for determining catalytic activity, desaturation efficiency and substrate specificity.

Chapter 6

Phylogenetic Analysis

Chapter 6 – Phylogenetic analysis

6.1. Aims

Two cDNAs were cloned and sequenced for salmon and carp, and in both cases one of the cDNAs produced a functional desaturase product and the other cDNA produced a product that had neither Δ5 or Δ6 desaturase activity. Both of the functional cDNAs were only capable of efficiently desaturating one fatty acid substrate. Only one cDNA was cloned, sequenced and functionally characterised for zebrafish, but it was capable of desaturating fatty acids at both the Δ5 and the Δ6 position in both *n*-3 and *n*-6 fatty acid substrates. This chapter aims:

- To examine the possibility of the non-functional "desaturase" cDNAs being pseudogenes.
- To determine if there is reasonable evidence for the existence of other desaturase genes by studying the possible number of whole or partial genome duplications and synteny with the human genome.
- To plot the newly sequenced and functionally characterised fish genes in the context of previously sequenced desaturase genes of mammals, with which fish have the greatest homology, and with other organisms such as *Caenorhabditis elegans*, *Borago officinalis* and *Mortierella alpina*.

6.2 NJ Tree

Before beginning the discussion on the number of Δ5 and Δ6 desaturase genes fish may have, and how gene duplication and evolution may have affected the evolution of these genes, it is important to first look at how the Δ5 and Δ6 desaturase genes of other organisms cluster with the fish desaturase genes, including both those that have been functionally characterised and those that have not. In Figure 26 the protein

sequence of the fish enzymes sequenced in chapter 3 have been aligned with human, rat, *C. elegans*, and *M. alpina* Δ5 and Δ6 desaturases and mouse and borage Δ6 desaturases using CLUSTALW. The sequence phylogenies were predicted using the neighbour joining method of Saitou and Nei (1987). This method was choosen as it uses the likelihood method where the distances between all pairwise alignments are used to generate the tree. Other methods such as the parsimony methods multiply aligned sequences themselves and attempt to produce the minimum number of changes required to produce identity in all sequences and are only advantageous where highly divergent sequences are being compared. Using the NJ method, confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping the data through 1,000 iterations. Thus, there are 20 operational taxonomic units (OTUs) (Saitou, 1996) in total in Figure 26, consisting of sequences from *Cyprinus carpio*, *Salmo salar*, *Sparus aurata*, *Oncorhynchus masou*, *Oncorhynchus mykiss*, *C. elegans*, *M. alpina*, rat, mouse, human and borage.

The Δ6 desaturase genes of human, rat and mouse group together and the Δ5 desaturase genes of rat and human group together. Therefore, the tree indicates that for the mammalian line, the Δ5 and Δ6 desaturases diverged before speciation occurred. The Δ5 and Δ6 desaturases in mammals and *C. elegans* are thought to have occurred by gene duplication as both the *C. elegans* and the human Δ5 and Δ6 desaturases lie very close together. The human desaturases are found on chromosome 11 and lie only 11,000 bp (11 Kb) apart (Cho *et al.*, 1999). However, the *C. elegans* genes lie even closer together and are separated by only 990 bp on chromosome 4 (Michaelson *et al.*, 1998). The data in Chapter 3 show that the fish genes have greater homology to the human Δ6 than to the human Δ5 desaturase and this is

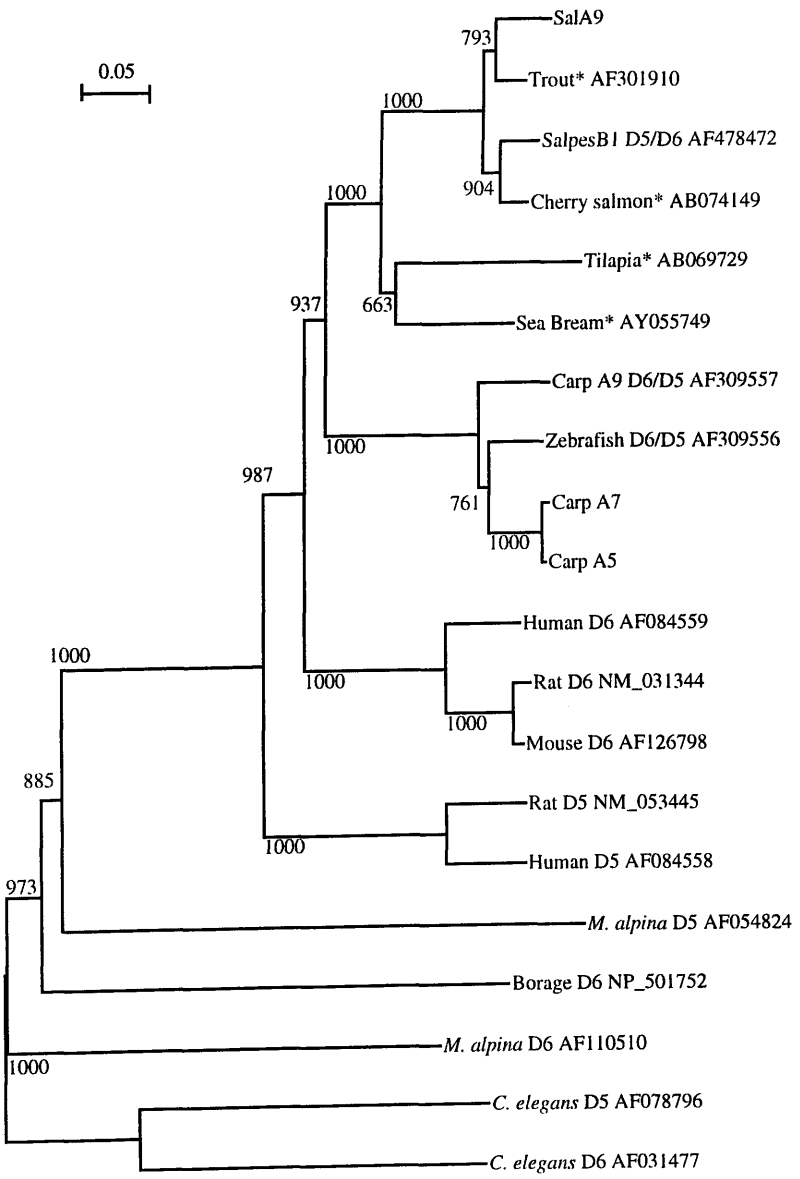


Figure 26: Phylogeny of desaturase deduced amino acid sequences. Sequences marked with an asterisk are not functionally characterised. Database accession numbers for the nucleic acid sequences are indicated. The conditions used for neighbour-joining tree construction are described in the materials and methods and were applied using CLUSTALW and NJPLOT. Horizontal branch lengths are proportional to the number of amino acid replacements per position, and the scale bar indicates this value. Numbers represent the percentage frequencies with which the tree topology presented here was replaced after 1,000 bootstrap iterations.

reflected in Figure 26. The fish genes, including the salmon SalpYesB1 cDNA, cluster closer to the mammalian Δ6 desaturases than to the Δ5 desaturases even though, as reported in chapter 4, SallivpYesB1 has predominantly Δ5 desaturase activity and very little Δ6 activity. All the salmonid cDNAs cluster together. An interesting feature of note in Figure 26 is that the non-functional SalA9 cDNA from Atlantic salmon clusters closer to the rainbow trout ortholog (*O. mykiss*) than to the functional Atlantic salmon clone, SallivpYesB1. The functional SallivpYesB1, which has Δ5 activity, clusters more closely with the sequence of the cherry salmon (*O. masou*). However, to date, neither the cherry salmon, the rainbow trout or the sea bream genes have been functionally characterised so it is difficult to determine the significance of this result.

The results from the phylogenetic analysis shown in Figure 26 prompt several questions:

i. How many Δ5 or Δ6 like desaturase genes exist in fish?

ii. Does zebrafish have more than one desaturase gene?

iii. Do the carp and salmon genomes contain further Δ5/6 like genes which have functions more specific for Δ6 activity and/or *n*-6 specificity in the instance of salmon, and Δ5 activity and/or *n*-6 specificity in the case of carp?

iv. Are the non-functional salmon and carp cDNAs pseudogenes or non-functional alleles or do they code for proteins with entirely different, so far uncharacterised, functions that have not been analysed?

6.3. More Genes in Fish?

In order to discuss the possibility of whether or not fish may possess more than one Δ5/6 like desaturase gene, it would be useful to know and understand fish in terms of their genome evolution.

There are presently approximately 25,000 known teleost fish species compared with only around 4,000 mammalian species (Wittbodt *et al.*, 1998, Vogel, 1998, Taylor *et al.*, 2001a). Most of the Teleostei species are believed to have arisen during the last 200 million years and the cause of the teleost radiation is believed to be genome duplication (Taylor *et al.*, 2001a). Specifically, genome duplication is believed to have provided the raw material for teleost radiation allowing them to adapt to the most divergent ecological conditions. However, this has not been proven. One convincing way to show that extra genes originating from genome duplication were responsible for the radiation of Teleostei would be to demonstrate that duplicated genes code for teleost specific traits (Taylor *et al.*, 2001b). Presently, the number of genome duplications, when they occurred and in which lineages, as well as their nature, whole or partial, is hotly debated. Much of the interest in this area in general stems from the fact that it is essential to understand the nature of genome duplications in fish because it is important to know how many genes fish species have, as they are often model organisms for helping to identify genes involved in disease and cancer which can be related to the human situation.

Over the last decade there have been a number of important studies that suggest that teleost fish contain more genes than mammals. One of the most important study genes in relation to discerning the number of genes in fish has been that of the multigene family of *Hox* genes. *Hox* genes are implicated in the control of axial patterning during embryonic development of many, and perhaps all, animals

(Holland & Garcia-Fernàndez, 1996). Human and mouse genomes are known to contain four *Hox* gene clusters and the two species probably possess the same 38 *Hox* genes (McGinnis and Krumlauf, 1992). However, there is strong evidence that zebrafish possess seven *Hox* gene clusters rather than the four clusters commonly observed among higher vertebrates (Amores *et al.*, 1998). Phylogenetic analysis revealed that the zebrafish had two *Hox* clusters closely related to each of the *Hoxa, Hoxb* and *Hoxc* clusters of mammals, plus a single *Hoxd* cluster ortholog. A possible second *Hoxd* may have been lost during subsequent evolution. Mapping studies confirmed that the zebrafish has two copies of each *Hox* chromosome segment of mammals, with the exception of *Hoxd,* which appears to have been deleted after duplication (Stellwag, 1999). The presence of additional *Hox* clusters in the zebrafish has led to the hypothesis that there was whole genome duplication at the origin of modern fish (Robinson-Rechavi *et al.*, 2001a). However, this contrasts with pufferfish (*Fugu rubripes*) which contains the four-cluster organisation common to mammals, but do not have the nine *Hox* genes found in mammals. The pufferfish also contains a *Hoxd-2* gene, and *Hoxc-1* and *Hoxc-3* pseudogenes not found in mammals (Aparicio *et al.*, 1997). Of particular interest was the loss of an entire *Hox* paralog group (group 7), in pufferfish with the implication being that this loss might be linked to the peculiar skeletal morphology of this fish (Holland, 1997).

The implication that zebrafish contains more genes than mammals is further supported by a study of 38 gene families (Robinson-Rechavi *et al.*, 2001a). Of the families that show at least one duplication in mouse or zebrafish, approximately two thirds have a duplication specific to zebrafish. For 80% of the gene families there is one gene in mouse and one in zebrafish but more than twice as many duplications are detected in zebrafish than in the mouse lineage (Robinson-Rechavi *et al.*, 2001a).

The synteny♦ between the zebrafish and human genomes is high. Evidence for the synteny between these genomes is given by the study of Barbazuk *et al.* (2000). Barbazuk mapped the positions for 523 zebrafish genes and ESTs with predicted human orthologs and revealed extensive contiguous blocks of synteny between the genomes (Barbazuk *et al.*, 2000). Of those mapped, 80% of genes and ESTs analysed belong to conserved synteny groups (two or more genes linked in both zebrafish and human) and 56% of all the genes analysed fell into 118 homology segments (uninterrupted segments containing two or more contiguous genes or ESTs) with conserved map order between the zebrafish and human genomes. The significant conservation of synteny between the human genome and the zebrafish is supported by the earlier study of Gates *et al.* (1999). Gates mapped 104 genes and ESTs and 275 previously mapped genes, microsatellites and sequence-tagged sites in a panel of haploid siblings. Phylogenetic analysis revealed likely mammalian orthologs of mapped zebrafish genes, and comparison of map positions in zebrafish and mammals revealed significant conservation of synteny (Gates *et al.*, 1999). The *Mhc* region is one example of the syntenic relationship between the zebrafish and human genomes (Sültmann *et al.*, 2000). The *Mhc* region is one of the most dense regions of the human genome, comprising 220 genes in a 4.6Mb segment of DNA on chromosome 6p21.3 (Sambrook et al., 2002). It is a region intimately associated with immune function, with approximately 40% of the gene products having some immunological role and contains genes essential to both the adaptive and innate immune systems (Sambrook *et al.*, 2002). It was discovered that the conserved synteny between human chromosome 6p and the zebrafish linkage group 19

♦ The term synteny (or syntenic) refers, originally, to gene loci on the same chromosome regardless of whether or not they are genetically linked by classic linkage analysis but nowadays refers to gene loci in different organisms on a chromosomal region of common evolutionary ancestry (Passarge *et al.*, 1999).

encompasses at least 27 loci of the *Mhc* region. This represents the largest conserved synteny between fishes and mammals recorded to date (Sültmann *et al.*, 2000).

The degree of synteny between fish and humans depends on which fish species are being compared. The ray and lobed fin fish diverged approximately 450 million years ago (Kumar & Hedges, 1998; Lee 1999). The tetrapods separated from the fish around 400 million years ago (Sültmann *et al.*, 2000). In contrast, the rodent/primate divergence is considered to have taken place only around 11 million years ago (Kumar and Hedges, 1998). Therefore, fish have had a much greater period of time over which to evolve and diverge from each other. Thus, synteny between fish and human genomes depends on where in the evolutionary tree the fish species is found. However, although synteny maybe conserved, often the gene orders are not. The genome of the pufferfish, *Fugu rubripes,* has considerable conservation of synteny with the human genome with conserved synteny observed in the PTEN locus which encodes a protein-tyrosine and lipid phosphatase (Yu *et al.*, 2001). However, gene orders are not well conserved with only very short sections of two to three adjacent genes being maintained in both organisms (Smith *et al.*, 2002).

6.3.1. Nature and number of genome duplications in fish

The conclusion of the above section is that there are more genes in fish than in mammals, and that this most likely occurred by genome duplication. However, the number of genome duplications that have occurred, and how have they occurred has been debated for many years now. It has been postulated that whole genome duplications in ancestral vertebrates generally appear to have occurred before the separation of fish and higher vertebrates (Sparrow & Neumann, 1976), whereas other duplications may have been lineage-specific (Wittbrodt *et al.*, 1998). Postlethwait *et al.* (1998) mapped 144 zebrafish genes and compared the resulting map with

mammalian maps. The comparison revealed large conserved chromosome segments. Since the duplicated chromosome segments in zebrafish often correspond with specific chromosome segments in mammals, it is conceivable that two polyploidisation[♦] events occurred prior to the divergence of fish and mammal lineages (Postlethwait *et al.*, 1998). The first polyploidisation event could have occurred in a common ancestor of all jawed and jawless vertebrates after the lineage leading to the present day amphioxus diverged. The second one could have occurred in the common ancestor of jawed vertebrates after the jawless ones diverged (Sidow, 1996). However, Holland and colleagues disagree with this and argue that, because various vertebrates have several *Hox* clusters, the two rounds of duplication occurred before the origin of jawed fishes (Holland *et al.*, 1994). Therefore, since zebrafish often have more members of multigene families than mammals, and assuming that most gene family members have been described in the intensely studied human and mouse genomes, the 'extra' genes in zebrafish could have arisen from either: (i) a greater propensity for tandem duplications in the fish lineage, (ii) less loss of paralogous gene copies in fish, or (iii) additional chromosome duplication or tetraploidisation (Postlethwait *et al.*, 1998). The abundance of gene duplication is not restricted to zebrafish or its order Cypriniformes, almost all euteleost fish groups share similar numbers of duplicate genes (Robinson-Rechavi *et al.*, 2001a).

However, a recent study concluded that most of the duplicates of fish genes arose more recently than the divergence of major fish groups and linkage data are unequivocal, with different origins of linkage groups, or even of linked genes (Robinson-Rechavi *et al.*, 2001b). These authors suggest that it is not possible to

♦ A polyploid is a cell or organism that has more than the normal diploid number of chromosomes; a multiple of the haploid number caused by chromosomal replication without nuclear division (BioTech Resources, 1998).

support the view that the abundance of duplicate genes in fish arose mainly through a unique whole-genome duplication. Rather, they conclude that it arose through several local duplications (Robinson-Rechavi *et al.*, 2001b), as suggested previously by Postlethwait *et al.* (1998) and again by Martin (2001). Thus chromosomal pattern of duplicate genes pleads in favour of complete or partial chromosome duplications rather than single gene duplications (Robinson-Rechavi *et al.*, 2001a). However, the question as to whether the duplications characterised in these studies were preceded by an ancestral tetraploidisation followed by massive gene loss, remains to be answered.

6.3.2. Nature and number of genome duplication in humans

The data from the Human Genome Sequencing Consortium (International Human Genome Sequencing Consortium, 2001) and the Celera project (Venter *et al.*, 2001) indicate that extensive segmental duplication can be detected in the human genome, but that current analyses are insufficient to determine whether these are the result of two whole-genome doublings or a larger number of unrelated duplications of chromosomal segments. The fact that invertebrate genomes contain one *Hox* cluster whereas the human genome contains 4 *Hox* clusters is believed to be evidence that several rounds of genome duplication has occurred in mammalian history. However, many researchers dispute the whole-genome doubling hypothesis. Hughes *et al.* (2001) has marshalled several lines of evidence most notably a type of phylogenetic analysis in which four duplicate genes descending from a single ancestor after two genome-doubling events should group as (AB)(CD) and not as A(BCD)). The former pattern does not show up more often than would be expected by chance and so Hughes reports that linked paralogous genes arose as a result of

independent gene duplication and translocation events, scattered at different times over the history of life (Hughes *et al.*, 2001).

In classes *Mammalia* and *Aves,* major quantitative genome variations have not taken place for a period of 150-250 million years (Ohno 1970a). This may be due to the pronounced sexual dimorphism of sex chromosomes established in these vertebrate species. A polyploidisation event occurring in sexually reproducing organisms with heteromorphic sex chromosomes should lead to sterile offspring with intersexual characteristics (Li, 1982). Therefore, a polyploidisation with evolutionary significance must have taken place before the establishment of heteromorphic sex chromosomes (Leipoldt, 1983). This would be evidence that any genome duplication after the divergence of the mammals from fish would be partial and not whole genome doubling.

As mentioned earlier, the human Δ5 and Δ6 desaturase genes lie close to each other on the same chromosome with only 11 Mb separating them. This suggests that they have evolved by genome duplication, possibly independent gene duplication as the exon organisation is nearly identical, each consisting of 12 exons and splice donor and acceptor sites interrupted at identical nucleotide positions within highly conserved codons (Marquardt *et al.*, 2000). Lynch and Force (2000) note that under the classical model of gene duplication, non-functionalisation of one member of the pair by degenerative mutation has generally been viewed as inevitable unless the fixation of a silencing mutation is preceded by a mutation to a novel beneficial function. However, there are several plausible mechanisms for the preservation of duplicate genes (Lynch & Force, 2000). One such mechanism is the duplication-degeneration-complementation (DDC) model which predicts that (i) degenerative mutations in regulatory elements can increase rather than reduce the probability of

duplicate gene preservation and (ii) the usual mechanism of duplicate gene preservation is the partitioning of ancestral functions rather than the evolution of new functions (Force *et al.*, 1999).

In respect to zebrafish, under the DDC model, it's feasible and plausible that the zebrafish represents a prototypic vertebrate pathway and the human Δ5 and Δ6 desaturases have 'partitioned' their ancestral functions.

Moreover, if there are more genes in fish than mammals, then it is possible that more than one gene exists in zebrafish and that the other genes simply remain to be cloned. However, the fact that the desaturase gene that has been cloned has significant Δ5 and Δ6 activity would suggest that no other Δ5/6 like gene is absolutely required. Thus, if the human Δ5 and Δ6 desaturases evolved before the separation of the fish and mammalian lineage then one would expect to find two genes for Δ5 and Δ6 activity as in humans. In relation to the discussion above on the nature and number of genome duplications, and to when they occurred is impossible to predict, therefore, it is impossible to determine the number of genes that exist in the zebrafish by using genome duplications as an indication of gene number.

As for Atlantic salmon and the common carp, if the functioning and non-functioning "desaturase" cDNAs sequenced from each species are separate genes then it can only be speculated upon as to whether they evolved by genome duplication due to gene duplication, or due to tetraploidisation. It is impossible to know without the full genomic sequence whether the non-functional clones are duplicated genes that have lost their function or whether they are non-functional alleles. Alleles from the same locus will be greater than 90% identical in their 5' and 3' UTR's and in their intron sequences and possibly have greater homology in their coding sequence. If they are duplicated genes then they may have greater sequence

divergence. Sequencing the introns of the genes and using Southern blots may help to resolve this and provide evidence as to what the different cDNAs are.

It is possible salmon or carp possess further functioning Δ5/6 like desaturase genes simply because they have undergone genome duplication and because it is known from biochemical enzymatic assays that salmon have a very active Δ6 desaturase and carp an active Δ5 desaturase.

The following section discusses the possibility of the non-functional "desaturase" cDNAs from carp and salmon being duplicated genes and in consequence what may have happened to their sequence over the period of time since duplication to cause their loss of desaturase function.

6.4. New Functions, Subfunctionalisation and Gene Silencing

There are three possible reasons for the loss of the original gene function: (i) the duplicated genes could have evolved new functions (ii) they could have gone through subfunctionalisation or (iii) they could have been silenced and become pseudogenes.

6.4.1. New functions

With respect to the salmon and carp functional and non-functional cDNAs, a number of lines of evidence suggest that several groups of fish including salmonids and castomids as well as the carp, *Cyprinus carpio* and some loaches have arisen from tetraploid ancestors. One observation of these studies is that a large percentage of loci have remained as functioning duplicates over long periods of time in these fishes (Bailey *et al.*, 1978). It has been estimated that salmonids appear to have retained 50-75% of their loci as duplicates since the tetraploidy occurred (Bailey *et al.*, 1978). Moreover, in the absence of selection, it would be expected that mutations and

genetic drift should have eliminated all or most of the gene duplicates since tetraploidy occurred.

The retention of 50% of the loci as functional duplicates may therefore result from the large effective size of salmonid populations. Bailey *et al.* (1978) studied the probability of the fixation of null alleles at unlinked duplicate loci. Under most conditions for populations of 2000-3000 or larger, unlinked duplicate loci will be sustained in the functional state longer than tandem (linked) duplicates and hence are available for evolution of new functions for a longer time (Bailey *et al.*, 1978). This may suggest that the two cDNAs, functional and non-functional, from both salmon and carp are linked, since in each case, one of their cDNAs doesn't produce a functioning desaturase.

It is also possible that the "non-functional" gene has a new function. Ohno (Ohno 1970) suggested that one of the two copies of duplicated genes may become less constrained by selection and thus be able to evolve toward a new function. However, recently Hughes and Hughes tested this hypothesis in the tetraploid *Xenopus laevis* and showed that both duplicate copies evolve at the same rate, with evidence of negative selection on both (Hughes & Hughes, 1993).

There are very few reports of genes evolving new functions. Duplicated genes evolving new functions include a pancreatic-trypsin like gene that produced a gene for antifreeze glycoproteins in Antarctic fish by expansion of repetitive regions in one copy of the original duplicated gene (Cheng and Chen, 1999). Mutations in duplicated opsin genes led to the evolution of trichromatic vision in New and Old world primates (Dulai *et al.*, 1999). One gene made redundant by tetraploidy evolved to encode a novel protein, ependymin. Ependymins are found only in the tetraploid species of teleosts. They are found in the cerebrospinal fluid of only cyprinid and

salmonid species of fish (Müller-Schmid *et al.*, 1992). Interestingly, the presence of ependymin has been demonstrated in the brain of man as well as mouse (Shashoua *et al.*, 1990). It has been implicated in synaptic changes associated with the consolidation of long-term memory formation. Moreover, if only tetraploid species among the teleosts are endowed with the gene encoding ependymin as implied (Müller-Schmid et al., 1992) it would constitute strong evidence that the mammalian ancestor, possibly fish, also underwent a round or two of tetraploidy (Ohno, 1993).

6.4.2. Subfunctionalisation

Subfunctionalisation is defined as the fixation of complementary loss-of-function alleles that result in the joint preservation of duplicate loci (Lynch & Force 1999). Possibly a simpler explanation is that subfunctionalisation occurs when duplicated genes divide the functions of their single ancestral gene. Stoltzfus has also suggested that partial loss-of-function mutations can lead to the preservation of duplicate genes with only a single function (Stoltzfus, 1999). Moreover, to quote Sidow 'a single unique function in an ocean of redundancy is enough to keep the gene afloat and prevent degenerative substitutions' (Sidow, 1996). Based on this hypothesis, it is conceivable that salmon and carp have other desaturase genes that encode products with different types of desaturase activity than those described in Chapter 4.

6.4.3. Gene silencing

In contrast to genome duplication facilitating the teleost radiation, the fate awaiting most duplicated genes appears to be loss or silencing due to degenerative mutations in coding and/or regulatory regions (Nei, 1969; Allendorf, 1978; Li, 1980). This is possibly the most likely explanation of what has happened in both the salmon and carp cases, where, assuming the non-functioning desaturase genes are separate

genes and not non-functioning alleles, the cDNAs could just be silenced genes. This has been discovered in extant tetraploid cyprinids where examinations of gene expression have shown frequent occurrences of "gene silencing" (Buth, 1979). However, if they are non-functioning alleles, then haploinsufficiency could occur. Haploinsufficiency occurs when the gene product from one functional allele is inadequate to support normal function (Lynch & Force, 2000). The salmonids are believed to be descended from a single tetraploid ancestor that underwent autotetraploidy 25-100 million years ago (Allendorf & Thorgaard, 1984). Disomic inheritance has become prevalent but salmonids are still undergoing diploidisation at least for a fraction of their genome (Hordvik, 1998). Thus salmonids carry a paralogous set of sequences representing the whole ancestral genome (Angers et al., 2002), and so salmonids may still have four alleles for a given locus. Therefore, the consequence of inheriting one functional allele can be as severe as inheriting only pseudogenes.

Recently, there have been two studies that have presented a model suggesting that loss or silencing of duplicated genes may be more important to the evolution of species diversity than the evolution of new functions in duplicated genes (Lynch and Conery, 2000; Lynch & Force, 2000). These studies describe how the loss of different genes in geographically separated populations could genetically isolate these populations should they become reunited. The model is as follows; individuals from a population are fixed for different unlinked copies of a duplicated gene. If these individuals mate, their hybrid progeny would be heterozygous, possessing a functional allele and a pseudogene at each locus of the duplicated gene. F2 individuals would either have only psuedogenes at both loci in question or receive between one and four functional alleles at these loci. The loss of different duplicates

in different populations is called 'divergent resolution'. Lynch and Conery stated that with tens to hundreds of gene duplicates present in most eukaryotic genomes, divergent resolution would result in the 'passive build-up of reproductive isolation' (Lynch & Conery, 2000).

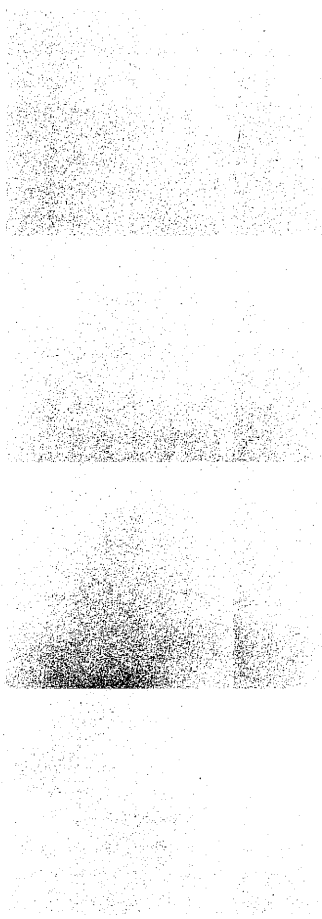
6.5. Summary

The high degree of gene duplication in fish by either tetraploidy or partial genome duplication is probably indicative of the scope of environments in which fish are found. The Teleostei have had 200 million years to evolve whereas the primates only diverged from the rodents approximately 41 million years ago (Kumar & Hedges, 1998). Consequently, the sequence similarities between the mammals show much greater homology than between each fish genus, and the functional and sequence differences in the fish desaturase genes emphasises this.

It is impossible to predict why the salmon and carp genomes contain non-functional "desaturase" cDNAs. They are either non-functional alleles at the same locus as the functioning alleles or duplicated genes at a different locus produced through some form of genome duplication which have incurred deleterious mutations rendering them either non-functional as desaturase genes or with new functions not observed in the experiments within this project. As to how they occurred, it is considered that, by and large, genes duplicated by tetraploidy show greater sequence diversion than tandemly duplicated genes (Ohno, 1993). Since the functional and non-functional "desaturase" cDNAs of both carp and salmon have high sequence homology, this would point to tandem duplication of the genes. However, tetrapolidisation has occurred recently and, therefore, it is also possible that they have occurred by tetraploidy. The occurrence of genome duplication may mean that carp and salmon have more than one functioning Δ5/6 like desaturase gene. This is of

great importance in the light of the fact that the functioning salmon desaturase is only efficient at desaturating *n*-3 fatty acids at the Δ5 position when it is known from enzymatic assays that salmon have high levels of Δ6 activity.

As for zebrafish, although it has been considered for many years that fish have more genes than mammals, this may not be the case for the zebrafish which, may only have one gene which codes for a protein capable of both Δ5 and Δ6 desaturation. However, as gene duplication has occurred in the zebrafish ancestral lineage, as shown with the *Hox* clusters, it is conceivable that they possess more than one gene, and that further desaturase genes remain to be isolated.



Chapter 7

Discussion and Conclusions

Chapter 7- Discussion and Conclusions

7.1. General considerations

The objectives of this study were to clone, sequence and functionally characterise the fatty acyl desaturase genes, involved in the biosynthesis of highly unsaturated fatty acids, of representative marine and freshwater teleosts in order to:

1. Further our understanding of the HUFA biosynthetic pathway in fish.
2. Attempt to relate desaturase gene structure with functional differences.
3. Establish how closely related fish desaturase genes are by comparison with other cloned and functionally characterised Δ5 and 6 desaturase genes from other organisms, and so contribute to our understanding of the evolution of fatty acid desaturation mechanisms and pathways.

Putative fatty acyl desaturase cDNAs were cloned from 4 fish species. These comprised two freshwater species, carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*), one anadromous species, the Atlantic salmon (*Salmo salar*) and one marine species, turbot (*Scophthalmus maximus*), and one partial fatty acyl desaturase sequence was cloned from another marine species, cod (*Gadus morhua*). Of those, the cDNAs of carp, zebrafish and salmon were functionally characterised in yeast (*Saccharomyces cerevisiae*). Three clones from carp were functionally characterised. Of these two proved to have no desaturase activity and one had significant Δ6 desaturase activity towards *n*-3 fatty acids, but considerably less Δ6 desaturase activity towards *n*-6 fatty acids. Only small amounts of Δ5 activity were observed for both *n*-3 and *n*-6 fatty acids. Three cDNAs were cloned from salmon and two were functionally characterised in yeast, of which one was found to have no Δ5 or Δ6 desaturase activity. The second clone was functional and in contrast to carp, had efficient Δ5 desaturase activity towards *n*-3 but not *n*-6 fatty acids, but showed little

Δ6 activity towards both *n*-3 and *n*-6 fatty acids. Only one clone was sequenced for the zebrafish, but unlike carp and salmon or any other Δ5 or Δ6 desaturase cloned to date including those from mammals, it had considerable Δ6 and Δ5 desaturase activity towards both *n*-3 and *n*-6 fatty acids. This prompted the hypothesis that the pathway in zebrafish represented a prototypic vertebrate pathway and that the subsequent separate mammalian Δ5 and Δ6 desaturases have resulted from gene or genome doubling of some sort since the separation of mammals from the vertebrate lineage.

Attempts were made to relate the structure of the coding sequence to the specific function of the cDNAs, for example what amino acid residue/s cause the zebrafish Δ5/6 fatty acids desaturase to have the specificity it has? What amino acids confer Δ5 or Δ6 desaturase activity? However, only speculations could be developed as to which areas and possible amino acid residues were important in determining the function of the resulting enzymes. It was concluded that the use of site directed mutagenesis and chimeras would be required to further elucidate the factors responsible for the specificity of the enzymes in relation to which fatty acids they desaturate.

However, some interesting results came from the phylogenetic analysis when it was apparent that all the fish desaturase cDNAs from the various fish species, no matter what their fatty acid specificity, showed greatest homology to mammalian Δ6 and Δ5 desaturases than to any other animal desaturase. It was also observed that all the fish cDNAs shared greater homology to the mammalian Δ6 desaturase than to the mammalian Δ5 desaturase, again no matter what their fatty acid specificity. This result reinforced the idea that by studying the structure and protein sequence of the

cDNA alone could give no clear indication as to the function of the resulting enzyme product.

7.2. Technical Considerations

Two major technical difficulties were encountered in the course of this study. The first was imposed by limitations inherent in the polymerase chain reaction, and the second arose from limitations of the yeast expression system used in the functional characterisation of the putative sequences.

The first major constraint was due to a general lack of nucleotide sequence information for the Δ5 and Δ6 desaturase enzymes of vertebrates. This information was essential for constructing degenerate primers which would allow the isolation of homologous genes from fish. Another constraint in the PCR method of isolation was the number of errors in the cloned sequences. A proofreading enzyme was used for isolation of the cDNAs to be inserted the yeast expression vector, and subsequent use in functional characterisation experiments. However, this may not eliminate all errors and only by sequencing from genomic DNA or DNA libraries could the number of errors be reduced. The PCR method, and restriction in time, also caused difficulties in obtaining the desaturase sequences of the marine fish species. Unfortunately, and for reasons unknown, the 5' end of the cod desaturase cDNA could not be cloned and insufficient time was available to resolve this problem. Time restrictions and problems with the PCR method also hampered the insertion of the turbot cDNA into the yeast expression vector. The insertion of cDNA could be reasonably simple, if the gene was highly expressed in the source animal. However, this was not the case for turbot, and as such, nested PCR was required to obtain sufficient product for insertion, but unforeseen technical difficulties arose and, due to time constraints, the

turbot cDNA could not be functionally characterised within the time frame of this project.

The second major constraint was due to the limitations of the yeast expression system. Firstly, in order to grow efficiently, the yeast were incubated and cultivated at 30°C. However, most of the fish used as sources for material to be cloned are usually found in temperate waters, with only the zebrafish found in tropical waters, and as such most of the enzymes coded for by the putative desaturase cDNAs would not be normally operate optimal, or indeed, at all in some cases at 30°C.

Secondly, the yeast expression system was constrained in that the entry of exogenous fatty acids into the yeast cells was proportional to chain length with the incorporation decreasing with increasing chain length. Thus, long chain C_{24} fatty acids were unable to enter the yeast cells to any significant degree and so testing for Δ6* desaturase activity proved very difficult to almost impossible. On only one occasion did appreciable amounts of C_{24} PUFA enter the yeast to allow analysis to show small amounts of Δ6* activity, but this was not robustly reproducible.

7.3. Contribution to knowledge of HUFA biosynthesis in fish

At the beginning of this study, none of the genes coding for the enzymes involved in the desaturation/elongation pathway to create HUFA from the C_{18} precursors had been cloned, sequenced or functionally characterised from any fish species. All knowledge of fatty acid desaturases came from studies on enzyme activities, most commonly from feeding trials or cell culture trials. These studies, however thorough, could not give definitive and fully conclusive answers as to what desaturase genes particular fish species possess, the number of genes, or how they function. This can only be achieved by genome analysis and with a molecular biological study of the

genes themselves. The present study went part of the way towards this, and included assessment of the specific function of individual genes. It was discovered, firstly, that the number of desaturase genes fish possess is perhaps not as straightforward to determine as that of the number of desaturase genes mammalian species possess. It is complicated by variations between fish species, the nature of their natural environments, their evolution in terms of number of genome duplications and the nature of their diets. Secondly, although still speculative, it appears that polyploidy may play an important role in determining the number of desaturase genes or in determining an individual fishes ability to biosynthesise HUFA due to differing alleles efficiencies. Finally, the present study has shown that marine fish do possess the necessary sequence information to code for the desaturase enzymes, but since the cDNAs from the marine species were not functionally characterised it is not possible to confirm that if they code for a functional desaturase product or whether they are pseudogenes or non-functioning alleles.

7.4. Contribution to the knowledge of fatty acyl desaturase evolution

The fatty acyl desaturases have been cloned and functionally characterised for microalgae, fungi and higher plants, but it's only in the past 4 to 5 years that there have there been extensive studies on the molecular structure of Δ6 and Δ5 fatty acyl desaturase genes from the animal kingdom. Now this information exists from nematodes to rodents and of course from humans. However, none of the Δ6 and Δ5 fatty acid desaturase genes, from any of the organisms above appear to have more than one type of desaturase activity. The zebrafish is not only the first fish desaturase gene to be entered onto the GenBank database, but the first published example of a desaturase having two types of activity, Δ5 and Δ6 that are both physiologically significant (Hastings *et al.*, 2001). However, even although a proofreading

polymerase enzyme was used in the cloning of the functionally characterised cDNA, there is still a chance that the zebrafish gene is an artefact generated by PCR. However, this is thought unlikely as no other sequences have been discovered that could account for more Δ5/6 like clones. This can only be ruled out by further functional characterisation of more clones. The carp and the salmon desaturases cloned here may also have both these activities, but only to a very limited extent. It is possible, as reported above, that the zebrafish represents a prototypic HUFA synthesis pathway and that the mammalian system has developed since the divergence of the mammalian line. The fact that humans and other mammals have two distinct enzymes for Δ6 and Δ5 desaturation may be an adaptation to a terrestrial diet providing relatively lower amounts of preformed C_{20} and C_{22} PUFAs than the diets of a vertebrate ancestor that they share with freshwater fish. This study has presented a new perspective on the evolution of the desaturase enzymes but further studies are required to discover if the zebrafish and other fish species have further genes.

7.5. Consequences for fish and human health

The inclusion of supplemental *n*-3 HUFA such as EPA and DHA in the human diet has been a much discussed topic in the last few years. The consumption of fish was the traditional way that *n*-3 HUFA were included in the human diet, but fish consumption has been steadily decreasing as a proportion of the diet in the UK and many "western" countries and so supplements delivered in the form of oral capsules have been produced as an alternative. The supplements usually comprise fish oils rich in *n*-3 HUFA such as cod liver oil or body oils from other fish species. However, recently there has been interest in microalgae, including diatoms and flagellates for the production of HUFA. Diatoms such as *Stauroneis amphioxys,* the Rhodophyte,

Olisthodiscus sp., and the Prymnesiophyte *Pavlova salina* contain AA, EPA and DHA. However, few possess AA, EPA and DHA all in significant amounts, and most diatoms contain only AA and EPA, but predominantly EPA. In most of the Dinoflagellates DHA is the major HUFA (Cohen *et al.*, 1995). The difficulty and cost in acquiring the oils from single cell organisms is great. Difficulties arise in retaining good growth conditions, with the cultures often becoming contaminated. There is also a high cost incurred with the extraction of the lipids (Ratledge, 1993; Cohen *et al.*, 1995). The direct feeding of these microorganisms to rats has been tested as an alternative to lipid extraction. It was discovered that undisrupted cells were difficult to digest and the only way to ensure they were digestible and therefore beneficial was by disrupting the cells (Sukenik *et al.*, 1994). Feeding microalgal and fungal oils rich in DHA from *Crypthecodium cohnii* and AA from *Mortierella alpina* is safe, but the way in which it is administered is obviously important (Innis and Hansen, 1996). Another alternative way to increase HUFA intake is by eating enriched eggs by feeding chickens oils such as cod liver oil (Scheideler *et al.*, 1997). The eggs the hen lays are then found to have greater levels of HUFA than non-enriched eggs. The type of increase in HUFA levels depends on the oil incorporated into the feed. However, eggs are high in the low density lipoprotein cholesterol (LDL-C) and this may be a negative effect if *n*-3 HUFA-enriched eggs are to be eaten on a regular basis for the provision of *n*-3 HUFA. In a consumer acceptance study little difference in the taste was found (Scheideler *et al.*, 1997). In any case, feeding fish oils to chickens does not address the problem of a lack of *n*-3 HUFA in the world in general.

Therefore, the consumption of fish remains the most efficient and acceptable way to increase *n*-3 HUFA, intake in the human diet, with marine fish being the most

beneficial. Therefore, one way of increasing n-3 HUFA supply in the world is to produce fish that can biosynthesise HUFA by desaturating 18:3*n*-3 fed in the form of vegetable oils. One way to do this is by transgenics, the introduction of foreign genes such as Δ5 and Δ6 desaturases into marine fish. At present GM food is unacceptable to a number of western societies, but this may not be the case for future generations. The zebrafish gene could be utilised in the GM modification of fish to produce a marine or freshwater fish capable of high levels of Δ5 and Δ6 desaturation of C_{18} precursors. It may be more acceptable to insert a fish desaturase gene into a fish rather than into a plant and or single cell organism. However, this is another possibility. Moreover it may be possible to insert into plants the Δ5/6 zebrafish desaturase gene along with zebrafish elongase gene that is also known to have a dual function (Agaba, M., pers. communication) into and produce a crop high in HUFA. As only two trans genes would be necessary it would make the process simpler. For the time being this remains a theoretical possibility.

However, another alternative is possible. If the salmon and carp cDNAs are poorly functioning alleles it may be that somewhere within the population are animals with highly active alleles. If this is the case then once the highly efficient alleles had been sequenced, selective breeding programmes could be adopted to breed animals and populations of fish with highly functioning alleles which would, therefore, require less HUFA in their diets and could be grown on higher levels of C18 precursors from vegetable oils.

7.6. Concluding remarks and prospects

The cloning and sequencing of the fish desaturase genes presents an opportunity to increase understanding at the molecular level of the process of HUFA biosynthesis. The results presented here represent the first instance of the functional

characterisation of the desaturase genes involved in HUFA biosynthesis in fish. This study is a first step in understanding how the genes operate out-with the environment in which they are usually found, the fish, and moreover, presents theories of how the genes operate within the animal. However, to fully understand the results of this study further cloning and functional characterisation of more cDNA clones is required in all of the fish species used in the studies described. To fully understand how these genes are controlled and function within the fish several other lines of investigation must be followed.

The genomic sequence must be determined to examine possible regulating sequences. In this project only the function of isolated cDNAs was tested, but it is highly likely that the expression and regulation of the genes involved in HUFA synthesis is modulated by promoter or other regulatory sequences within the genes. However, external regulating factors such as those due to functions of other genes may be responsible for the differing levels of activity observed in the functionally characterised desaturases of fish from different environments.

This project has opened up the field of fatty acyl desaturase molecular biology by describing some novel fatty acid desaturase genes. In particular, the gene from zebrafish codes for an enzyme with a fatty acid specificity not reported in animals hitherto. In addition to some answers, it has led to more questions, all of which present exciting challenges for future investigations.

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Appendix I - Formulation of Solutions, Reagents and Media

LB Medium (1litre)

25 g LB broth (MERCK)

Water to 1 litre

LB/Ampicillin/X-gal/IPTG plates (1 litre)

25 g LB broth (MERCK)

15 g agar bacteriological No.1 (Oxoid, Hampshire, U.K.)

1 mg X-gal (5-bromo-4-chloro-3-indoyl-β-galactopyranoside)

0.8 mg IPTG (isoproylthio-β-D-galactoside)

10 X TBE electrophoresis buffer, per litre

108 g Tris (BioRad labs, Hercules, CA, U.S.A.)

55 g Boric acid (BioRad labs, Hercules, CA, U.S.A.)

8.3 g EDTA (BDH)

YPD (Yeast extract peptone dextrose) medium/plates, per litre

10 g yeast extract (Oxoid, Hampshire, U.K.)

20 g peptone (GibcoBRL, NY, U.S.A.)

20 g dextrose (BDH, Poole, U.K.)

Yeast -Uracil Selective Medium (1 litre)

7 g nitrogen base (Sigma-Aldrich, Dorset, U.K.)

1.92 g –uracil yeast synthetic drop out media supplement (Sigma-Aldrich, Dorset, U.K.).

10 ml Tergitol NP-40 (Sigma-Aldrich, Dorset U.K.)

Water to 1 litre

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25% Galactose, per 50 ml

12.5 g galactose (Sigma-Aldrich, Dorset, U.K.)

Water to 50 ml

20% Raffinose

10 g	raffinose (Sigma-Aldrich, Dorset, U.K.)
Water to 50 ml	

Methylation Reagent

1ml H_2SO_4

100 ml MeOH

Picolinyl Derivatising Reagent

450 µls	3-(hydroxymethyl) pyridine (Sigma-Aldrich, Dorset, U.K.)
4.5 mls	dichloromethane (Fisher, distol grade)
4.5 mls	triethylamine (Sigma-Aldrich, Dorset, U.K.)
2-3 mm	anhydrous sodium sulphate (Fisher)

1,1'-carbonyldiimidazole Solution

0.5 mg	1,1'-carbonyldiimidazole (Sigma-Aldrich, Dorset, U.K.)
5 ml	dichloromethane (Fisher)

Loading Dye

30%	glycerol in water
0.25%	Bromophenol blue (Sigma-Aldrich, Dorset, U.K.)
0.25%	Xylene cyanole (Sigma-Aldrich, Dorset, U.K.)

Fatty Acid Desaturase Substrates

C18 fatty acid substrates

30 mg	18:2*n*-6 or 18:3*n*-3 (Sigma-Aldrich, Dorset, U.K.)
200 µl	1 M NaOH (BDH, Poole, U.K.)
800 µl	5.6% tergitol NP-40

C20 fatty acid substrates

45 mg	20:3*n*-6 (>98% purity) (Sigma-Aldrich, Dorset, U.K.) or 20:4*n*-3 (>98% purity); Cayman Chemicals,AnnArbor, MI, U.S.A.). (98-99% purity)

250 µl	1 M NaOH
750 µl	5.6% tergitol NP-40

C22 fatty acid substrates

60 mg	22:4*n*-6 or 22:5*n*-3 (>98% purity) (Cayman Chemicals, AnnArbor, MI, U.S.A.)
300 µl	1 M NaOH
700 µl	5.6% tergitol NP-40

C24 fatty acid substrates

90 mg	24:4*n*-6 or 24:5*n*-3 (Dr. A.E.A. Porter, Department of Biological Sciences, University of Stirling)
500 µl	1 M NaOH
1150 µl	5.6% tergitol NP-40

Appendix II – Kit Components and Basic Instructions

GFX™ Micro Plasmid Prep Kit (Amersham Biosciences)

Components of kit

Solution I:	100 mM Tris-HCl (pH 7.5), 10 mM EDTA, 400 μg/ml Rnase I.
Solution II:	1 M NaOH, 5.3% (w/v) SDS (5.3X concentrated solution).
Solution III:	Buffered solution containing acetate and chaotrope.
GFX columns:	MicroSpin columns pre-packed with a fibre glass matrix.
Collection tubes:	2 ml capless microcentrifuge tubes.
Wash buffer:	Tris-EDTA buffer in 80% ethanol.

Cell Lysis

- 1.5 ml of an overnight *E. coli* culture was transferred to a 1.5 ml microcentrifuge tube then centrifuged at full speed in a microcentrifuge for 30 seconds to pellet the cells.
- As much supernatant as possible was removed by aspiration without disturbing the cell pellet.
- An additional aliquot of culture (to give 2-3 ml total volume of culture) was added to the microcentrifuge tube, centrifuged at full speed for 30 seconds and the supernatant removed by aspiration as described above.
- The pellet was then resuspended in 300 μl solution I with vigorous vortexing.
- 300 μl of solution II was added to the microcentrifuge tube and mixed by inverting the tube 10-15 times.
- 600 μl of solution III was added and mixed by inverting the tube until a flocculent precipitate appeared with inverting continuing until the precipitate was evenly dispersed.
- The microcentrifuge tube containing the flocculent precipitate was then centrifuged for 5 minutes at room temperature to pellet the cells.
- One GFX™ column for each preparation was prepared by placing the column into a collection tube.

DNA purification

- Approximately half of the supernatant was transferred to the prepared GFX™ column, incubated for 1 minute and centrifuged at full speed for 30 seconds.
- The flow-through was discarded by emptying the collection tube and the remaining supernatant transferred to the same GFX™ column.
- The GFX column was incubated for a further minute at room temperature before being centrifuged for 30 seconds at full speed.
- The flow-through was once again discarded as above.
- 400 µl of wash buffer was added to the column. The column was centrifuged at full speed for 30 seconds to remove the buffer and dry the matrix prior to elution.
- The GFX column was transferred to a clean microcentrifuge tube, not a collection tube, and 50 to 75 µl of H_2O added to the top of the glass fibre matrix in the GFX column.
- The column was incubated at room temperature for 1 minute before being centrifuged for 1 minute to remove the purified plasmid DNA.

GFX™ PCR DNA and Gel Band Purification Kit

Purification of DNA from Gel Bands

Components of Kit

Capture Buffer: Buffered solution containing acetate and chaotrope.

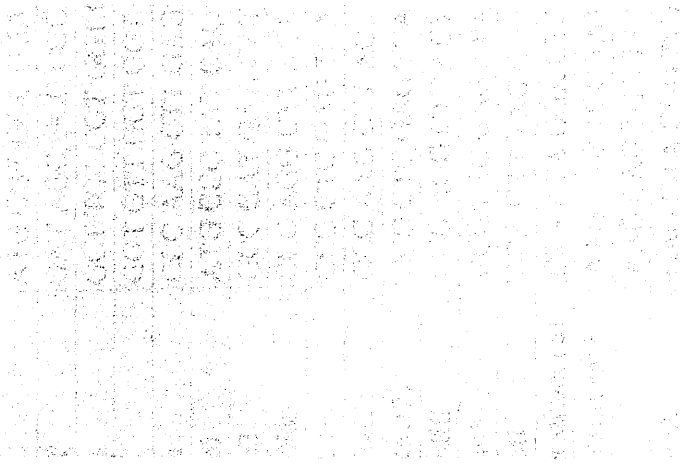
GFXTM Columns: MicroSpin (2) columns pre-packed with a glass fibre matrix.

Collections Tubes: 2 ml capless microcentrifuge tubes

Wash Buffer: Tris-EDTA buffer in 80% ethanol.

- An empty 1.5 ml microcentrifuge tube was weighed to the nearest 10 mg.
- Using a clean scalpel a slice of the agarose gel containing the DNA band to be purified was excised. This was then transferred to the microcentrifuge tube and the tube weighed again to determine the weight of the slice.
- To the slice of agarose 10 µl of capture buffer for each 10 mg of gel slice was added.
- The tube was closed, vortexed vigorously and incubated at 60°C for 5-15 minutes.

- During the incubation one GFX column for each purification was placed into a collection tube.
- Once the agarose was completely dissolved the microcentrifuge was centrifuged briefly to collect the sample at the bottom of the tube.
- Once complete the sample was transferred to the prepared GFX column and incubated at room temperature for 1 minute then centrifuged at full speed for 30 seconds.
- The flow-through was discarded by emptying the collection tube.
- 500 µl of wash buffer was added to the GFX column. The column was centrifuged once again at full speed for 30 seconds.
- The collection tube was discarded and the GFX column was transferred to a fresh 1.5 ml microcentrifuge tube.
- 25-50 µl of H_2O was added to the column and incubated for 1 minute at room temperature.
- Finally, the column in the microcentrifuge tube was centrifuged at full speed for 1 minute to recover the purified DNA.



Appendix III - Primer Sequences

Primer Name	Primer Sequence (5'-3')	Length
ZF2A	ATG GGT GGC GGA GGA CAG C	22-mer
Not1polyT	GAT AGC GCC CGC GTT TTT TTT TTT TTT TTV	30-mer
Cod1Rev	GCT TCT CGT GGT CTA TGT TCA T	22-mer
Cod3Rev	GGT GTT GGT GGT GAT AGG GCA T	22-mer
FishD6For	CCC AAG CTT GAG GAT GGG DGG	21-mer
Cod3Race1	ATG CCC TAT CAC CAC CAA CAC C	22-mer
Cod3Race3	GCC GAT GAA CAT AGA CCA CG	20-mer
TurbpYesFor	CCC AGG CTT ACT ATG GGA GGT GGG GGC C	28-mer
TurbpYesRev	CCG CTC GAG TCA TTT ATG GAG ATA TGC AT	27-mer
SalpYesFor(2)	CCC AAG CTT ACT ATG GGG GGC GGA GGC G	28-mer
SalpYesRev(2)	CCG CTC GAG TCA TTT ATG GAG ATA TGC AT	29-mer
ZF2AHindIII	CCG AGG CTT ACT ATG GGT GGC GGA GGA CAG	31-mer
ZFRevXhoI	CCG CTC GAG TTA TTT GTT GAG ATA CGC	27-mer
CpRevNotI	ATA GTT TAG CGG CCG CTT ATT CGT TGA GGT ACG C	34-mer
UPM (constituent a)	CTA ATA GGA CTC ACT ATA GGG CAA GCA GTG GTA TCA ACG CAG AGT	45-mer
UPM (constituent b)	AAG CAG TGG TAT CAA CGC AGA GT	23-mer
NUP	AAG CAG TGG TAT CAA CGC AGA GT	23-mer
Turb3RaceA	GAG CAA GGG TCT GTT TCA AGC	21-mer
Turb3RaceB	CCG CTG GAA TCA CTT GTT GC	20-mer
Smart II A oligo	AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG	30-mer
SalFor5	GGA GCA TTT TCC GCA TTC C	19-mer
T7 promoter	TAA TAC GAC TCA CTA TAG GG	20-mer
UniD56-2A	TTY CAR CAY CAY GCN AAR CC	20-mer

Appendix III - Primer Sequences

Uni5/63A	CAR CAY CAY GCN AAR CCN AA	20-mer
Uni5/63B	RAA NAR RTG YTC DAT YTG	18-mer
M13FOR	GTA AAA CGA CGG CCA GT	17-mer
M13REV	GGA AAC AGC TAT GAC CAT G	19-mer
FishD6rev	TYA TTT RTK GAG ATA YGC AYC CAG CCA	21-mer
CPpYesFor1	CCC TGA TGT TGT TGT GGT ACT	21-mer
5'-RACE CDS Primer	5'-(T)25N-1N-3' (N=A,C,G, or T; N-1+A,G, or C)	

Appendix IV – Zebrafish (*Danio rerio*) nucleotide sequences

ZFD6 1 atgggtggcggaggacagcagacagaccgaatcaccgacaccaacggcag

ZFD6 51 attcagcagctacacctgggaggaggtgcagaaacacaccaaacatggag

ZFD6 101 atcagtgggtggtggtggagaggaaggtttataacgtcagccagtgggtg

ZFD6 151 aagagacaccccggaggactgaggatcctcggacactatgctggagaaga

ZFD6 201 cgccacggaggcgttcactgcgtttcatccaaaccttcagctggtgagga

ZFD6 251 aatacctgaagccgctgctaatcggagagctggaggcgtctgaacccagt

ZFD6 301 caggaccggcagaaaaacgctgctctcgtggaggatttccgagccctgcg

ZFD6 351 tgagcgtctggaggctgaaggctgttttaaaacgcagccgctgtttttcg

ZFD6 401 ctctgcatttgggccacattctgctcctggaggccatcgctttcatgatg

ZFD6 451 gtgtggtatttcggcaccggttggatcaacacgctcatcgtcgctgttat

ZFD6 501 tctggctactgcacagtcacaagctggatggttgcagcatgacttcggtc

ZFD6 551 atctgtccgtgtttaaaacctctggaatgaatcatttggtgcacaaattt

ZFD6 601 gtcatcggacacctgaaggggagcgtctgcgggctggtggaaccatcggca

ZFD6 651 cttccagcatcacgctaaacccaacatcttcaagaaggacccggacgtca

ZFD6 701 acatgctgaacgccttttgtggtgggaaacgtgcagcccgtggagtatggc

ZFD6 751 gttaagaagatcaagcatctgccctacaaccatcagcacaagtacttctt

ZFD6 801 cttcattggtcctccccctgctcatcccagtgtatttccagttccaaatct

ZFD6 851 ttcacaatatgatcagtcatggcatgtgggtggacctgctgtggtgtatc

ZFD6 901 agctactacgtccgatacttcctttgttacacgcagttctacggcgtctt

ZFD6 951 ttgggctattatcctctttaatttcgtcaggtttatggagagccactggt

ZFD6 1001 ttgtttgggtcacacagatgagccacatccccatgaacattgactatgag

ZFD6 1051 aaaaatcaggactggctcagcatgcagctggtcycgacctgtaacatcga

ZFD6 1101 gcagtctgccttcaacgactggttcagcggacacctcaacttccagatcg

ZFD6 1151 agcatcatctctttccacagtgcctcggcacaactactggcgcgccgct

ZFD6 1201 ccacgggtgcgaggcgttgtytgagaaatacgyygtcaaataccaagagaa

ZFD6 1251 gacottgtacggcgcatttgcgggtatcattaggtctttggagaaatctg

ZFD6 1301 gcgagctctggctggatgcgtatctcaacaaataaagcatgacttctccc

ZFD6 1351 taaatagaaaaaaagcggagccgaatcctccaatcagagcgagccttcac

ZFD6 1401 agactagacatcaggaagaatcgcgactaaaccccccagtcaatcagttca

ZFD6 1451 gtaaaatgacgcgtagcactttgtttttgatatctgcaagataattgtg

ZFD6 1501 gcactttgaatgcgttttaaagagacggctcaaagatgtgtgtctcgctc

ZFD6 1551 ctaataaaggaagaatctgaaaggaaaaaaaaaaaaaaaa

Appendix V – Carp (*Cyprinus carpio*) nucleotide sequences

A: Putative Open Reading Frames

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CPpYESA5   1    ATGGGTGGCGGAGGACAGCAGACGGACCGGATCACCGGGACCAACGCGAG
CPpYESA7   1    ATGGGTGGCGGAGGACAGCAGACGGACCGGATCACCGGGACCAACGCGAG
CPpYESA9   1    ATGGGTGGCGGAGGACAGCAGACGGACCGGATCGCCGGTACCAATGCGAG

CPpYESA5   51   GTTCAGCACTTACACCTGGGAGGAGGTACAGAAACACACTAAGTCTGGAG
CPpYESA7   51   GTTCAGCACTTACACCTGGGAGGAGGTACAGAAACACACTAAGTCTGGAG
CPpYESA9   51   GTTCGGCACATACACCTGGGAGGAGGTGCAGAAACACACTAAGTCTGGAG

CPpYESA5   101  ATCAGTGGATCGTGGTGGAAAGGAAGGTGTATAATGTGAGCCAGTGGGTG
CPpYESA7   101  ATCAGTGGATCGTGGTGGAAAGGAAGGTGTATAATGTGAGCCAGTGGGTG
CPpYESA9   101  ATCAGTGGATCGTGGTGGAAAGGAAGGTTTATAATGTGAGCCAGTGGGTG

CPpYESA5   151  AAGAGACACCCCGGAGGACTAAGGATCATCGGACACTATGCTGGAGAAGA
CPpYESA7   151  AAGAGACACCCCGGAGGACTAAGGATCATCGGACACTATGCTGGAGAAGA
CPpYESA9   151  AAGAGACACCCCGGAGGACTGAGGATCATCGGACACTATGCTGGAGAAGA

CPpYESA5   201  TGCCACGGAGGCGTTTACTGCGTTTCGTCCAGACCTTCCGCTGGTGAGGA
CPpYESA7   201  TGCCACGGAGGCGTTTACTGCGTTTCATCCAGACCTTCCGCTGGTGAGGA
CPpYESA9   201  TGCCACGGATGCATTTCATGCGTTTCATCCAAACATTCAACTCGTGAGGA

CPpYESA5   251  AATACATGAAGCCGCTGTTAATCGGGGAGCTGGAGGCGTCTGAACCCAGT
CPpYESA7   251  AATACATGAAGCTGCTGTTAATCGGGGAGCTGGAGGCGTCTGAACCCAGT
CPpYESA9   251  AATACATGAAGCCGCTGTTAATTGGGGAGCTTGAGGCATCTGAACCCAGT

CPpYESA5   301  CAAGACCGCCAGAAAAACGCTGCTCTTGTGGAGGATTTCCGAGCCCTTCG
CPpYESA7   301  CAAGACCGCCAGAAAAACGCTGCTCTTGTGGAGGATTTCCGAGCCCTTCG
CPpYESA9   301  CAAGACCGCCAGAAAAACGCGGCTCTTGTGGAGGATTTCCGAGCCCTTCG

CPpYESA5   351  TGAGCGTCTGGAAGCTGAGGGGTGTTTCAAAACCCAGCCCCTGTTTTTCC
CPpYESA7   351  TGAGCGTCTGGAAGCTGAGGGGTGTTTCAAAACCCAGCCCCTGTTTTTCC
CPpYESA9   351  TGAGCGTCTGGAGGCTGAGGGGTGTTTCAAAACCCAGCCCCTGTTTTTCA

CPpYESA5   401  TCCTGCATCTGGGTCACATCCTGCTCCTGGAGGTCATCGCCCTGATGCTG
CPpYESA7   401  TCCTGCATCTGGGTCACATCCTGCTCCTGGAGGTCATCGCCCTGATGCTG
CPpYESA9   401  TCCTGCATCTGGGTCACATCCTGCTCCTGGAGGCCATCGCCCTGATGTTG

CPpYESA5   451  GTGTGGTACTTTGGAACCGGCTGGATCAACACGGCCATCGTTGCTGTTTT
CPpYESA7   451  GTGTGGTACTTTGGAACCGGCTGGATCAACACGGCCATCGTTGCTGTTTT
CPpYESA9   451  TTGTGGTACTTTGGAACCGGCTGGATCAACACGGCCATCGTTTCTGTTAT

CPpYESA5   501  AATGGCTACCGCACAGTCGCAGGCTGGATGGCTGCAGCATGACTTCGGTC
CPpYESA7   501  AATGGCTACCGCACAGTCGCAGGCTGGATGGCTGCAGCATGACTTCGGTC
CPpYESA9   501  ACTGGCTACCGCACAGTCGCAGGCTGGATGGTTGCAGCATGACTTCGGTC

CPpYESA5   551  ATCTGTCTGTCTGTAAATCCTCTCGATGGAATCACTTAGTGCACAAATTT
CPpYESA7   551  ATCTGTCTGTCTGTAAATCCTCTCGATGGAATCACTTAGTGCACAAATTT
CPpYESA9   551  ATCTGTCCGTGTTCAAAAACTCACGATGGGATCACTTACTGCACAAATTT
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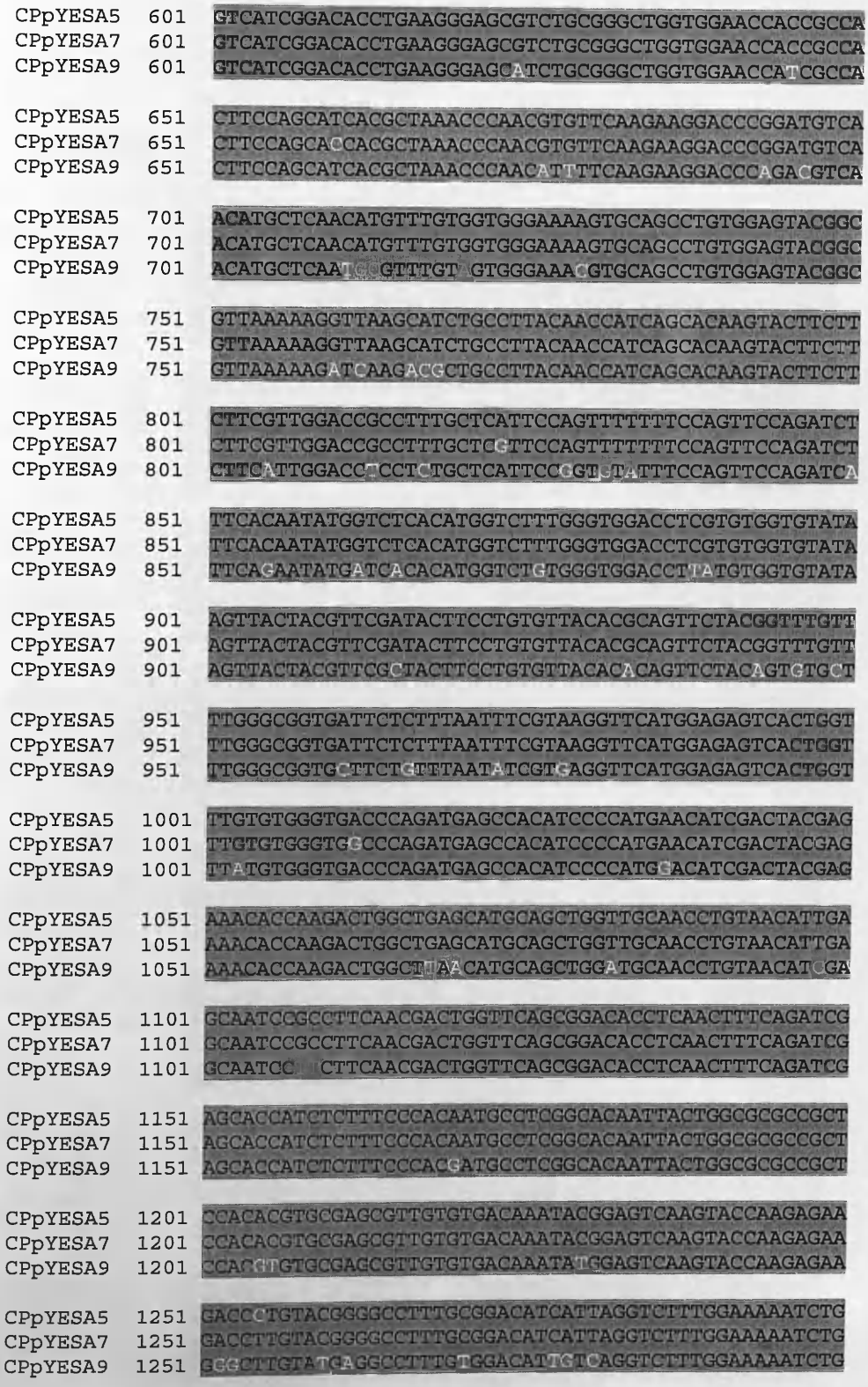
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CPpYESA9 601 GTCATCGGACACCTGAAGGGAGCATCTGCGGGCTGGTGGAACCATCGCCA
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CPpYESA7 651 CTTCCAGCACCACGCTAAACCCAACGTGTTCAAGAAGGACCCGGATGTCA
CPpYESA9 651 CTTCCAGCATCACGCTAAACCCAACATTTTCAAGAAGGACCCAGACGTCA
CPpYESA5 701 ACATGCTCAACATGTTTGTGGTGGGAAAAGTGCAGCCTGTGGAGTACGGC
CPpYESA7 701 ACATGCTCAACATGTTTGTGGTGGGAAAAGTGCAGCCTGTGGAGTACGGC
CPpYESA9 701 ACATGCTCAATCCGTTTGTAGTGGGAAACGTGCAGCCTGTGGAGTACGGC
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CPpYESA7 751 GTTAAAAAGGTTAAGCATCTGCCTTACAACCATCAGCACAAGTACTTCTT
CPpYESA9 751 GTTAAAAAGATCAAGACGCTGCCTTACAACCATCAGCACAAGTACTTCTT
CPpYESA5 801 CTTCGTTGGACCGCCTTTGCTCATTCCAGTTTTTTTCCAGTTCCAGATCT
CPpYESA7 801 CTTCGTTGGACCGCCTTTGCTCGTTCCAGTTTTTTTCCAGTTCCAGATCT
CPpYESA9 801 CTTCATTGGACCTCCTCTGCTCATTCCGGTGTATTTCCAGTTCCAGATCA
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CPpYESA9 851 TTCAGAATATGATCACACATGGTCTGTGGGTGGACCTTATGTGGTGTATA
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CPpYESA7 901 AGTTACTACGTTCGATACTTCCTGTGTTACACGCAGTTCTACGGTTTGTT
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CPpYESA9 951 TTGGGCGGTGCTTCTGTTTAATATCGTGAGGTTCATGGAGAGTCACTGGT
CPpYESA5 1001 TTGTGTGGGTGACCCAGATGAGCCACATCCCCATGAACATCGACTACGAG
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CPpYESA9 1001 TTATGTGGGTGACCCAGATGAGCCACATCCCCATGGACATCGACTACGAG
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CPpYESA7 1101 GCAATCCGCCTTCAACGACTGGTTCAGCGGACACCTCAACTTTCAGATCG
CPpYESA9 1101 GCAATCC CTTCAACGACTGGTTCAGCGGACACCTCAACTTTCAGATCG
CPpYESA5 1151 AGCACCATCTCTTTCCCACAATGCCTCGGCACAATTACTGGCGCGCCGCT
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CPpYESA9 1151 AGCACCATCTCTTTCCCACGATGCCTCGGCACAATTACTGGCGCGCCGCT
CPpYESA5 1201 CCACACGTGCGAGCGTTGTGTGACAAATACGGAGTCAAGTACCAAGAGAA
CPpYESA7 1201 CCACACGTGCGAGCGTTGTGTGACAAATACGGAGTCAAGTACCAAGAGAA
CPpYESA9 1201 CCACGTGTGCGAGCGTTGTGTGACAAATATGGAGTCAAGTACCAAGAGAA
CPpYESA5 1251 GACCCTGTACGGGGCCTTTGCGGACATCATTAGGTCTTTGGAAAAATCTG
CPpYESA7 1251 GACCTTGTACGGGGCCTTTGCGGACATCATTAGGTCTTTGGAAAAATCTG
CPpYESA9 1251 GGGCTTGTATGAGGCCTTTGTGGACATTGTCAGGTCTTTGGAAAAATCTG

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CPpYESA5   1301 GAGAACTCTGGCTGGATCCGTACCTCAACGAA
CPpYESA7   1301 GAGAACTCTGGCTGGATCCGTACCTCAACGAA
CPpYESA9   1301 GAGAACTCTGGCTGGATCCGTACCTCAACGAA
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B: 3'UTRs

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CP3'B3   1    CACAGTCGCAGGCTGGATGGTTGCGGCATGACTTCGGTCATCTGTCCGTG
CP3'C9   1    CACAGTCGCAGGCTGGATGGTTGCGGCATGACTTCGGTCATCTGTCCGTG
CP3'C7   1    CACAGTCGCAGGCTGGATGGTTGCAGCATGACTTCGGTCATCTGTCCGTG
CP3'B2
CP3'C6

CP3'B3   51   TTCAAAAACTCACGATGGGATCACTTACTGCACAAATTTGTCATCGGACA
CP3'C9   51   TTCAAAAACTCACGATGGGATCACTTACTGCACAAATTTGTCATCGGACA
CP3'C7   51   TTCAAAAACTCACGATGGGATCACTTACTGCACAAATTTGTCATCGGACA
CP3'B2
CP3'C6

CP3'B3   101  CCTGAAGGGAGCATCTGCGGGCTGGTGGAACCATCGCCACTTCCAGCATC
CP3'C9   101  CCTGAAGGGAGCATCTGCGGGCTGGTGGAACCATCGCCACTTCCAGCATC
CP3'C7   101  CCTGAAGGGAGCATCTGCGGGCTGGTGGAACCATCGCCACTTCCAGCATC
CP3'B2
CP3'C6

CP3'B3   151  ACGCTAAACCCAACATTTTCAAGAAGGACCCAGACGTCAACATGCTCAAT
CP3'C9   151  ACGCTAAACCCAACATTTTCAAGAAGGACCCAGACGTCAACATGCTCAAT
CP3'C7   151  ACGCTAAACCCAACATTTTCAAGAAGGACCCAGACGTCAACATGCTCAAT
CP3'B2
CP3'C6

CP3'B3   201  GCGTTTGTAGTGGGAAACGTGCAGCCTGTGGAGTACGGCGTTAAAAAGAT
CP3'C9   201  GCGTTTGTAGTGGGAAACGTGCAGCCTGTGGAGTACGGCGTTAAAAAGAT
CP3'C7   201  GCGTTTGTAGTGGGAAACGTGCAGCCTGTGGAGTACGGCGTTAAAAAGAT
CP3'B2
CP3'C6

CP3'B3   251  CAAGACGCTGCCTTACGACCATCAGCACAAGTACTTCTTCTTCATTGGAC
CP3'C9   251  CAAGACGCTGCCTTACGACCATCAGCACAAGTACTTCTTCTTCATTGGAC
CP3'C7   251  CAAGACGCTGCCTTACAACCATCAGCACAAGTACTTCTTCTTCATTGGAC
CP3'B2
CP3'C6

CP3'B3   301  CTCCTCTGCTCATTCCGGTGTATTTCCAGTTCCAGATCATTCAGAATATC
CP3'C9   301  CTCCTCTGCTCATTCCGGTGTATTTCCAGTTCCAGATCATTCAGAATATC
CP3'C7   301  CTCCTCTGCTCATTCCGGTGTATTTCCAGTTCCAGATCATTCAGAATATC
CP3'B2
CP3'C6

CP3'B3   351  ATCACACATGGTCTGTGGGTGGACCTTATGTGGTGTATAAGTTACTACGT
CP3'C9   351  ATCACACATGGTCTGTGGGTGGACCTTATGTGGTGTATAAGTTACTACGT
CP3'C7   351  ATCACACATGGTCTGTGGGTGGACCTTATGTGGTGTATAAGTTACTACGT
CP3'B2   1    ----------TATGTGGTGTATAAGTTACTACGT
CP3'C6
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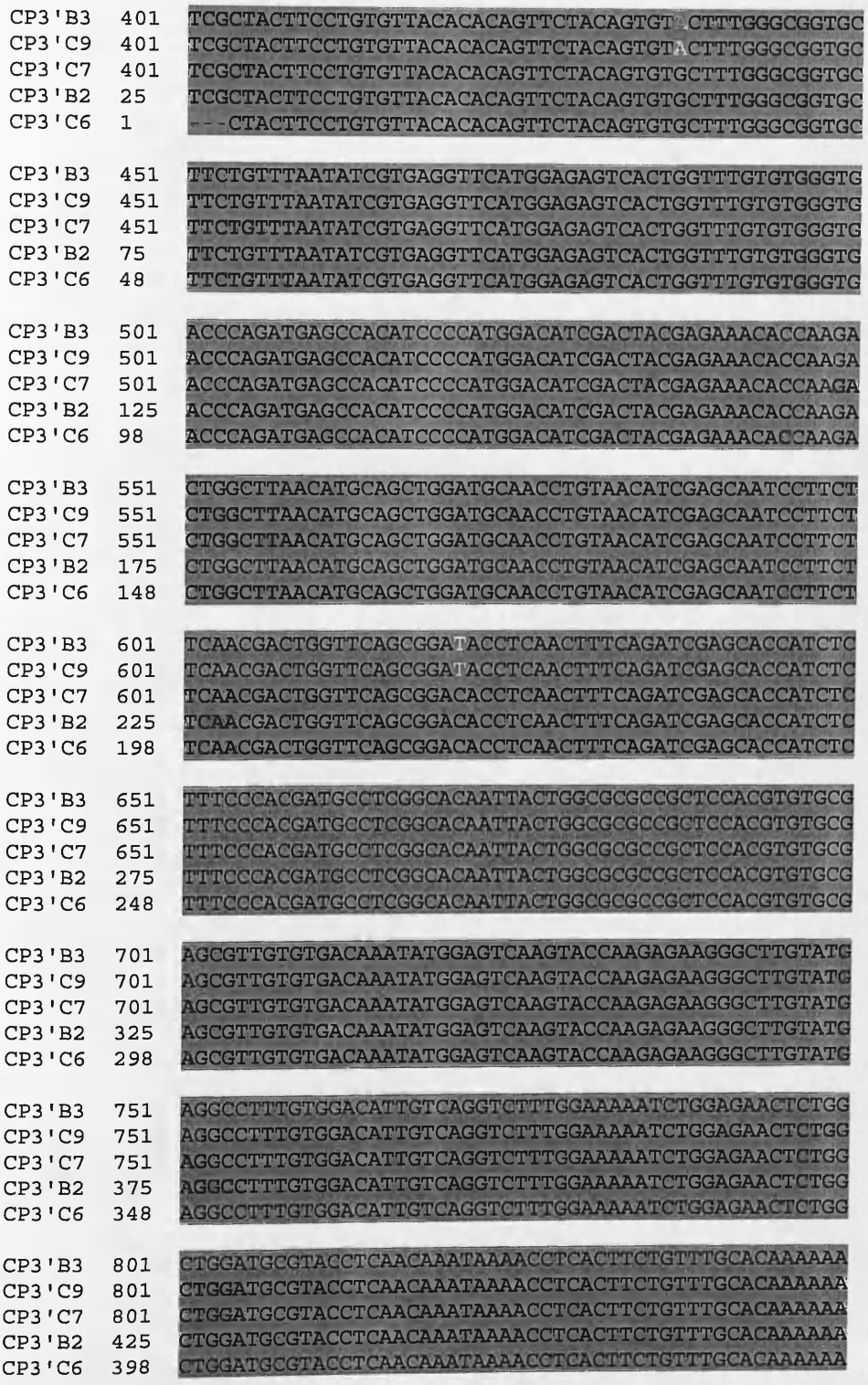
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CP3'C9 401 TCGCTACTTCCTGTGTTACACACAGTTCTACAGTGTACTTTGGGCGGTGC
CP3'C7 401 TCGCTACTTCCTGTGTTACACACAGTTCTACAGTGTGCTTTGGGCGGTGC
CP3'B2 25 TCGCTACTTCCTGTGTTACACACAGTTCTACAGTGTGCTTTGGGCGGTGC
CP3'C6 1 ---CTACTTCCTGTGTTACACACAGTTCTACAGTGTGCTTTGGGCGGTGC
CP3'B3 451 TTCTGTTTAATATCGTGAGGTTCATGGAGAGTCACTGGTTTGTGTGGGTG
CP3'C9 451 TTCTGTTTAATATCGTGAGGTTCATGGAGAGTCACTGGTTTGTGTGGGTG
CP3'C7 451 TTCTGTTTAATATCGTGAGGTTCATGGAGAGTCACTGGTTTGTGTGGGTG
CP3'B2 75 TTCTGTTTAATATCGTGAGGTTCATGGAGAGTCACTGGTTTGTGTGGGTG
CP3'C6 48 TTCTGTTTAATATCGTGAGGTTCATGGAGAGTCACTGGTTTGTGTGGGTG
CP3'B3 501 ACCCAGATGAGCCACATCCCCATGGACATCGACTACGAGAAACACCAAGA
CP3'C9 501 ACCCAGATGAGCCACATCCCCATGGACATCGACTACGAGAAACACCAAGA
CP3'C7 501 ACCCAGATGAGCCACATCCCCATGGACATCGACTACGAGAAACACCAAGA
CP3'B2 125 ACCCAGATGAGCCACATCCCCATGGACATCGACTACGAGAAACACCAAGA
CP3'C6 98 ACCCAGATGAGCCACATCCCCATGGACATCGACTACGAGAAACACCAAGA
CP3'B3 551 CTGGCTTAACATGCAGCTGGATGCAACCTGTAACATCGAGCAATCCTTCT
CP3'C9 551 CTGGCTTAACATGCAGCTGGATGCAACCTGTAACATCGAGCAATCCTTCT
CP3'C7 551 CTGGCTTAACATGCAGCTGGATGCAACCTGTAACATCGAGCAATCCTTCT
CP3'B2 175 CTGGCTTAACATGCAGCTGGATGCAACCTGTAACATCGAGCAATCCTTCT
CP3'C6 148 CTGGCTTAACATGCAGCTGGATGCAACCTGTAACATCGAGCAATCCTTCT
CP3'B3 601 TCAACGACTGGTTCAGCGGATACCTCAACTTTCAGATCGAGCACCATCTC
CP3'C9 601 TCAACGACTGGTTCAGCGGATACCTCAACTTTCAGATCGAGCACCATCTC
CP3'C7 601 TCAACGACTGGTTCAGCGGACACCTCAACTTTCAGATCGAGCACCATCTC
CP3'B2 225 TCAACGACTGGTTCAGCGGACACCTCAACTTTCAGATCGAGCACCATCTC
CP3'C6 198 TCAACGACTGGTTCAGCGGACACCTCAACTTTCAGATCGAGCACCATCTC
CP3'B3 651 TTTCCCACGATGCCTCGGCACAATTACTGGCGCGCCGCTCCACGTGTGCG
CP3'C9 651 TTTCCCACGATGCCTCGGCACAATTACTGGCGCGCCGCTCCACGTGTGCG
CP3'C7 651 TTTCCCACGATGCCTCGGCACAATTACTGGCGCGCCGCTCCACGTGTGCG
CP3'B2 275 TTTCCCACGATGCCTCGGCACAATTACTGGCGCGCCGCTCCACGTGTGCG
CP3'C6 248 TTTCCCACGATGCCTCGGCACAATTACTGGCGCGCCGCTCCACGTGTGCG
CP3'B3 701 AGCGTTGTGTGACAAATATGGAGTCAAGTACCAAGAGAAGGGCTTGTATG
CP3'C9 701 AGCGTTGTGTGACAAATATGGAGTCAAGTACCAAGAGAAGGGCTTGTATG
CP3'C7 701 AGCGTTGTGTGACAAATATGGAGTCAAGTACCAAGAGAAGGGCTTGTATG
CP3'B2 325 AGCGTTGTGTGACAAATATGGAGTCAAGTACCAAGAGAAGGGCTTGTATG
CP3'C6 298 AGCGTTGTGTGACAAATATGGAGTCAAGTACCAAGAGAAGGGCTTGTATG
CP3'B3 751 AGGCCTTTGTGGACATTGTCAGGTCTTTGGAAAAATCTGGAGAACTCTGG
CP3'C9 751 AGGCCTTTGTGGACATTGTCAGGTCTTTGGAAAAATCTGGAGAACTCTGG
CP3'C7 751 AGGCCTTTGTGGACATTGTCAGGTCTTTGGAAAAATCTGGAGAACTCTGG
CP3'B2 375 AGGCCTTTGTGGACATTGTCAGGTCTTTGGAAAAATCTGGAGAACTCTGG
CP3'C6 348 AGGCCTTTGTGGACATTGTCAGGTCTTTGGAAAAATCTGGAGAACTCTGG
CP3'B3 801 CTGGATGCGTACCTCAACAAATAAAACCTCACTTCTGTTTGCACAAAAAA
CP3'C9 801 CTGGATGCGTACCTCAACAAATAAAACCTCACTTCTGTTTGCACAAAAAA
CP3'C7 801 CTGGATGCGTACCTCAACAAATAAAACCTCACTTCTGTTTGCACAAAAAA
CP3'B2 425 CTGGATGCGTACCTCAACAAATAAAACCTCACTTCTGTTTGCACAAAAAA
CP3'C6 398 CTGGATGCGTACCTCAACAAATAAAACCTCACTTCTGTTTGCACAAAAAA

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CP3'B3   851  ATTCAAAGCTGAGCAGAATCCACCAATCAGAGCAAGAACGTCGCGGACTA
CP3'C9   851  ATTCAAAGCTGAGCAGAATCCACCAATCAGAGCAAGAACGTCGCGGACTA
CP3'C7   851  ATTCAAAGCTGAGCAGAATCCACCAATCAGAGCAAGAACGTCGCGGACTA
CP3'B2   475  ATTCAAAGCTGAGCAGAATCCACCAATCAGAGCAAGAACGTCGCGGACTA
CP3'C6   448  ATTCAAAGCTGAGCAGAATCCACCAATCAGAGCAAGAACGTCGCGGACTA

CP3'B3   901  GGCATTAAAAAGTCTCATAACTAAAAGACCAGTCAATCAGATTGCTAAAA
CP3'C9   901  GGCATTAAAAAGTCTCATAACTAAAAGACCAGTCAATCAGATTGCTAAAA
CP3'C7   901  GGCATTAAAAAGTCTCATAACTAAAAGACCAGTCAATCAGATTGCTAAAA
CP3'B2   525  GGCATTAAAAAGTCTCATAACTAAAAGACCAGTCAATCAGATTGCTAAAA
CP3'C6   498  GGCATTAAAAAGTCTCATAACTAAAAGACCAGTCAATCAGATTGCTAAAA

CP3'B3   951  TGACACGTAGCACTTTTGTTTTTGGTATTTGAAGGGTAATTAAATGTGTT
CP3'C9   951  TGACACGTAGCACTTTTGTTTTTGGTATTTGAAGGGTAATTAAATGTGTT
CP3'C7   951  TGACACGTAGCACTTTTGTTTTTGGTATTTGAAGGGTAATTAAATGTGTT
CP3'B2   575  TGACACGTAGCACTTTTGTTTTTGGTATTTGAAGGGTAATTAAATGTGTT
CP3'C6   548  TGACACGTAGCACTTTTGTTTTTGGTATTTGAAGGGTAATTAAATGTGTT

CP3'B3   1001 TACAAAGAGACTGAGATGTATAGGTCTTGCTCCTAATAAAGGACATATCT
CP3'C9   1001 TACAAAGAGACTGAGATGTATAGGTCTTGCTCCTAATAAAGGACATATCT
CP3'C7   1001 TACAAAGAGACTGAGATGTATAGGTCTTGCTCCTAATAAAGGACATATCT
CP3'B2   625  TACAAAGAGACTGAGATGTATAGGTCTTGCTCCTAATAAAGGACATATCT
CP3'C6   598  TACAAAGAGACTGAGATGTATAGGTCTTGCTCCTAATAAAGGACATATCT

CP3'B3   1051 GAAGGTACAAGAACGTGCATGAATGACCTGGAAGTTGTGTGATTGCTGGA
CP3'C9   1051 GAAGGTACAAGAACGTGCATGAATGACCTGGAAGTTGTGTGATTGCTGGA
CP3'C7   1051 GAAGGTACAAGAACGTGTATGAATGACCTGGAAGTTGTGTGATTGCTGGA
CP3'B2   675  GAAGGTACAAGAACGTGCGTGAATGACCTGGAAGTTGTGTGATTGCTGGA
CP3'C6   648  GAAGGTACAAGAACGTGCGTGAATGACCTGGAAGTTGTGTGATTGCTGGA

CP3'B3   1101 TTACAATTGCTGAAGTTTAGATTTGCGTAGTTGCTAGATGCATTGAATAT
CP3'C9   1101 TTACAATTGCTGAAGTTTAGATTTGCGTAGTTGCTAGATGCATTGAATAT
CP3'C7   1101 TTACAATTGCTGAAGTTTAGATTTGCGTAGTTGCTAGATGCATTGAATAT
CP3'B2   725  TTACAATTGCTGAAGTTTAGATTTGCGTAGTTGCTAGATGCATTGAATAT
CP3'C6   698  TTACAATTGCTGAAGTTTAGATTTGCGTAGTTGCTAGATGCATTGAATAT

CP3'B3   1151 TTTGAGGCACAATCTTTATGGTAGTCACTTTATTTCTGAAATTTACAGCG
CP3'C9   1151 TTTGAGGCACAATCTTTATGGTAGTCACTTTATTTCTGAAATTTACAGCG
CP3'C7   1151 TTTGAGGCACAATCTTTATGGTAGTCACTTTATTTCTGAAATTTACAGCG
CP3'B2   775  TTTGAGGCACAATCTTTATGGTAGTCACTTTATTTCTGAAATTTACAGCG
CP3'C6   748  TTTGAGGCACAATCTTTATGGTAGTCACTTTATTTCTGAAATTTACAGCG

CP3'B3   1201 ATTTGGTTTTTATAAATAACATTTGGAGAAACTAGCAGAGGATAATACTA
CP3'C9   1201 ATTTGGTTTTTATAAATAACATTTGGAGAAACTAGCAGAGGATAATACTA
CP3'C7   1201 ATTTGGTTTTTATAAATAACATTTGGAGAAACTAGCAGAGGATAATACTA
CP3'B2   825  ATTTGGTTTTTATAAATAACATTTGGAGAAACTAGCAGAGGATAATACTA
CP3'C6   798  ATTTGGTTTTTATAAATAACATTTGGAGAAACTAGCAGAGGATAATACTA

CP3'B3   1251 AGAGGTTGTACTTCTGGTTTAATATGACATTTAATTTGATTTCCTACAAT
CP3'C9   1251 AGAGGTTGTACTTCTGGTTTAATATGACATTTAATTTGATTTCCTACAAT
CP3'C7   1251 AGAGGTTGTACTTCTGGTTTAATATGACATTTAATTTGATTTCCTACAAT
CP3'B2   875  AGAGGTTGTACTTCTGGTTTAATATGACATTTAATTTGATTTCCTACAAT
CP3'C6   848  AGAGGTTGTACTTCTGGTTTAATATGACATTTAATTTGATTTCCTACAAT
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CP3'B3   1301 TGTTATCCGATTAAACTGATTAAATCTCA-------------AAAAAAAA
CP3'C9   1301 TGTTATCCGATTAAACTGATTAAATCTCA-------------AAAAAAAA
CP3'C7   1301 TGTTATCCGATTAAACTGATTAAATCTCA-------------AAAAAAAA
CP3'B2   925  TGTTATCCGATTAGACTGATTAAATCTCATCTGTGTGTCTTCAAAAAAAA
CP3'C6   898  TGTTATCCGATTAGACTGATTAAATCTCATCTGTGTGTCTTCAAAAAAAA

CP3'B3   1338 AAAAAAA
CP3'C9   1338 AAAAAAA
CP3'C7   1338 AAAAAAA
CP3'B2   975  AAAAAAA
CP3'C6   948  AAAAAAA
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Appendix VI – Salmon (*Salmon salar*) nucleotide sequences

A: Putative Open Reading Frames

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SalpYESB1   1    ATGGGGGGCGGAGGCCAGCAGACGGAGTCAGGCGAGCCGGCCAAGGGTGA
SalA10      1    ATGGGGGGCGGAGGCCAGCAGACGGAGTCAGCGAGCCGGCCAAGGGTGA
SalA9       1    ATGGGAGGCGGAGGCCAGCAGAATGATTCAGGAGAGCCGGCCAAGGGTGA

SalpYESB1   51   CGGGCTTGAGCCCGATGGAGGGCAAGGTGGCAGTGCAGTCTACACCTGGG
SalA10      51   CGGGCTTGAGCCCGATGGAGGGCAAGGTGGCAGTGCAGTCTACACCTGGG
SalA9       51   CAGGGCTGCGCCCGGTGGAGGGCTAGGTGGCAGTGCAGTCTACACCTGGG

SalpYESB1   101  AAGAGGTCCAGAGGCACTCCCACAGAAGCGACCAGTGGTTGGTCATCGAC
SalA10      101  AAGAGGTCCAGAGGCACTCCCACAGAAGCGACCAGTGGTTGGTCATCGAC
SalA9       101  AAGAGGTCCAGAGGCACTGCCACAGAAGCGACCAGTGGTTGGTCATCGAC

SalpYESB1   151  AGGAAGGTCTATAATATTACCCAGGGGGCAAAGAGACACCCGGGTGGCAT
SalA10      151  AGGAAGGTCTATAATATTACCCAGTGGGCAAAGAGACACCCGGGTGGCAT
SalA9       151  AGGAAGGTCTATAATATTACCCAGTGGGTGAAGAGACACCCGGGTGGCAC

SalpYESB1   201  CAGGGTCATCAGTCACTTTGCTGGAGAAGATGCCACGGAAGCATTTTCCG
SalA10      201  CAGGGTCATCAGTCACTTTGCTGGAGAAGATGCCACGGAAGCATTTTCCG
SalA9       201  CAGGGTCATCAGTCACCTTGCTGGAGAAGATGCCACGGACGCATTTGTCG

SalpYESB1   251  CATTCCATCTTGATGCTAATTTTGTCAGGAAGTTTCTGAAGCCGTTGCTG
SalA10      251  CATTCCATCTTGATGCTAATTTTGTCAGGAAGTTTCTGAAGCCGTTGCTG
SalA9       251  CATTCCATCCCAATGCTAATTTTGTCAGGAAGTTGCTGAAGCCGTTGCTG

SalpYESB1   301  ATTGGAGAGCTGGCACCGACAGAGCCCAGCCAGGACCATGGGAAAAATGC
SalA10      301  ATTGGAGAGCTGGCACCGACAGAGCCCAGCCAGGACCATGGGAAAAATGC
SalA9       301  ATTGGAGAGCTGGCACCGACAGAGCCCAGCCAGGACCATGGGAAAAATGC

SalpYESB1   351  AGCTCTGGTGCAGGACTTCCAGGCCTTGCGTGACCATGTGGAGAGGGAGG
SalA10      351  AGCTCTGGTGCAGGACTTCCAGGCCTTGCGTGACCATGTGGAGAGGGAGG
SalA9       351  AGTACTGGTGCAGGATTCCAGGCCTTGCGCAACCGTGTGGAGAGGGAGG

SalpYESB1   401  GTCTCCTCCGTGCCCGCCTCCTGTTCTTCAGCCTCTACCTGGGCCACATC
SalA10      401  GTCTCCTCCGTGCCCGCCTCCTGTTCTTCAGCCTCTACCTGGGCCACATC
SalA9       401  GTTTGCTCCGTGCCCGCCCCCTGTTCTTCAGCCTCTACCTGGGCCACATC

SalpYESB1   451  CTGCTACTAGAGGCCCTGGCTTTGGGCCTGCTCTGGGTCTGGGGGACCAG
SalA10      451  CTGCTACTAGAGGCCCTGGCTTTGGGCCTGCTCTGGGTCTGGGGGACCAG
SalA9       451  CTGCTACTAGAGGCCCTGGCTTTGGGCCTGCTCTGGGTCTGGGGGACCAG

SalpYESB1   501  CTGGAGCCTCACACTGCTCTGTTCCCTCATGCTGGCCACGTCTCAGGCCC
SalA10      501  CTGGAGCCTCACACTGCTCTGTTCCCTCATGCTGGCCACGTCTCAGGCCC
SalA9       501  CTGGAGCCTCACACTGCTCTGTTCCCTCATGCTGGCCACGTCTCAGTCCC

SalpYESB1   551  AGGCTGGCTGGCTGCAGCATGACTACGGCCACCTATCAGTCTGCAAGAAA
SalA10      551  AGGCTGGCTGGCTGCAGCATGACTACGGCCACCTGTCAGTCTGCAAGAAA
SalA9       551  AGGCTGGCTGGCTGCAGCATGACTACGGCCACCTGTCAGTCTGCAAGAAA
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SalpYESB1  601  TCCAGCTGGAACCACAAACTGCACAAGTTTGTCATTGGACACCTAAAGGG
SalA10     601  TCCAGCTGGAACCACAAACTGCACAAGTTTGTCATTGGACACCTAAAGGG
SalA9      601  TCCAGCTGGAATCACGTACTGCACAAGTTTGTCATTGGACACCTAAAGGG

SalpYESB1  651  TGCCTCTGCTAACTGGTGGAACCATCGTCACTTCCAGCACCACGCTAAAC
SalA10     651  TGCCTCTGCTAACTGGTGGAACCATCGTCACTTCCAGCACCACGCTAAGC
SalA9      651  TGCCTCTGCTAACTGGTGGAACCATCGTCACTTCCAGCACCACGCTAAAC

SalpYESB1  701  CCAACGTGTTTCGTAAAGATCCTGATATCAACTCACTGCATGTCTTCGTC
SalA10     701  CCAACGTGTTTCGTAAAGATCCTGATATCAACTCACTGCCTGTCTTCGTC
SalA9      701  CCAACGTGTTGAGTAAAGATCCTGATGTTAATATGCTGCATGTTTTCGTC

SalpYESB1  751  CTGGGAGACACACAGCCTGTAGAGTATGGTATAAAGAAGTTGAAGTACAT
SalA10     751  CTGGGAGACACACAGCCTGTAGAGTATGGTATAAAGAAGTTGAAGTACAT
SalA9      751  CTGGGAGACAAGCAGCCTGTAGAGTATGGTATAAAGAAGTTGAAGTACAT

SalpYESB1  801  GCCCTACCATCACCAACACCAGTACTTCTTCCTCATTGGACCTCCACTAA
SalA10     801  GCCCTACCATCACCAACACCAGTACTTCTTCCTCATTGGACCTCCACTAA
SalA9      801  GCCCTACCATCACCAACACCAGTACTTCTTCCTCATTGGACCTCCACTAC

SalpYESB1  851  TCGTTCCAGTGTTTTTCAACATCCAGATATTCCGGACCATGTTTTCACAA
SalA10     851  TCGTTCCAGTGTTTTTCAACATCCAGATATTCCGGACCATGTTTTCACAA
SalA9      851  TTATTCCAGTGTTTTTCACCATCCAGATGTTCCAGACCATGTTTTCACAA

SalpYESB1  901  CGGGACTGGGTGGATCTGGCGTGGTCGATGAGTTTCTACCTTCGCTTCTT
SalA10     901  CGGGACTGGGTGGATCTGGCGTGGTCGATGAGTTTCTACCTTCGCTTCTT
SalA9      901  CGGAACTGGGTGGATCTGGCGTGGTCGATGACTTTCTACCTTCGCTTCTT

SalpYESB1  951  CTGCTGTTACTATCCCTTCTTTGGTTTCTTTGGCTCAGTAGCATTGATCA
SalA10     951  CTGCTGTTACTATCCCTTCTTTGGTTTCTTTGGCTCAGTAGCATTGATCA
SalA9      951  CTGCTCTTACTATCCCTTCTTTGGTTTCTTTGGCTCAGTAGCATTGATCA

SalpYESB1  1001 GCTTCGTCAGGTTTTTGGAAAGCCACTGGTTTGTATGGGTGACCCAGATG
SalA10     1001 GCTTCGTCAGGTTTTTGGAAAGCCACTGGTTTGTATGGGTGACCCAGATG
SalA9      1001 CCTTCGTCAGGTTTTTGGAAAGCCACTGGTTTGTATGGGTGACCCAGATG

SalpYESB1  1051 AATCACCTTCCTATGGAGATGGATCATGAGAGACACCAGGACTGGCTCAC
SalA10     1051 AATCACCTTCCTATGGAGATGGATCATGAGAGACACCAGGACTGGCTCAC
SalA9      1051 AATCACCTTCCTATGGAGATAGATCATGAGAGACACCAGGATTGGCTCAC

SalpYESB1  1101 CATGCAGTTGAGCGCTACTTGCAACATTGAACAGTCAACCTTCAACGACT
SalA10     1101 CATGCAGTTGAGCGCTACTTGCAACATTGAACAGTCAACCTTCAACGACT
SalA9      1101 CATGCAGTTGAGTGGTACTTGCAACATTGAACAGTCAACCTTCAACGACT

SalpYESB1  1151 GGTTCAGTGGACACCTCAACTTTCAGATTGAACACCATCTGTTTCCTACC
SalA10     1151 GGTTCAGTGGACACCTCAACTTTCAGATTGAACACCATCTGTTTCCTACC
SalA9      1151 GGTTCAGTGGACACCTCAACTTTCAGATTGGACACCATCTGTTTCCTACC

SalpYESB1  1201 ATGCCCCGTCATAACTACCACCTGGTGGCTCCTCTGGTGCGTACTTTGTG
SalA10     1201 ATGCCCCGTCATAACTACCACCTGGTGGCTCCTCTGGTGCGTACTTTGTG
SalA9      1201 ATGCCCCGTCATAACTACCACCTGGTGGCTCCTCTGGTGCGTACTTTGTG

SalpYESB1  1251 TGAGAAACATGGAGTTCCCTATCAGGTCAAGACTTTGCAGAAAGGCATGA
SalA10     1251 TGAGAAACATGGAGTTCCCTATCAGGTCAAGACTTTGCAGAAAGGCATGA
SalA9      1251 TGAGAAACATGGAATTCCCTATCAGGTCAAGACTTTGCAGAAAGCCATCA
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SalpYESB1   1301 CTGATGTTGTCAGGTCACTGAAGAAGTCAGGGGATCTGTGGCTGGATGCA
SalA10      1301 CTGATGTTGTCAGGTCACTGAAGAAGTCAGGGGATCTGTGGCTGGATGCA
SalA9       1301 TTGATGTTGTCAGGTCACTGAAGAAGTCAGGGGATCTGTGGCTGGATGCA

SalpYESB1   1351 TATCTCCATAAATGA
SalA10      1351 TATCTCCATAAATGA
SalA9       1351 TATCTCAATAAGGGC
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B: 3'UTR's

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Sal3'A4     1    TTTTTGGAAAGCCACTGGTTTGTATGGGTGACCCAGATGAATCACCTTCC
Sal3'A1     1    TTTTTGGAAAGCCACTGGTTTGTATGGGTGACCCAGATGAATCACCTTCC
Sal3'A10    1    TTTTTGGAAAGCCACTGGTTTGTATGGGTGACCCAGATGAATCACCTTCC

Sal3'A4     51   TATGGAGATAGATCATGAGAGACACCAGGATTGGCTCACCATGCAGTTGA
Sal3'A1     51   TATGGAGATAGATCATGAGAGACACCAGGATTGGCTCACCATGCAGTTGA
Sal3'A10    51   TATGGAGATGGATCATGAGAGACACCAGGACTGGCTCACCATGCAGTTGA

Sal3'A4     101  GTGGTACTTGCAACATTGAACAGTCAACCTTCAACGACTGGTTCAGTGGA
Sal3'A1     101  GTGGTACTTGCAACATTGAACAGCCAACCTTCAACGACTGGTTCAGTGGA
Sal3'A10    101  GCGCTACTTGCAACATTGAACAGTCAACCTTCAACGACTGGTTCAGTGGA

Sal3'A4     151  CACCTCAACTTTCAGATTGAACACCATCTGTTTCCTACCATGCCCCGTCA
Sal3'A1     151  CACCTCAACTTTCAGATTGAACACCATCTGTTTCCTACCATGCCCCGTCA
Sal3'A10    151  CACCTCAACTTTCAGATTGAACACCATCTGTTTCCTACCATGCCCCGTCA

Sal3'A4     201  TAACTACCACCTGGTGGCTCCTCTGGTGCGTACTTTGTGTGAGAAACATG
Sal3'A1     201  TAACTACCACCTGGTGGCTCCTCTGGTGCGTACTTTGTGTGAGAAACATG
Sal3'A10    201  TAACTACCACCTGGTGGCTCCTCTGGTGCGTACTTTGTGTGAGAAACATG

Sal3'A4     251  GAATTCCCTATCAGGTCAAGACTTTGCAGAAAGCCATCATTGATGTTGTC
Sal3'A1     251  GAATTCCCTATCAGGTCAAGACTTTGCAGAAAGCCATCATTGATGTTGTC
Sal3'A10    251  GAGTTCCCTATCAGGTCAAGACTTTGCAGAAAGGCATGACTGATGTTGTC

Sal3'A4     301  AGGTCACTGAAGAAGTCAGGGGATCTGTGGCTAGATGCATATCTCCATAA
Sal3'A1     301  AGGTCACTGAAGAAGTCAGGGGATCTGTGGCTAGATGCATATCTCCATAA
Sal3'A10    301  AGGTCACTGAAGAAGTCAGGGGATCTGTGGCTGGATGCATATCTCCATAA

Sal3'A4     351  ATAAATCCCTTCCTGACTCTGGACGGGATTT--AATCCATCGCATATTAA
Sal3'A1     351  ATAAATCCCTTCCTGACTCTGGACGGGATTT--AATCCATCGCATATTAA
Sal3'A10    351  ATAAATCCCTTCCTGACTCTGGATGGGATTTTAAATCCATCGCAGATTAA

Sal3'A4     399  CTACCTGTGAACAGAGATAGTTTCCCCAGACGTTTGTGTCAGATGCTGTA
Sal3'A1     399  CTACCTGTGAACAGAGATAGTTTCCCCAGACGTTTGTGTCAGACGCTGTA
Sal3'A10    401  CAACCTGCGAACAGAGAAGACATTTCCTAGATGTTTTTGATGATAATGAT

Sal3'A4     449  TTTTGTTGTGACGTAATTGTTTTAATCATTTTTTGGACCCCAGGAAGAGT
Sal3'A1     449  TTTTGTTGTGACGTAATTGTTTTAATCATTTTTTGGACCCCAGGAAGAGT
Sal3'A10    451  CGCTGCAAACATTATTGTGATATAATTGTTTTAATCCTTGGCAGCAGTGA

Sal3'A4     499  AGCTGCTGCCTTGGCAGGAATTAGTGGGAATCCATAGTAAACCCCAGGAA
Sal3'A1     499  AGCTGCTGCCTTGGCAGGAATTAATGGGAATCCATAATAAACCCCAGGAA
Sal3'A10    501  ATGCGGAATCCATAGCATAAATAGAGGTTTATATAGTAGGGCGAGGCGTT
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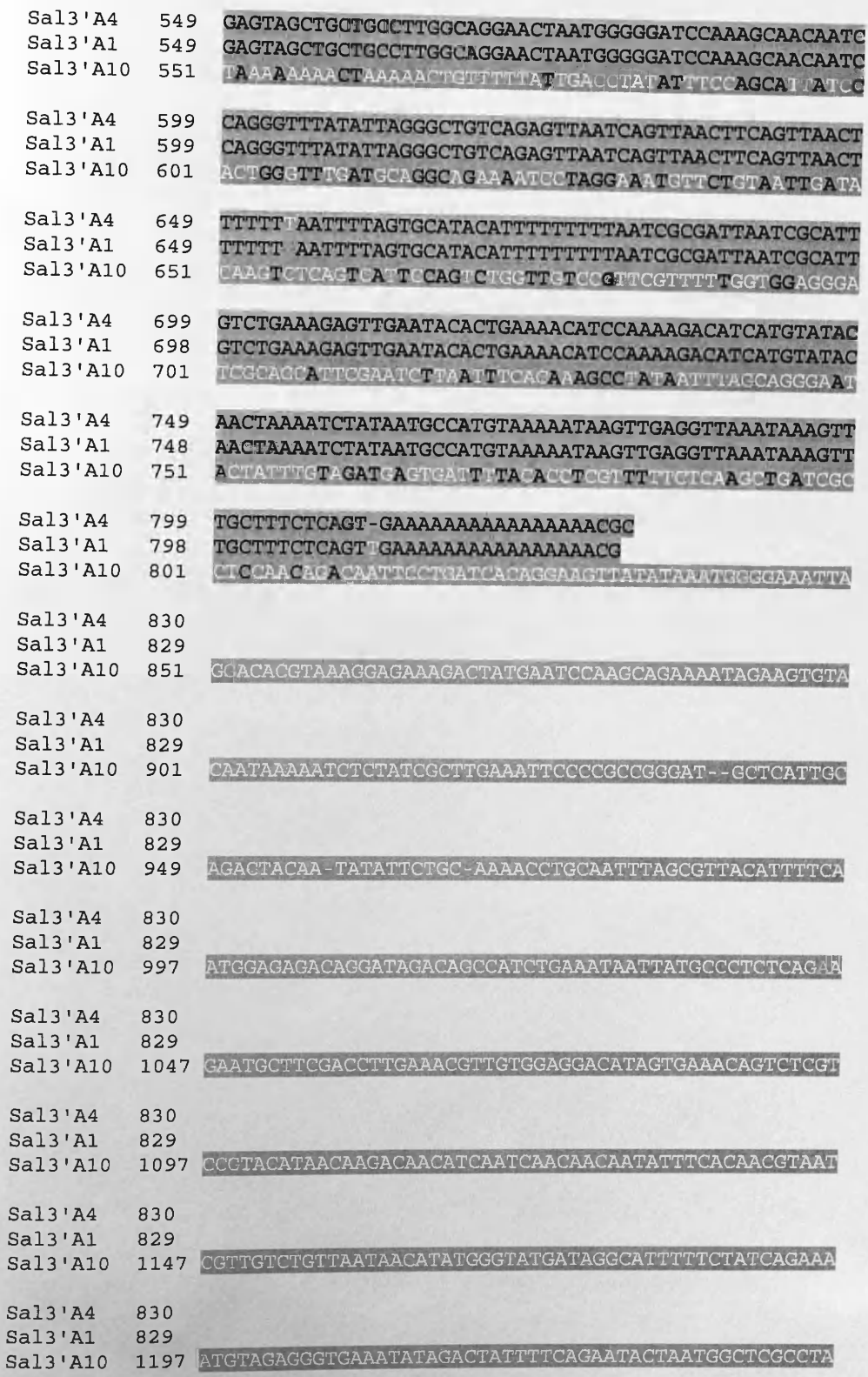
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Sal3'A1 549 GAGTAGCTGCTGCCTTGGCAGGAACTAATGGGGGATCCAAAGCAACAATC
Sal3'A10 551
Sal3'A4 599 CAGGGTTTATATTAGGGCTGTCAGAGTTAATCAGTTAACTTCAGTTAACT
Sal3'A1 599 CAGGGTTTATATTAGGGCTGTCAGAGTTAATCAGTTAACTTCAGTTAACT
Sal3'A10 601
Sal3'A4 649 TTTTTTAATTTTAGTGCATACATTTTTTTTTAATCGCGATTAATCGCATT
Sal3'A1 649 TTTTT AATTTTAGTGCATACATTTTTTTTTAATCGCGATTAATCGCATT
Sal3'A10 651
Sal3'A4 699 GTCTGAAAGAGTTGAATACACTGAAAACATCCAAAAGACATCATGTATAC
Sal3'A1 698 GTCTGAAAGAGTTGAATACACTGAAAACATCCAAAAGACATCATGTATAC
Sal3'A10 701
Sal3'A4 749 AACTAAAATCTATAATGCCATGTAAAAATAAGTTGAGGTTAAATAAAGTT
Sal3'A1 748 AACTAAAATCTATAATGCCATGTAAAAATAAGTTGAGGTTAAATAAAGTT
Sal3'A10 751
Sal3'A4 799 TGCTTTCTCAGT-GAAAAAAAAAAAAAAAAACGC
Sal3'A1 798 TGCTTTCTCAGTTGAAAAAAAAAAAAAAAACG
Sal3'A10 801
Sal3'A4 830
Sal3'A1 829
Sal3'A10 851
Sal3'A4 830
Sal3'A1 829
Sal3'A10 901
Sal3'A4 830
Sal3'A1 829
Sal3'A10 949
Sal3'A4 830
Sal3'A1 829
Sal3'A10 997
Sal3'A4 830
Sal3'A1 829
Sal3'A10 1047
Sal3'A4 830
Sal3'A1 829
Sal3'A10 1097
Sal3'A4 830
Sal3'A1 829
Sal3'A10 1147
Sal3'A4 830
Sal3'A1 829
Sal3'A10 1197

```
Sal3'A4    830
Sal3'A1    829
Sal3'A10   1247 TTTTCATTCACTATAGAAATAACCCAATTTATCCATCTCTGTTTTTAACG

Sal3'A4    830
Sal3'A1    829
Sal3'A10   1297 TCTTCACCCAGCTCTCATACAAATGTGTGTCAGTGGATTTGTCCGTTAC

Sal3'A4    830
Sal3'A1    829
Sal3'A10   1347 TATGCTTTCCGTTGTGTTTTTACCGGACTGCGGCTCCGTTCTTTACGTGT

Sal3'A4    830
Sal3'A1    829
Sal3'A10   1397 GCTAATGAATAAACGCAATTTGGGATTGTCAAAAAAAAAAAAAAAACGC
```

Appendix VII – Cod (*Gadus morhua)* nucleotide sequences

A: Putative Open Reading Frames

```
CodF4     1   CGTCTTCAGCAAGGACCCTGACGTCAACATGCTGCATGTCTTTGTGGTTG
CodG6     1   CGTCTTCAGCAAGGACCCTGACGTCAACATGCTGCATGTCTTTGTGGTTG
CodG11    1   CGTCTTCAGCAAGGACCCTGACGTCAACATGCTGCATGTCTTTGTGGTTG
CodF7     1   CGTCTTCAGCAAGGACCCTGACGTCAACATGCTGCATGTCTTTGTGGTTG
CodF6     1   CGTCTTCAGCAAGGACCCTGACGTCAACATGCTGCATGTCTTTGTGGTTG
CodF8     1   CGTCTTCAGCAAGGACCCTGACGTCAACATGCTGCATGTCTTTGTGGTTG

CodF4    51   GGGATATCCAGCCAGTGGAGTATGGCATTAAAAAGATTAAATACATGCCC
CodG6    51   GGGATATCCAGCCAGTGGAGTATGGCATTAAAAAGATTAAATACATGCCC
CodG11   51   GGGATATCCAGCCAGTGGAGTATGGCATTAAAAAGATTAAATACATGCCC
CodF7    51   GGGATATCCAGCCAGTGGAGTATGGCATTAAAAAGATTAAATACATGCCC
CodF6    51   GGGATATCCAGCCAGTGGAGTATGGCATTAAAAAGATTAAATACATGCCC
CodF8    51   GGGATATCCAGCCAGTGGAGTATGGCATTAAAAAGATTAAATACATGCCC

CodF4   101   TATCACCACCAACACCAGTACTTCTTTTTAGTCGGACCCCCAC GCTCAT
CodG6   101   TATCACCACCAACACCAGTACTTCTTTTTAGTCGGACCCCCACTGCTCAT
CodG11  101   TATCACCACCAACACCAGTACTTCTTTTTAGTCGGACCCCCACTGCTCAT
CodF7   101   TATCACCACCAACACCAGTACTTCTTTTTAGTCGGACCCCCACTGCTCAT
CodF6   101   TATCACCACCAACACCAGTACTTCTTTTTAGTCGGACCCCCACTGCTCAT
CodF8   101   TATCACCACCAACACCAGTACTTCTTTTTAGTCGGACCCCCACTGCTCAT

CodF4   151   CCCGGTGTATTTCCACATCCAGATATTACGGGCCATGTTCTCTCGACGGG
CodG6   151   CCCGGTGTATTTCCACATCCAGATATTACGGGCCATGTTCTCTCGACGGG
CodG11  151   CCCGGTGTATTTCCACATCCAGATATTACGGGCCATGTTCTCTCGACGGG
CodF7   151   CCCGGTGTATTTCCACATCCAGATATTACGGGCCATGTTCTCTCGACGGG
CodF6   151   CCCGGTGTATTTCCACATCCAGATATTACGGGCCATGTTCTCTCGACGGG
CodF8   151   CCCGGTGTATTTCCACATCCAGATATTACGGGCCATGTTCTCTCGACGGG

CodF4   201   ACTGGGTGGACCTGGCCTGGTCCATGTCGTACTACCTGCGCTACTTCTGC
CodG6   201   ACTGGGTGGACCTGGCCTGGTCCATGTCGTACTACCTGCGCTACTTCTGC
CodG11  201   ACTGGGTGGACCTGGCCTGGTCCATGTCGTACTACCTGCGCTACTTCTGC
CodF7   201   ACTGGGTGGACCTGGCCTGGTCCATGTCGTACTACCTGCGCTACTTCTGC
CodF6   201   ACTGGGTGGACCTGGCCTGGTCCATGTCGTACTACCTGCGCTACTTCTGC
CodF8   201   ACTGGGTGGACCTGGCCTGGTCCATGTCGTACTACCTGCGCTACTTCTGC

CodF4   251   TGCTACGCTCCGTTCTACGGCCTGCTGGGCTCTGTGGCCCTCATCAGCTT
CodG6   251   TGCTACGCTCCGTTCTACGGCCTGCTGGGCTCTGTGGCCCTCATCAGCTT
CodG11  251   TGCTACGCTCCGTTCTACGGCCTGCTGGGCTCTGTGGCCCTCATCAGCTT
CodF7   251   TGCTACGCTCCGTTCTACGGCCTGCTGGGCTCTGTGGCCCTCATCAGCTT
CodF6   251   TGCTACGCTCCGTTCTACGGCCTGCTGGGCTCTGTGGCCCTCATCAGCTT
CodF8   251   TGCTACGCTCCGTTCTACGGCCTGCTGGGCTCTGTGGCCCTCATCAGCTT

CodF4   301   TGTCAGGTTCCTGGAGAGCCACTGGTTCGTGTGGGTGACCCAGATGAATC
CodG6   301   TGTCAGGTTCCTGGAGAGCCACTGGTTCGTGTGGGTGACCCAGATGAATC
CodG11  301   TGTCA
CodF7   301   TGTCAGGTTCCTGGAGAGCCACTGGTTCGTGTGGGTGACCCAGATGAATC
CodF6   301   TGTCAGGTTCCTGGAGAGCCACTGGTTCGTGTGGGTGACCCAGATGAATC
CodF8   301   TGTCAGGTTCCTGGAGAGCCACTGGTTCGTGTGGGTGACCCAGATGAATC
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CodF4    351 ATCTGCCGATGAACATAGACCACGAGAAGCAGCAGGACTGGCTCAGCATG
CodG6    351 ATCTGCCGATGAACATAGACCACGAGAAGCAGCAGGACTGGCTCAGCATG
CodG11   305
CodF7    351 ATCTGCCGATGAACATAGACCACGAGAAGCAGCAGGACTGGCTCAGCATG
CodF6    351 ATCTGCCGATGAACATAGACCACGAGAAGCAGCAGGACTGGCTCAGCATG
CodF8    351 ATCTGCCGATGAACATAGACCACGAGAAGCAGCAGGACTGGCTCAGCATG

CodF4    401 CAGCTGAGCGCCACCTGTAATATAGAACAGTCCTGCTTCAACGACTGGTT
CodG6    401 CAGCTGAGCGCCACCTGTAATATAGAACAGTCCTGCTTCAACGACTGGTT
CodG11   305
CodF7    401 CAGCTGAGCGCCACCTGTAATATAGAACAGTCCTGCTTCAACGACTGGTT
CodF6    401 CAGCTGAGCGCCACCTGTAATATAGAACAGTCCTGCTTCAACGACTGGTT
CodF8    401 CAGCTGAGCGCCACCTGTAATATAGAACAGTCCTGCTTCAACGACTGGTT

CodF4    451 CAGCGGACAT
CodG6    451 CAGCGGACAT
CodG11   305
CodF7    451 CAGCGGACAT
CodF6    451 CAGCGGACAT
CodF8    451 CAGCGGACAT
```

B: 3'UTRs

```
Cod3'A11   1   CGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAAGCTAACGGCTACT
Cod3'B3    1   CGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAAGCTAACGGCTACT

Cod3'A11   51  TGTATCTGGTGCTCTTCGTCGTCCTGACCGAGTCGTACGTCGACTCGCGG
Cod3'B3    51  TGTATCTGGTGCTCTTCGTCGTCCTGACCGAGTCGTCCGTCGACTCGCGG

Cod3'A11   101 TGGACATTATATCTTGTCAGGACGAAGTTGCTGACCAAGTCGCCTGTAGA
Cod3'B3    101 TGGACATTATATCTTGTCAGGACGAAGTTGCTGACCAAGTCGCCTGTAGA

Cod3'A11   151 CTTGAAAGTTTAGCTCGTGGTGAACAAAGGGTGGTACGGCTCCGTGTTGA
Cod3'B3    151 CTTGAAAGTTTAGCTCGTGGTGAACAAAGGGTGGTACGGCTCCGTGTTGA

Cod3'A11   201 TGGTCCACGACCGAGGCGACCACGCTCGCGACACACTCTTCGTGTCGTAG
Cod3'B3    201 TGGTCCACGACCGAGGCGACCACGCTCGCGACACACTCTTCGTGTCGTAG

Cod3'A11   251 GGCATGGTCCTCTTCTGCGACACCGCCCCGCACCGGCTGCAGCACGCCAG
Cod3'B3    251 GGCATGGTCCTCTTCTGCGACACCGCCCCGCACCGGCTGCAGCACGCCAG

Cod3'A11   301 CGAATTCTTGAGTCCTCTGGAGACCTACCTACGAATAGAGGTATTCACTC
Cod3'B3    301 CGAATTCTTGAGTCCTCTGGAGACCTACCCACGAATAGAGGTATTCACTC

Cod3'A11   351 CTCTTGTTGTTCCTTAAGTTGTGTTTAACTTTGGTACGACAAACTTTAAA
Cod3'B3    351 CTCTTGTTGTTCCTTAAGTTGTGTTTAACTTTGGTACGACAAACTTTAAA

Cod3'A11   401 TGGTTTTTCTTTTTGCAGTACTTGATGGATTCTCTATTTACTACATGCGC
Cod3'B3    401 TGGTTTTTCTTTTTGCAGTACTTGATGGATTCTCTATTTACTACATGCAC

Cod3'A11   451 ACCGAAACATTATGCCACATCACGTCCTTGTTAGATAAGAATGATAACAT
Cod3'B3    451 ACCGAAACATTATGCCACATCACGTCCTTGTTAGATAAGAATGATAACAT
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Cod3'A11   501 AAATATCAGTTAGACCAAATGAAACTACACGTCCTAAAGGTTGCTACACG
Cod3'B3    501 AAATATCAGTTAGACCAAATGAAACTACACGTCCTAAAGGTTGCTACACG

Cod3'A11   551 ATCTCGAGGAACCCATAAGATGCTACGGAATGTCAAGACACGTTCTTACT
Cod3'B3    551 ATCTCGAGGAACCCATAAGATGCTACGGAATGTCAAGACACGTTCTTACT

Cod3'A11   601 AAAATTTATTGTAAAACACGTAAATAACAATGAACAAGTTTGTCACTTAC
Cod3'B3    601 AAAATTTATTGTAAAACACGTAAATAACAATGAACAAGTTTGTCACTTAC

Cod3'A11   651 AAAAAAAGATTAGCTAGTACCTGCTATTCAAATAGTTATTTCAAAACTGA
Cod3'B3    651 AAAAAAAGATAAGCTAGTACCTGCTATTCAAATAGTTATTTCAAAACTGA

Cod3'A11   701 CTTATCACTTTTTTTTTTTTTTT
Cod3'B3    701 CTTATCACTTTTTTTTTTTTTTT
```

Appendix VIII – Turbot (*Scophthalmus maximus)* nucleotide sequences

A: Putative Open Reading Frames

```
Turb5'B1   1   GTTTGCGATAAAAATGGACCTGAACGGATCGGGGGCAAATAAAGTTATCT
Turb5'B6   1   GTTTGCGATAAAAATGGACCTGAACGGATCGGGGGCAAATAAAGTTATCT
Turb5'C9   1   GTTTGCGATAAAAATGGACCTGAACGGATCGGGGGCAAATAAAGTTATCT

Turb5'B1   51  GCTCTGCTGACGTGACCGGGACCCGGCGAGTGTAGCGCAAAAAATCGGGA
Turb5'B6   51  GCTCTGCTGACGTGACCGGGACCCGGCGAGTGTAGCGCAAAAAATCGGGA
Turb5'C9   51  GCTCTGCTGACGTGACCGGGACCCGGCGAGTGTAGCGCAAAAAATCGGGA

Turb5'B1   101 AACTGTGTAAGAGTTTGTGGCTGAGAGGCCCTGCAGTGCGCAGGTGGATC
Turb5'B6   101 AACTGTGTAAGAGTTTGTGGCTGAGAGGCCCTGCAGTGCGCAGGTGGATC
Turb5'C9   101 AACTGTGTAAGAGTTTGTGGCTGAGAGGCCCTGCAGTGCGCAGGTGGATC

Turb5'B1   151 ACGGCCAGACACAGCAGTGAGGTGAAGATGGGAGGTGGAGGCCAGCTGAC
Turb5'B6   151 ACGGCCAGACACAGCAGTGAGGTGAAGATGGGAGGTGGAGGCCAGCTGAC
Turb5'C9   151 ACGGCCAGACACAGCAGTGAGGTGAAGATGGGAGGTGGAGGCCAGCTGAC

Turb5'B1   201 GGAGCAAGGGGAGACGGGCAGCAAGCGAGCCGGATGTGTTTACACCTGGG
Turb5'B6   201 GGAGCAAGGGGAGACGGGCAGCAAGCGAGCCGGATGTGTTTACACCTGGG
Turb5'C9   201 GGAGCAAGGGGAGACGGGCAGCAAGCGAGCCGGATGTGTTTACACCTGGG

Turb5'B1   251 AGGAGGTGCAGAGCCACAGCAGCAGGACCGACCAGTGGCTGGTCATAGAT
Turb5'B6   251 AGGAGGTGCAGAGCCACAGCAGCAGGACCGACCAGTGGCTGGTCATAGAT
Turb5'C9   251 AGGAGGTGCAGAGCCACAGCAGCAGGACCGACCAGTGGCTGGTCATAGAT

Turb5'B1   301 CGGAAAGTTTACAACACTACTCAGTGGGCCAAAAGACACCCAGGAGGGTT
Turb5'B6   301 CGGAAAGTTTACAACACTACTCAGTGGGCCAAAAGACACCCAGGAGGGTT
Turb5'C9   301 CGGAAAGTTTACAACTCTACTCAGTGGGCCAAAAGACACCCAGGAGGGTT

Turb5'B1   351 TCATGTCATCAGCCACTATGCTGGACAGGACGCCACGGAGGCATTTACTG
Turb5'B6   351 TCATGTCATCAGCCACTATGCTGGACAGGACGCCACGGAGGCATTTACTG
Turb5'C9   351 TCATGTCATCAGCCACTATGCTGGACAGGACGCCACGGAGGCATTTACTG

Turb5'B1   401 CTTTTCATCCCGACCTAAAGTTTGTCCAGAAGTTTCTGAAGCCCCTGCTG
Turb5'B6   401 CTTTTCATCCCGACCTAAAGTTTGTCCAGAAGTTTCTGAAGCCCCTGCTG
Turb5'C9   401 CTTTTCATCCCGACCTAAAGTTTGTCCAGAAGTTTCTGAAGCCCCTGCTG

Turb5'B1   451 ATTGGAGAACTGGCGGCAACAGAGCCCAGCCAGGACCGAAACAAAAATGC
Turb5'B6   451 ATTGGAGAACTGGCGGCAACAGAGCCCAGCCAGGACCGAAACAAAAATGC
Turb5'C9   451 ATTGGAGAACTGGCGGCAACAGAGCCCAGCCAGGACCGAAACAAAAATGC

Turb5'B1   501 AGCACTCGTACAGGATTTCCACACTTTACGTGTCAAGGCAGAGAGCAAGG
Turb5'B6   501 AGCACTCGTACAGGATTTCCACACTTTACGTGTCAAGGCAGAGAGCAAGG
Turb5'C9   501 AGCACTCGTACAGGATTTCCACACTTTACGTGTCAAGGCAGAGAGCAAGG


Turb5'B1   551 GTCTGTTCCAAGCTCGGCCTTTGTTCTTCTGCCTCCACCTGGATCACATT
Turb5'B6   551 GTCTGTTTCAAGCTCGGCCTTTGTTCTTCTGCCTCCACCTGGGTCACATT
Turb5'C9   551 GTCTGTTTCAAGCTCGGCCTTTGTTCTTCTGCCTCCACCTGGGTCACATT
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Turb5'B1   601 GTGCTGCTGGAGGCCCTCGCCTGGTTGATCATATGGGTCTGGGGAACCAA
Turb5'B6   601 GTGCTGCTGGAGGCCCTCGCCTGGTTGATCATATGGGTCTGGGGAACCAA
Turb5'C9   601 GTGCTGCTGGAGGCCCTCGCCTGGTTGATCATATGGGTCTGGGGAACCAA

Turb5'B1   651 CTGGATACTCACATTTCTCTGTGCACTCTTGATGACAATTGCTCAGTCGC
Turb5'B6   651 CTGGATACTCACATTTCTCTGTGCACTCTTGATGACAATTGCTCAGTCGC
Turb5'C9   651 CTGGATACTCACATTTCTCTGTGCACTCTTGATGACAATTGCTCAGTCGC

Turb5'B1   701 AGGCTGGTTGGCTGCAGCACGACTTTGGCCACCTGTCTGTCTTCAAGCAG
Turb5'B6   701 AGGCTGGTTGGCTGCAGCACGACTTTGGCCACCTGTCTGTCTTCAAGCAG
Turb5'C9   701 AGGCTGGTTGGCTGCAGCACGACTTTGGCCACCTGTCTGTCTTCAAGCAG

Turb5'B1   751 TCCCGCTGGAATCACTTGTTGCAAAAGTTTGCTATCGGCCATTTAAAGGG
Turb5'B6   751 TCCCGCTGGAATCACTTGTTGCAAAAGTTTGCTATCGGCCATTTAAAGGG
Turb5'C9   751 TCCCGCTGGAATCACTTGTTGCAAAAGTTTGCTATCGGCCATTTAAAGGG

Turb5'B1   801 AGCTTCTGCCAACTGGTGGAATCATCGACATTTCCAGCATCATGCTAAAA
Turb5'B6   801 AGCTTCTGCCAACTGGTGGAATCATCGACATTTCCAGCATCATGCTAAAA
Turb5'C9   801 AGCTTCTGCCAACTGGTGGAATCATCGACATTTCCAGCATCATGCTAAAA

Turb5'B1   851 CAAACATTTTCAGAAAGGACCCTGATGTCAACATGTTGAACATCTTTGTA
Turb5'B6   851 CAAACATTTTCAGAAAGGACCCTGATGTCAACATGTTGAACATCTTTGTA
Turb5'C9   851 CAAACATTTTCAGAAAGGACCCTGATGTCAACATGTTGAACATCTTTGTA

Turb5'B1   901 ATTGGTGCCACTCAGCCGGTTGAGTATGGCGTAAAAAAAGATCAAACATAT
Turb5'B6   901 ATTGGTGCCACTCAGCCGGTTGAGTATGGCGTAAAAAAAGATCAAACATAT
Turb5'C9   901 ATTGGTGCCACTCAGCCGGTTGAGTATGGCGT...................

Turb5'B1   951 GCCCTATCACCACCAACACCAATCAAGCTTATCGATA
Turb5'B6   951 GCCCTATCACCACCAACACCAATCAAGCTTATCGATA
Turb5'C9   932 ..................................
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B: 3' UTRs

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Turb3'4    1   AGGGAGCTTCTGCCAGTTGGTGGAATCATCGACATTTCCAGCATCATGCT
Turb3'5    1   AGGGAGCTTCTGCCAACTGGTGGAATCATCGACATTTCCAGCATCATGCT
Turb3'8    1   AGGGAGCTTCTGCCAACTGGTGGAATCATCGACATTTCCAGCATCATGCT
Turb3'9    1   AGGGAGCTTCTGCCAACTGGTGGAATCATCGACATTTCCAGCATCATGCT
Turb3'10   1   AGGGAGCTTCTGCCAACTGGTGGAATCATCGACATTTCCAGCATCATGCC
Turb3'13   1   AGGGAGCTTCTGCCAACTGGTGGAATCATCGACATTTCCAGCATCATGCT

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Turb3'5   51   AAAACAAACATTTTCAGAAAGGACCCTGATGTCAACATGTTGAACATCTT
Turb3'8   51   AAAACAAACATTTTCAGAAAGGACCCTGATGTCAACATGTTGAACATCTT
Turb3'9   51   AAAACAAACATTTTCAGAAAGGACCCTGATGTCAACATGTTGAACATCTT
Turb3'10  51   AAAACAAACATTTTCAGAAAGGACCCTGATGTCAACATGTTGAACATCTT
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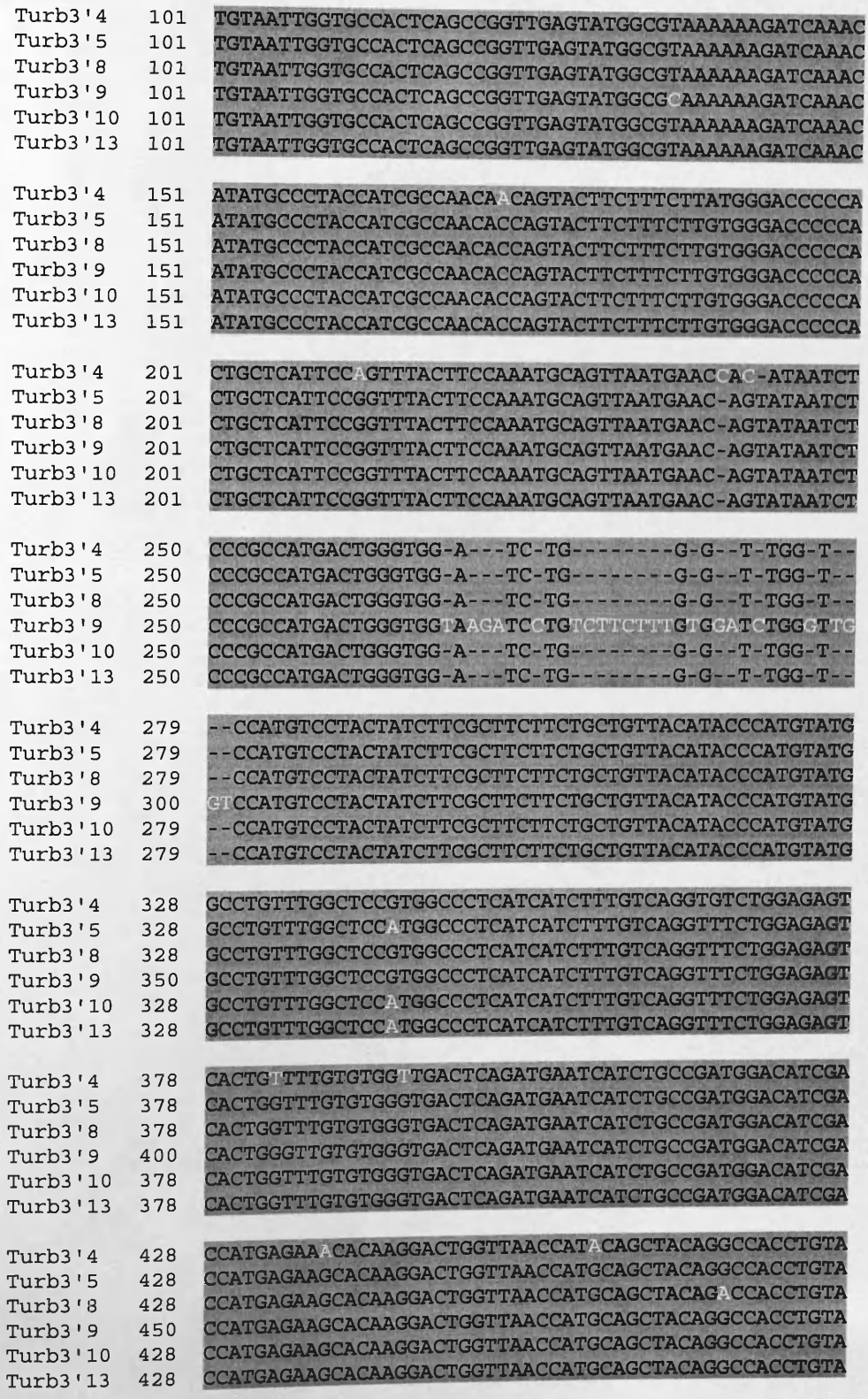
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Turb3'9 151 ATATGCCCTACCATCGCCAACACCAGTACTTCTTTCTTGTGGGACCCCCA
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Turb3'9 201 CTGCTCATTCCGGTTTACTTCCAAATGCAGTTAATGAAC-AGTATAATCT
Turb3'10 201 CTGCTCATTCCGGTTTACTTCCAAATGCAGTTAATGAAC-AGTATAATCT
Turb3'13 201 CTGCTCATTCCGGTTTACTTCCAAATGCAGTTAATGAAC-AGTATAATCT
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Turb3'9 250 CCCGCCATGACTGGGTGGTAAGATCCTGTCTTCTTTGTGGATCTGGGTTG
Turb3'10 250 CCCGCCATGACTGGGTGG-A---TC-TG--------G-G--T-TGG-T--
Turb3'13 250 CCCGCCATGACTGGGTGG-A---TC-TG--------G-G--T-TGG-T--
Turb3'4 279 --CCATGTCCTACTATCTTCGCTTCTTCTGCTGTTACATACCCATGTATG
Turb3'5 279 --CCATGTCCTACTATCTTCGCTTCTTCTGCTGTTACATACCCATGTATG
Turb3'8 279 --CCATGTCCTACTATCTTCGCTTCTTCTGCTGTTACATACCCATGTATG
Turb3'9 300 GTCCATGTCCTACTATCTTCGCTTCTTCTGCTGTTACATACCCATGTATG
Turb3'10 279 --CCATGTCCTACTATCTTCGCTTCTTCTGCTGTTACATACCCATGTATG
Turb3'13 279 --CCATGTCCTACTATCTTCGCTTCTTCTGCTGTTACATACCCATGTATG
Turb3'4 328 GCCTGTTTGGCTCCGTGGCCCTCATCATCTTTGTCAGGTGTCTGGAGAGT
Turb3'5 328 GCCTGTTTGGCTCCATGGCCCTCATCATCTTTGTCAGGTTTCTGGAGAGT
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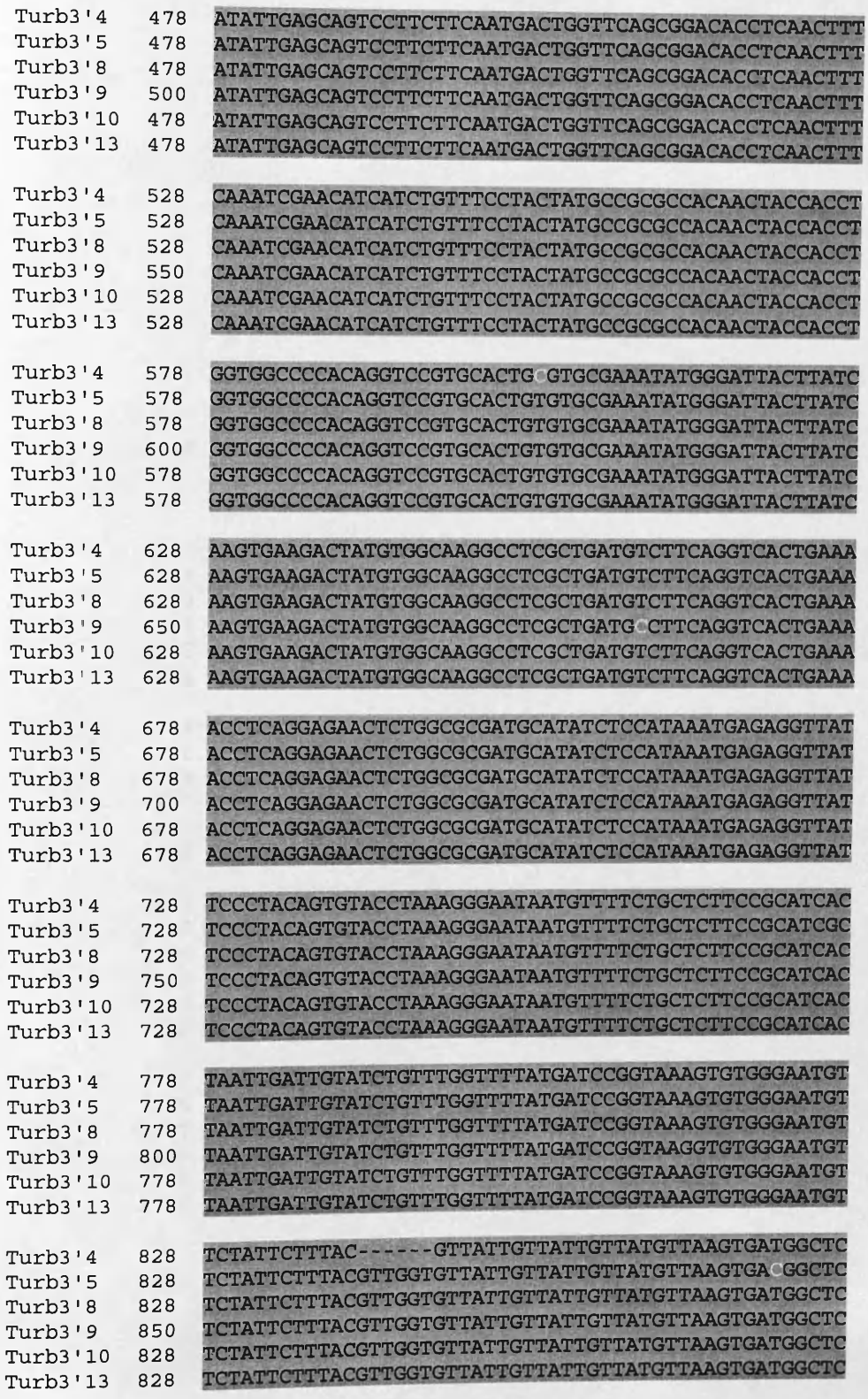
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Appendix IX– Publications

A: Conference Poster Presentations and Abstracts

A Comparison of the Delta 6 Desaturase Genes of Marine and Freshwater Teleosts

27th International Conference on Animal Genomics (ISAG), 2000, Minnesota, USA.

Harden Biochemical Conference, 2000, Wye, England.

N. Hastings, M. Agaba, D.R. Tocher, J.R. Sargent & A. Teale.

Our objective is to determine the genetic and molecular basis for the fact that marine teleosts, unlike their freshwater counterparts, have a repressed ability to synthesise long chain highly unsaturated fatty acids (HUFA). These include the omega-3 HUFA's eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexenoic acid (22:6*n*-3) which, in non-marine species, are synthesised from the 18-carbon precursor linolenic acid (18:3*n*-3). The deficiency in HUFA biosynthesis in marine fish is of considerable practical significance because, in consequence, farmed marine species require a dietary source of presynthesised HUFA. This is provided by processed products of "feed" species of marine fish. These include sand eels and other "industrial" fish, which themselves obtain HUFA through the marine food chain. Indicators suggest that the wild fishery supporting the aquaculture industry is unsustainable at current levels of exploitation. Consideration of the complex HUFA synthetic pathway indicates several steps which could be compromised in marine fish. However, we have chosen to examine first the delta 6 desaturase step as it is known to be rate limiting in mammals. To this end, we have cloned and compared the delta 6 desaturase genes of representative marine and freshwater teleost species. The genes are being compared with a view to relating structural and potential functional differences with different HUFA synthesis phenotypes.

Fatty Acyl Desaturase Genes of Marine and Freshwater Teleosts

Plant, Animal & Microbe Conference X (PAGX), 2002, San Diego, CA, USA

Nicola Hastings, Morris K. Agaba, Dougas, R. Tocher, John R. Sargent, Alan J. Teale.

Marine teleosts, unlike their freshwater counterparts, have a repressed ability to synthesise long chain highly unsaturated fatty acids (LC-HUFA). In competent species the delta 6 and delta 5 fatty acid desaturases are critical in the biosynthetic pathway that produces LC-HUFA.'s arachidonic acid (20:4*n*-6; AA) and eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexenoic acid (22:6n-3; DHA). As part of our effort to understand the reason for the differences in LC-HUFA synthesis ability between marine and freshwater teleosts we are comparing the desaturase genes of representative marine and freshwater species. The genes are being studied with a view to relating structural and potential functional differences with different HUFA synthesis phenotypes. Among the genes so far studied is a desaturase of zebrafish (*Danio rerio*). The 1590 bp cDNA transcript has close sequence similarity to mammalian delta 6 desaturase genes. However, the clone encodes a novel desaturase. When expressed in *Saccharomyces cerevisiae* the zebrafish gene confers the ability to convert linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3) to their corresponding delta 6 and delta 5 products, 18:3n-6 and 18:4n-3, respectively. Therefore, the zebrafish gene encodes a bifunctional enzyme having both delta 5 and delta 6 desaturase activity. This is the first report of a functionally characterised desaturase enzyme of fish, and of a fatty acid desaturase with both delta 5 and delta 6 activity.

Cloning, characterisation and nutritional regulation of fatty acid desaturase gene regulation in Atlantic salmon.

10th International Symposium on Nutrition & Feeding in Fish, 2002, Rhodes, Greece.

N. Hastings, A. Agaba, D. Tocher, X. Zheng, A. Teale.

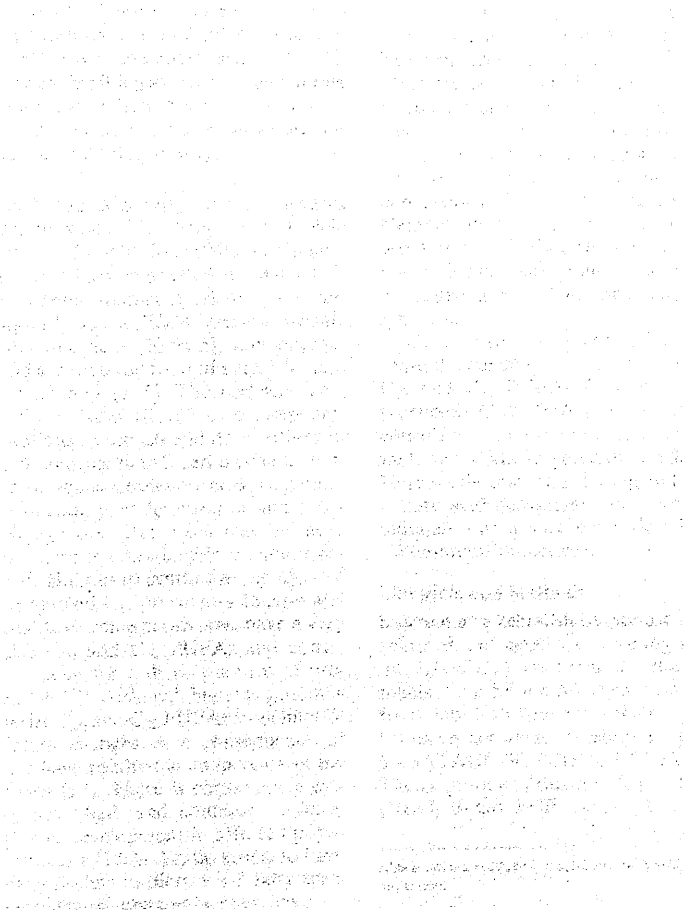
Salmon culture is extremely important in several countries including Norway, Chile, Canada and the UK. In 2000, approximately 127,000 tonnes of Atlantic salmon was produced in Scotland out a world total of 900,000 tonnes with a total value of $3 billion. Presently, salmon culture relies completely on supplies of fish oil and fish meal derived entirely from industrial fisheries. Increased demand on the limited global supplies of fish oil as well as the increasing ecological and ethical objections to exploiting a non-sustainable resource means that the financial and logistical position of salmon farming is vulnerable. However, in addition there is debate within the EU regarding drastic reductions in permitted dioxin levels (up to 80%) in marketed fish and their feeds. If these dioxin levels are agreed and enforced the present use of cheap northern hemisphere fish oils and meals were be severely curtailed. For the reasons above it would be particularly advantageous to replace fish oils with cheaper, freely available and sustainable oils derived from plants. However, salmon have a limited ability to convert alpha-linolenic (18:3*n*-3) and linoleic (18:2*n*-6) acids, abundant in many vegetable oils, to the long chain highly unsaturated fatty acids (20:5*n*-3, 22:6*n*-3 and 20:4*n*-6) which are essential components of all cell membranes. The overall aim of this study is to investigate whether salmon can be grown on diets containing vegetable oils without deleterious effects on the fish itself or its value as an important nutritious food source. Therefore, we have cloned, sequenced and functionally characterised an Atlantic salmon fatty acid desaturase gene homologous to other animal delta 6 desaturase genes. This was then used to design primers for sensitive quantitative Real-time PCR studies to investigate how the expression of desaturase activity was affected by feeding diets containing vegetable oil (VO) compared to diets containing fish oil (FO). Atlantic salmon were fed from first feeding diets in which 100% of the fish oil was replaced by a 1:1 blend of linseed and rapeseed oil. Livers of these fish were sampled at various time points throughout their life cycle for lipid and fatty acid composition and desaturase enzyme activity and gene expression. The desaturation/elongation of [1-14C]18:3*n*-3 in isolated hepatocytes was significantly greater in fish fed the VO

diets and lowest in fish fed the highest level of FO. Liver 18:2*n*-6, and to a lesser extent 18:3*n*-3, contents were significantly increased, and 22:6*n*-3 levels were reduced, in fish fed the VO diets compared to fish fed the FO diets. The trial if ongoing and so the data set is not yet complete. However, the results will determine relationships between gene expression and activity and how this correlates with tissue fatty acid composition.

B: Journal Publications

Hastings, N. Agaba, M., Tocher, D. R., Leaver, M. J., Dick, J. R., Sargent, J. R., Teale, A. J. (2001). A vertebrate fatty acid desaturase with delta 5 and delta 6 activities. *Proc Natl Acad Sci U S A* **98**:14304-14309.

Tocher, D. R., Agaba, M., Hastings, N., Bell, J. G., Dick, J. R., and Teale, A. J. (2002). Nutritional regulation of hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition in zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*). *Fish Physiol Biochem* **24**:309-320.



A vertebrate fatty acid desaturase with Δ5 and Δ6 activities

Nicola Hastings, Morris Agaba, Douglas R. Tocher, Michael J. Leaver, James R. Dick, John R. Sargent, and Alan J. Teale*

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Communicated by John E. Halver, University of Washington, Seattle, WA, September 27, 2001 (received for review August 5, 2001)

Δ5 and Δ6 fatty acid desaturases are critical enzymes in the pathways for the biosynthesis of the polyunsaturated fatty acids arachidonic, eicosapentaenoic, and docosahexaenoic acids. They are encoded by distinct genes in mammals and *Caenorhabditis elegans*. This paper describes a cDNA isolated from zebrafish (*Danio rerio*) with high similarity to mammalian Δ6 desaturase genes. The 1,590-bp sequence specifies a protein that, in common with other fatty acid desaturases, contains an N-terminal cytochrome b_5 domain and three histidine boxes, believed to be involved in catalysis. When the zebrafish cDNA was expressed in *Saccharomyces cerevisiae* it conferred the ability to convert linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3) to their corresponding Δ6 desaturated products, 18:3*n*-6 and 18:4*n*-3. However, in addition it conferred on the yeast the ability to convert di-homo-γ-linoleic acid (20:3*n*-6) and eicosatetraenoic acid (20:4*n*-3) to arachidonic acid (20:4*n*-6) and eicosapentaenoic acid (20:5*n*-3), respectively, indicating that the zebrafish gene encodes an enzyme having both Δ5 and Δ6 desaturase activity. The zebrafish Δ5/Δ6 desaturase may represent a component of a prototypic vertebrate polyunsaturated fatty acids biosynthesis pathway.

Vertebrates lack the Δ12 and Δ15 fatty acid desaturases responsible for converting oleic acid (18:1*n*-9) into linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3) and thus are unable to biosynthesize polyunsaturated fatty acids (PUFA) *de novo* (1, 2). PUFAs therefore are essential dietary nutrients for vertebrates (1, 2). The physiologically active PUFAs are arachidonic acid (20:4*n*-6), eicosapentaenoic acid (20:5*n*-3), and docosahexaenoic acid (22:6*n*-3) and are required for optimal health and normal development of vertebrates (3–6). The pathway from 18:2*n*-6 to arachidonic acid and from 18:3*n*-3 to eicosapentaenoic acid involves desaturations at the Δ6 and Δ5 positions in the carbon backbone, and an intermediate 2-carbon chain elongation step (7). Production of docosahexaenoic acid from eicosapentaenoic acid requires an additional desaturation and 2-carbon chain elongation, although the mechanism may be more complicated (8). However, there is considerable variation between animal species in their abilities to synthesize the C_{20} and C_{22} PUFAs from the plant-derived C_{18} precursors 18:2*n*-6 and 18:3*n*-3. Some animals, notably extreme carnivores, have a very limited ability to synthesize C_{20} and C_{22} PUFAs and consequently have a strict requirement for a dietary source of preformed C_{20} and C_{22} PUFAs (9–12). Although humans generally possess the ability to synthesize C_{20} and C_{22} PUFAs from linoleic and α-linolenic acids, dietary changes as a consequence of intensification of agriculture have resulted in an increase in the 18:2*n*-6/18:3*n*-3 ratio in foods (13). There is considerable evidence that these changes have had, and continue to have, negative impacts on health and development in affected populations (4, 13). The dietary 18:2*n*-6/18:3*n*-3 ratio seems to have increased because of a steady decline in dietary *n*-3 fatty acids over a period of several hundred years, and this increase has been compounded by an increased dietary intake of 18:2*n*-6 in recent decades, particularly in Western societies (13–16).

Meeting the dietary demands of a burgeoning human population for a correct dietary balance of PUFAs, and at levels required for normal health and development, is a major challenge. Clearly, an understanding of the molecular basis of PUFA biosynthesis could underpin efforts to meet this challenge. However, until recently, and although the biochemical pathways involved in PUFA synthesis were described, little was known of the enzymes involved and of the factors affecting their function(s). Some progress has been made recently in characterizing the elongase and desaturases involved in PUFA synthesis (17). Full-length cDNAs for Δ6 desaturases have been isolated from the nematode *Caenorhabditis elegans* (18), rat (19), mouse, and human (20). Fatty acid Δ5 desaturase genes have been isolated from *C. elegans* (21, 22) and humans (23, 24).

We have undertaken to study the PUFA synthesis pathway in fish for two reasons. First, fish are an important source of PUFA, especially of the long chain C_{20} and C_{22} *n*-3 PUFAs that often are deficient in human diets (14–16). Second, there is wide variation between fish species in their ability to synthesize PUFA (25–27). Many freshwater species such as trout, tilapia, and carp are able to convert dietary C_{18} precursor fatty acids to arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid. However, marine species such as turbot and sea bream, which are inherently piscivorous, have very limited abilities to perform these conversions (26–31). A comparison of the genes encoding key elements in the fatty acid desaturation pathway between freshwater and marine species may, therefore, increase knowledge of the molecular components of the pathway itself and of the molecular genetic basis of phenotypic variation in PUFA biosynthesis.

We have demonstrated recently that zebrafish (*Danio rerio*) share the capacity of other freshwater fish species to synthesize C_{20} and C_{22} PUFAs from vegetable oil-derived C_{18} dietary precursors (32). Therefore, because Δ6 desaturation generally is considered to be the rate-limiting step in PUFA synthesis (33), we have initially targeted the Δ6 desaturase gene(s) of zebrafish. Here we describe the cloning and functional characterization of a fatty acid desaturase gene obtained from zebrafish, which, uniquely among such genes described to date, has both Δ5 and Δ6 desaturation activities.

Materials and Methods

Isolation of a Zebrafish Desaturase cDNA and Sequence Analysis. A zebrafish expressed sequence tag sequence (GenBank accession no. AI497337) was identified that displayed high homology to mammalian Δ5 and Δ6 desaturase genes. cDNA was synthesized from zebrafish liver total RNA by using Moloney murine leukemia virus reverse transcriptase primed by the oligonucleotide 5′-GATAGCGGCCGCGTTTTTTTTTTTTTTT(AGC)-3′. Then a portion of this cDNA was subjected to PCR amplification (Ready-to-Go PCR beads, Amersham Pharmacia) with the

Abbreviations: PUFA, polyunsaturated fatty acid; FAME, fatty acid methyl ester; EI, electron ionization.

Data deposition: The sequence reported in this paper for zebrafish Δ5/Δ6 desaturase cDNA has been deposited in the GenBank database (accession no. AF309556).

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primer described above and an oligonucleotide (5′-ATGGGT-GGCGGAGGACAGC-3′) predicted from the zebrafish expressed sequence tag sequence to contain the protein initiation codon. Amplification involved an initial denaturation step at 94°C for 1 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 3 min. The products were cloned into the pYES2 plasmid (Invitrogen) by using standard methods, and nucleotide sequences were determined by using a Perkin–Elmer ABI-377 DNA sequencer. Deduced amino acid sequences were aligned by using CLUSTALX, and sequence phylogenies were predicted by using the neighbor-joining method of Saitou and Nei (34). Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping the data through 1,000 iterations.

Expression of the Zebrafish Desaturase cDNA. The coding sequence of the zebrafish cDNA was amplified by using the forward primer 5′-CCCAAGCTTACTATGGGTGGCGGAGGACAGC-3′ and reverse primer 5′-CCGCTGGAGTTATTTGTTGAGAT-ACGC-3′ containing *Hin*dIII and *Xho*I sites, respectively. The amplified product was ligated into the *Hin*dIII and *Xho*I sites of the pYES yeast expression vector (Invitrogen). The resulting plasmid construct, pYESZFB10, was transformed into *Saccharomyces cerevisiae* (strain INVSc1) by using the EasyComp transformation kit (Invitrogen). Yeast transformed with either the pYES vector or pYESZFB10 were cultured overnight in 2% raffinose, 0.67% nitrogen base, 1% tergitol type Nonidet P-40, and 0.19% uracil dropout medium at 30°C. The cultures then were diluted to an OD_{600} of 0.4 and grown until they reached an OD_{600} of 1, after which expression of the transgene was induced by the addition of galactose to 2% (wt/vol). At this point the cultures were supplemented with one of 0.5 mM 18:2(*n*-6), 18:3(*n*-3), 20:3(*n*-6), or 20:4(*n*-3) and then maintained at 30°C in a shaking incubator. Samples were taken for analysis 48 h after galactose induction.

Fatty Acid Analysis. Approximately equal amounts of yeast cells were transferred into glass conical test tubes after determination of culture densities at OD_{600}. The cells were collected by centrifugation at 500 × *g* for 2 min, and the pellets were washed twice with 5 ml of ice-cold Hanks' balance salt solution and dried under a stream of oxygen-free nitrogen. Fatty acid methyl esters (FAMEs) were prepared by incubating the dried yeast cells directly with 1 ml of methylation reagent containing 10% (vol/vol) concentrated HCl, 5% (vol/vol) 2,2-dimethoxypropane, and 85% (vol/vol) dry methanol for 1 h at 85°C. After incubation, FAMEs were extracted by the addition of 1 ml of 1% NaCl solution and 0.5 ml of hexane containing 0.01% butylated hydroxytoluene as antioxidant. The mixture was mixed vigorously and centrifuged at 600 × *g* for 5 min to promote phase separation. The top phase was removed carefully and filtered through Whatman No. 1 filter paper into a clean glass test tube, and the solvent evaporated under a stream of oxygen-free nitrogen. The FAMEs were purified by TLC and then resuspended in hexane, all as described previously (35). FAMEs were separated in a Fisons GC8160 gas chromatograph equipped with a chemically bonded CP Wax 52CB fused silica wall coated capillary column (30 m × 0.32 mm i.d., Chrompack U.K., London) with an on-column injection system and flame ionization detection. Hydrogen was used as carrier gas with an oven thermal gradient from an initial 50 to 180°C at 40°C/min and then to a final temperature of 235°C at 2°C/min. Individual FAMEs were identified by comparison with known standards, with a well characterized fish oil, and by reference to published data as described previously (35). FAMEs were quantified by using a directly linked PC operating CHROM-CARD software (Thermo-Quest Italia S.P.A., Milan, Italy). All solvents contained 0.01% butylated hydroxytoluene as an antioxidant.

GC-MS. The identities of fatty acids and positions of their double bonds were confirmed by subjecting the picolinyl esters to electron ionization (EI) GC-MS. Free fatty acids were prepared from FAMEs by alkaline hydrolysis as described by Christie (36). Picolinyl esters were prepared by the method of Balazy and Nies (37). This method involves activating the free fatty acid by reaction with 1,1′-carbonyldiimidazole to form the imidazolide, which then reacts with 3-(hydroxymethyl)pyridine under basic conditions to form the picolinyl ester. GC-MS of the picolinyl esters was performed by using a Fisons GC8000 gas chromatograph coupled to an MD800 mass spectrometer (Fisons Instruments, Crawley, U.K.). The gas chromatograph was equipped with a fused silica capillary column (60 m × 0.32 mm i.d., 0.25-mm internal film thickness) coated with Zebron ZB-Wax (Phenomenex, Macclesfield, U.K.) and used helium as carrier gas. Samples were applied by using on-column injection with the oven temperature programmed to rise from 80 to 250°C at 40°C/min.

Materials. Eicosatetraenoic acid (20:4*n*-3, >98% pure) was purchased from Cayman Chemicals (Ann Arbor, MI). Linoleic (18:2*n*-6), α-linolenic (18:3*n*-3), and eicosatrienoic (20:3*n*-6) acids (all >99% pure), BHT, 1,1′-carbonyldiimidazole, 2,2-dimethoxypropane, fatty acid-free BSA, galactose, 3-(hydroxymethyl)pyridine, and Hanks' balanced salt solution, nitrogen base, raffinose, tergitol Nonidet P-40, and uracil dropout medium were obtained from Sigma. TLC (20 × 20 cm × 0.25 mm) plates precoated with silica gel 60 (without fluorescent indicator) were purchased from Merck. All solvents were HPLC grade and obtained from Rathburn Chemicals (Peebleshire, U.K.).

Results

Sequencing revealed that the zebrafish cDNA (GenBank accession no. AF309556) comprised 1,590 bp, which included an ORF specifying a protein of 444 amino acids. The protein sequence included a number of characteristic features of microsomal fatty acid desaturases including three histidine boxes (Fig. 1). The protein sequence also contained an N-terminal cytochrome b_5 domain containing the heme-binding motif, H-P-G-G, similar to that of other fatty acid desaturases. Further, the third histidine box contained a glutamine residue substituted for the first histidine. The amino acid sequence predicted by the zebrafish ORF indicated that the desaturase candidate possessed 64% identity and 78% similarity to human Δ6 desaturase (GenBank accession no. AF126799) and possessed 58% identity and 75% similarity to human Δ5 desaturase (GenBank accession no. AF199596). Phylogenetic analysis, comparing a variety of Δ5 and Δ6 desaturases, clustered the zebrafish sequence with mammalian Δ6 desaturase sequences and with an uncharacterized, putative fish Δ6 desaturase sequence (Fig. 2).

The zebrafish desaturase cDNA was functionally characterized by determining the fatty acid profiles of transformed *S. cerevisiae* containing either the pYES vector alone or the vector with the zebrafish cDNA insert (pYESZFB10) grown in the presence of 18:2*n*-6, 18:3*n*-3, 20:3*n*-6, or 20:4*n*-3. The fatty acid composition of the yeast transformed with the vector alone showed the four main fatty acids normally found in *S. cerevisiae*, namely 16:0, 16:1*n*-7, 18:0, and 18:1*n*-9, together with the four exogenously derived fatty acids (data not shown). This result is consistent with the fact that *S. cerevisiae* does not possess Δ5 or Δ6 fatty acid desaturase activities. Additional peaks were observed in the profiles of pYESZFB10-transformed yeast grown in the presence of the Δ6 desaturase substrate fatty acids, 18:2*n*-6 and 18:3*n*-3, and also in the profiles of pYESZFB10-transformed yeast grown in the presence of the Δ5 desaturase substrate fatty acids, 20:3*n*-6 and 20:4*n*-3 (Fig. 3 *A–D*). Based on GC retention times, the additional peaks associated with the presence of the

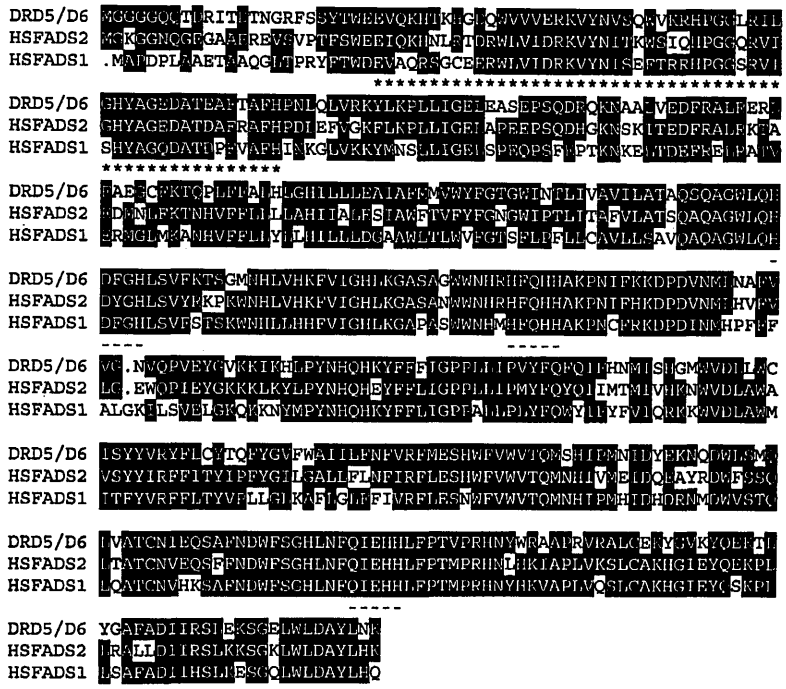

Fig. 1. Alignment of the predicted amino acid sequences of the zebrafish desaturase (DRD5/6) and human Δ5 (HSFADS1) and Δ6 desaturases (HSFADS2). The three ORFs encode 444 amino acid residues. Identical amino acids are in black, the cytochrome b_5-like domain is underlined by asterisks, and the three histidine-rich domains are underlined.

zebrafish cDNA, indicated in Fig. 3 *A–D*, were identified as 18:3*n*-6, 18:4*n*-3, 20:4*n*-6, and 20:5*n*-3, respectively.

The FAME samples from the transformed yeast incubated with the exogenous PUFAs were converted to picolinyl esters and subjected to EI GC-MS to positively identify the structures represented by the additional PUFA peaks produced in cultures containing pYESZFB10. The samples all showed prominent ions at m/z = 92, 108, 151, and 164, which are characteristic of picolinyl esters representing fragments about the pyridine ring (Fig. 4; ref. 38). The EI spectra of the additional fatty acid in pYESZFB10p-transformed yeast incubated with 18:2*n*-6 showed a fragmentation pattern with a mass ion of 369 m/z and prominent peaks at 354, 340, 326, 312, 298, 272, 258, 232, 218, and 192 m/z (Fig. 4*A*). The initial interval of 15 (369–354) represented the terminal methyl and was followed by four intervals of 14, indicating four methylene groups. The intervals of 26 (298–272, 258–232, and 218–192) denoted the positions of three double bonds, indicating that this fatty acid is $^{\Delta 12,9,6}$18:3 = 18:3*n*-6. The EI spectra of the additional fatty acid from cells incubated with 18:3*n*-3 showed a mass ion of 367 m/z and fragments at 338, 312, 298, 272, 258, 232, 218, and 192 m/z, confirming this fatty acid as $^{\Delta 15,12,9,6}$18:4 = 18:4*n*-3 (Fig. 4*B*). The EI spectra of the additional fatty acid produced in cells incubated with 20:3*n*-6 showed a fragmentation pattern with a mass ion of 395 m/z and prominent ions at intervals of 26 (324–298, 284–258, 244–218, and 204–178 m/z), denoting the position of the double bonds and indicating that this fatty acid is $^{\Delta 14,11,8,5}$20:4 = 20:4*n*-6 (Fig. 4*C*). Similarly, the EI spectra of the additional fatty acid in cells incubated with 20:4*n*-3 showed a fragmentation pattern with a mass ion of 393 m/z with prominent ions at intervals of 26 (364–338, 324–298, 284–258, 244–218, and 204–178 m/z), confirming that this fatty acid is $^{\Delta 17,14,11,8,5}$20:5 = 20:5*n*-3 (Fig. 4*D*). The GC-MS data confirmed that the zebrafish clone is a fatty acid desaturase that introduces double bonds into 18:2*n*-6 and 18:3*n*-3 at the Δ6 position and also into 20:3*n*-6 and 20:4*n*-3 at the Δ5 position.

Thus, the analyses revealed that the cells transformed with pYESZFB10 had acquired functional Δ6 and Δ5 fatty acid

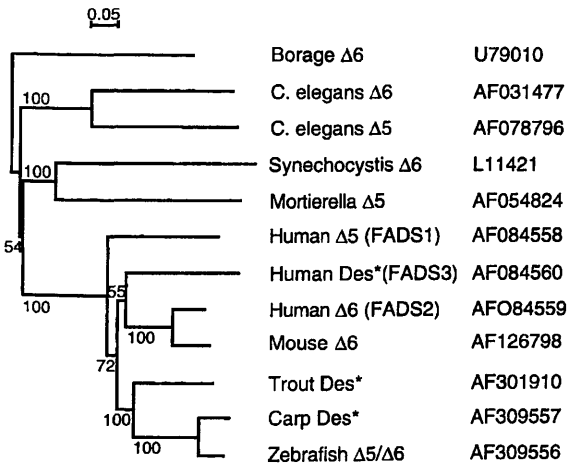

Fig. 2. Phylogeny of desaturase deduced amino acid sequences. Sequences marked with an asterisk are not functionally characterized. Database accession numbers for the nucleic acid sequences are indicated. The conditions used for neighbor-joining tree construction are described in *Materials and Methods* and were applied by using CLUSTALW and NJPLOT. Horizontal branch lengths are proportional to the number of amino acid replacements per position, and the scale bar indicates this value. Numbers represent the percentage frequencies with which the tree topology presented here was replicated after 1,000 bootstrap iterations.

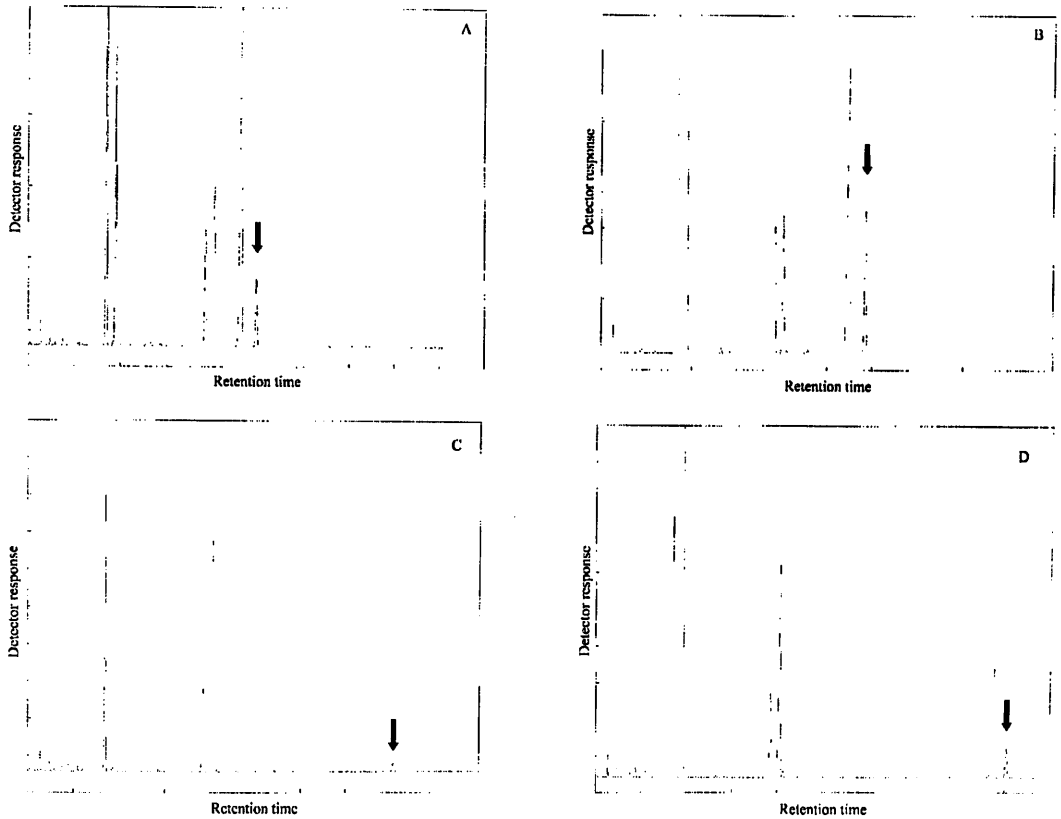

Fig. 3. Identification of fatty acid desaturation products in transgenic yeast. FAMEs were extracted from yeast transformed with pYES2FB10 grown in the presence of 18:2*n*-6 (*A*), 18:3*n*-3 (*B*), 20:3*n*-6 (*C*), or 20:4*n*-3 (*D*). The first four peaks in *A–D* are 16:0, 16:1*n*-7, 18:0, and 18:1*n*-9, respectively. The fifth peaks in each panel are the exogenously added fatty acids 18:2*n*-6 (*A*), 18:3*n*-3 (*B*), 20:3*n*-6 (*C*), and 20:4*n*-3 (*D*), respectively. The sixth peaks in each panel (arrowed) were identified tentatively (based on retention times) as 18:3*n*-6 (*A*), 18:4*n*-3 (*B*), 20:4*n*-6 (*C*), and 20:5*n*-3 (*D*), respectively.

desaturase activity. On the basis of the percentages of substrate fatty acids converted to product, the zebrafish gene is more active with Δ6 desaturase substrates than with Δ5 substrates and preferentially converts *n*-3 fatty acids rather than *n*-6 fatty acids (Table 1).

Discussion

The 1,590-bp ORF of the zebrafish cDNA encodes a protein with substantial similarity to vertebrate Δ6 desaturases. Overall amino acid identities are 64% to human Δ6 desaturase and 58% to human Δ5 desaturase. In addition, the zebrafish protein contains a similar N-terminal cytochrome b_5-like domain and the three catalytically important histidine boxes conserved in all members of the desaturase gene family. It also includes the variant third histidine box that seems typical of Δ5 and Δ6 desaturase genes described to date.

The analyses demonstrated unequivocally that the zebrafish cDNA encodes a polypeptide with both Δ5 and Δ6 fatty acid desaturase activities with slight biases toward *n*-3 substrates and Δ6 function.

Phylogenetic analysis indicates that with respect to functionally characterized genes, the zebrafish sequence has highest homology with mammalian Δ6 desaturases, with human Δ5 desaturase appearing to be distinct from the Δ6 desaturase sequences. Human Δ5 and Δ6 desaturase genes (*FADS1* and *FADS2*, respectively) are clustered together with a related gene (*FADS3*) of unknown function on chromosome 11 and have arisen presumably from a gene-duplication event (39). The fact that the Δ5 and *FADS3* sequences fall on distinct branches of the phylogenetic tree from that occupied by the Δ6 sequences suggests that this gene-duplication event(s) predates the divergence of mammalian and fish evolutionary lines. This interpretation suggests that a homologue of the human Δ5 gene should exist in fish species. Interestingly, the nematode *C. elegans* also possesses a cluster of Δ5 and Δ6 desaturase genes that seem to have arisen by gene duplication. However, this duplication clearly occurred after the divergence of nematodes and vertebrates (18, 21). Both the mammalian and nematode Δ5 and Δ6 desaturase enzymes have distinct, nonoverlapping substrate specificities. It therefore is remarkable that the zebrafish desaturase, when expressed in *S. cerevisiae*, exhibits both Δ5 and Δ6 fatty acid desaturase activities with a distinct preference for *n*-3 compared with *n*-6 substrates.

Although more fatty acid desaturase genes may be found in both zebrafish and mammals, it is conceivable that the bifunctional desaturase described here is a component of a prototypic vertebrate PUFA biosynthetic pathway that has persisted in a freshwater fish species. That humans and other mammals have two distinct enzymes for Δ5 and Δ6 desaturation may be an adaptation to a terrestrial diet providing relatively lower amounts of preformed C_{20} and C_{22} PUFAs than the diets of a vertebrate ancestor that they share with freshwater fish. Func-

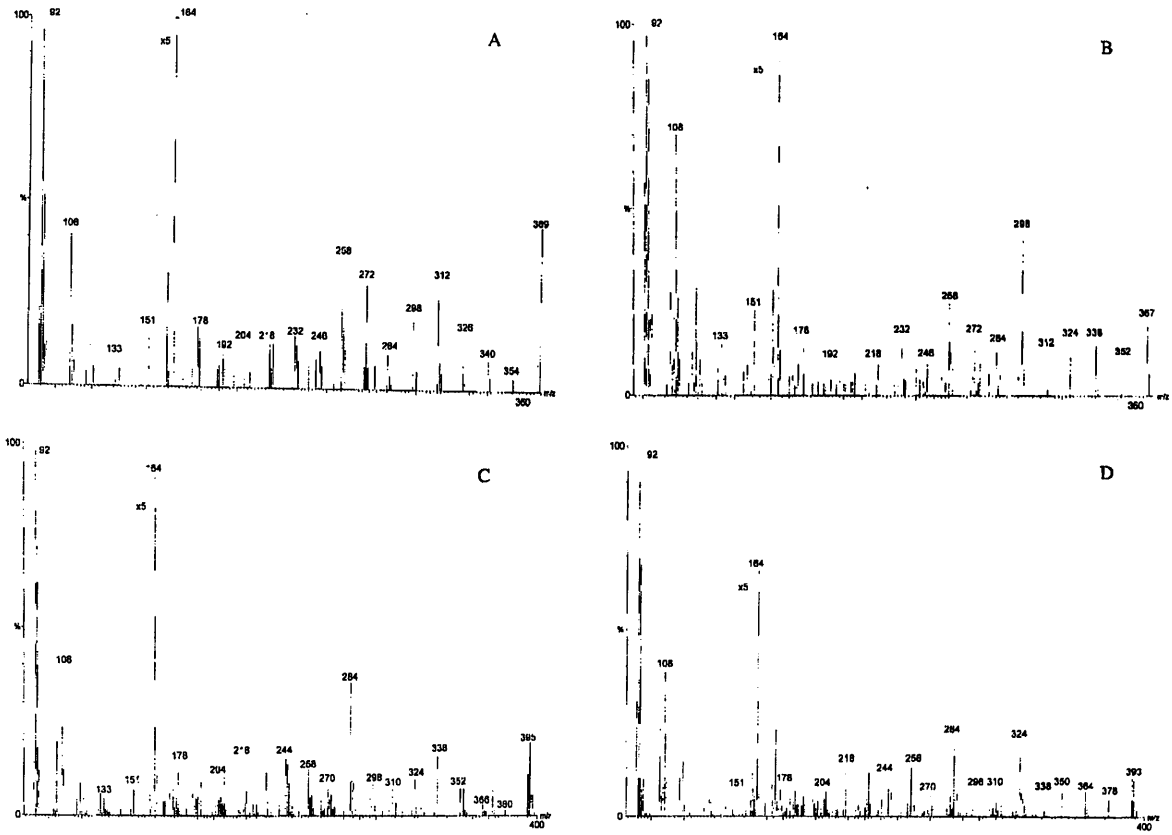

Fig. 4. Mass spectra of the arrowed peaks in Fig. 3. Picolinyl esters were prepared from FAMEs extracted from yeast transformed with pYESZFB10 grown in the presence of 18:2*n*-6 (*A*), 18:3*n*-3 (*B*), 20:3*n*-6 (*C*), and 20:4*n*-3 (*D*) and analyzed by GC-MS as described in *Materials and Methods*. The identities of the peaks were confirmed as 18:3*n*-6 (*A*), 18:5*n*-3 (*B*), 20:4*n*-6 (*C*), 20:5*n*-3 (*D*).

tional divergence of the products of a putative ancient gene-duplication event is a possible mechanism underlying adaptation to such a dietary change.

Conversely, the apparent deficiencies in the fatty acid desaturation pathway in some marine fish species, many of which are strictly piscivorous, may be a result of relaxation of constraints on a prototypic pathway in an environment providing a diet that is naturally rich in C_{20} and C_{22} PUFAs. Certainly the discovery of a bifunctional enzyme in zebrafish is consistent with findings in the piscivorous marine species turbot (*Scophthalmus maximus*). In turbot, a deficiency in the fatty acid desaturation/elongation pathway has been ascribed to a lack of C_{18-20} elongase function, while both Δ6 and Δ5 desaturase capabilities have been retained (40). However, in another marine species, gilthead sea bream (*Sparus aurata*), a block in the fatty acid desaturation/elongation pathway has been identified as a deficiency in Δ5 desaturase activity (41). In this case it is possible that a homologous desaturase enzyme has lost significant Δ5 desaturase activity, thus limiting the efficiency of the PUFA biosynthetic pathway in gilthead sea bream.

Table 1. pYESZFB10 substrate conversion and activity type

Substrate fatty acid	Substrate conversion, %	Desaturase activity
18:2*n*-6	11.7	Δ6
18:3*n*-3	29.4	Δ6
20:3*n*-6	8.3	Δ5
20:4*n*-3	20.4	Δ5

The ability of fish to provide 20:5*n*-3 and 22:6*n*-3 for human and other animal diets has become an increasingly important subject in recent years after the global stagnation and decline of capture marine fisheries (42). These fisheries hitherto have been the major source of 20:5*n*-3 and 22:6*n*-3 in human and animal diets (13). Increased fish farming potentially can offset the effects of this decline in a key marine resource. However, where farmed marine species are concerned, a dietary source of 20:5*n*-3 and 22:6*n*-3 is required, and this hitherto has been provided by fish oils from captured marine "feed species" such as sand eels, capelin, and anchovies (42). Such feed species are themselves finite resources that, in the case of anchovies, is reduced markedly by climatic phenomena such as El Niño. Understanding the molecular basis of the differences between marine and freshwater fish in their abilities to synthesize 20:5*n*-3 and 22:6*n*-3 may suggest ways in which the reliance of farmed marine species on marine fish oils could be reduced.

Here we report a fatty acid desaturase with both Δ6 and Δ5 activities. Such an enzyme could contribute to a biotechnological solution for 20:5*n*-3 and 22:6*n*-3 production, ultimately relieving pressure for unsustainable extraction of these key nutrients from natural marine sources.

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Nutritional regulation of hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition in zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*)

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Abstract

The desaturation and elongation of [1-^{14}C]18:3n-3 was investigated in hepatocytes of the tropical warm freshwater species, zebrafish (*Danio rerio*) and Nile tilapia (*Oreochromis niloticus*). The hepatocyte fatty acid desaturation/elongation pathway was assayed before and after the fish were fed two experimental diets, a control diet containing fish oil (FO) and a diet containing vegetable oil (VO; a blend of olive, linseed and high oleic acid sunflower oils) for 10 weeks. The VO diet was formulated to provide 1% each of 18:2n-6 and 18:3n-3, and so satisfy the possible EFA requirements of zebrafish and tilapia. At the end of the dietary trial, the lipid and fatty acid composition was determined in whole zebrafish, and liver, white muscle and brain of tilapia. Both zebrafish and tilapia expressed a hepatocyte fatty acid desaturation/elongation pattern consistent with them being freshwater and planktonivorous fish. The data also showed that hepatic fatty acid desaturation/elongation was nutritionally regulated with the activities being higher in fish fed the VO diet compared to fish fed the FO diet. In zebrafish, the main effect of the VO diet was increased fatty acid Δ6 desaturase activity resulting in the production of significantly more 18:4n-3 compared to fish fed the FO diet. In tilapia, all activities in the pathway were greater in fish fed the VO diet resulting in increased amounts of all fatty acids in the pathway, but primarily eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). However, the fatty acid compositional data indicated that despite increased activity, desaturation of 18:3n-3 was insufficient to maintain tissue proportions of EPA and DHA in fish fed the VO diet at the same level as in fish fed the FO diet. Practically, these results indicate that manipulation of tilapia diets in commercial culture in response to the declining global fish oil market would have important consequences for fish fatty acid composition and the health of consumers. Scientifically, zebrafish and tilapia, both the subject of active genome mapping projects, could be useful models for studies of lipid and fatty acid metabolism at a molecular biological and genetic level.

Abbreviations: AA – arachidonic acid; BHT – butylated hydroxytoluene; DHA – docosahexaenoic acid; EFA – essential fatty acid; EPA – eicosapentaenoic acid; FAF-BSA – fatty acid free bovine serum albumin; FO – fish oil; HBSS – Hanks balanced salt solution; HUFA – highly unsaturated fatty acids acids (carbon chain length $\geq C_{20}$ with $\geq$ 3 double bonds); PL, polar lipid; PUFA, polyunsaturated fatty acids; VO, vegetable oil; TLC, thin-layer chromatography.

Introduction

The polyunsaturated fatty acids (PUFA), linoleate (18:2n-6) and linolenate (18:3n-3) cannot be biosynthesized *de novo* by most animals, including fish, and so they are termed essential fatty acids (EFA) (Holman 1986). The precise EFA requirements of fish varies both quantitatively and qualitatively between species (Watanabe 1982; Kanazawa 1985; Sargent et al. 1995, 1999). Thus, fish species displaying a so-called 'freshwater' pattern are able to convert the C_{18} EFA, 18:3n-3 and 18:2n-6, to the longer chain, more unsaturated and physiologically important highly unsaturated fatty acids (HUFA), eicosapentaenoic acid (20:5n-3; EPA), docosahexaenoic acid (22:6n-3; DHA) and arachidonic acid (20:4n-6; AA), via a series of fatty acid desaturation and elongations, and so only require the C_{18} PUFA (Sargent et al. 1989, 1995; Henderson and Tocher 1986). In contrast, fish species displaying a typical 'marine' pattern cannot perform these conversions at an appreciable rate and so require a dietary source of the essential HUFA (Sargent et al. 1989, 1995). However, the 'marine' pattern may actually be associated with adaptation to a carnivorous or, more specifically, a piscivorous, lifestyle where consumption of a predominantly fish diet, naturally rich in HUFA, has resulted in an evolutionary down-regulation of the desaturase and/or elongase enzyme activities required for the conversion of C_{18} PUFA to HUFA (Mourente and Tocher 1993, 1994; Sargent et al. 1995). These hypotheses are based on data from relatively few species including rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), pike (*Esox lucius*), turbot (*Scophthalmus maximus*) and sea bream (*Sparus aurata*) (Owen et al. 1975; Mourente and Tocher 1994; Sargent et al. 1995, 1999; Buzzi et al. 1997; Henderson et al. 1997). The fatty acid desaturation pathways in fish are currently of great interest as there is an urgent need to replace the $C_{20/22}$ HUFA-rich fish oils, derived from non-sustainable marine fisheries, with vegetable oils, rich in C_{18} PUFA, in the diets of aquacultured fish species (Sargent and Tacon 1999).

Diet is also known to directly affect desaturase enzyme activities in mammals (Brenner 1981), and we have shown that the hepatic desaturation of 18:3n-3 and 18:2n-6 was significantly greater in Atlantic salmon fed diets containing vegetable oils rich in 18:2n-6 and 18:3n-3 compared to fish fed diets containing fish oils rich in the long-chain HUFA, EPA and DHA (Bell et al. 1997; Tocher et al. 1997, 2000). Increased hepatic fatty acid desaturase activities were also induced in rainbow trout fed a diet containing olive oil compared to a diet containing fish oil (Buzzi et al. 1996) and the conversion of intraperitoneally injected ^{14}C-labelled 18:2n-6 and 18:3n-3 to HUFA was increased in Arctic charr (*Salvelinus alpinus*) fed diets containing only C_{18} PUFA compared to commercial diets containing fish oil (Olsen and Ringo 1992). However, the effects of feeding dietary vegetable oils on hepatic fatty acid desaturation in fish has been restricted almost exclusively to these studies on salmonids.

In the present study we investigated the desaturation and elongation of [1-^{14}C]18:3n-3 in hepatocytes of two species of warm freshwater tropical fish, zebrafish (*Danio rerio*), the most important fish model species, and tilapia (*Oreochromis niloticus*), a species commercially important due to its extensive aquaculture. Fish were sampled initially immediately prior to being fed two diets, a control diet containing fish oil (FO) and a diet containing vegetable oils (VO; a blend of olive, linseed and high oleic acid sunflower oils) for 10 weeks. The VO diet was formulated to provide approximately 1% each of 18:2n-6 and 18:3n-3 and so satisfy the possible EFA requirements of zebrafish and tilapia (Sargent et al. 1995). At each sampling point, fatty acyl desaturation and elongation activities were determined in isolated hepatocytes and at the end of the trial, whole zebrafish and samples of tilapia liver, white muscle and brain were collected for analyses of lipid and fatty acid composition.

Materials and methods

Animals and diets

Zebrafish (of approximately 2 months of age) were obtained from a wholesale tropical fish supplier (Vale Aquatics, Edinburgh, Scotland) and were maintained in duplicate 50 l aquaria (60 × 30 × 30 cm) containing filtered freshwater which was maintained at a constant temperature of 26±1 °C. The tanks were cleaned daily with approximately one third of the water replaced each day. Fish were fed twice a day by hand to satiety. Initially the zebrafish were fed a commercial flake diet (Tetraflake, Tetra Werke, Germany).

Tilapia were bred in the Institute of Aquaculture and were male F1s produced by natural mating of an XX female with a YY male (these parents being diploid, homozygous products of gynogenesis). Fish

Table 1. Components (g/Kg of dry diet) of experimental diets

Component	Fish oil (FO) diet	Vegetable oil (VO) diet
Vitamin-free casein[1]	480	480
Potato starch[2]	150	150
Fish meal[3]	50	50
Mineral mix[4]	47	47
Vitamin mix[5]	10	10
Arginine	4	4
Leucine	4	4
Methionine	3	3
Cystine	2	2
Orange G	1	1
α-cellulose	139.6	139.6
Fish oil[6]	110	0
Olive oil[7]	0	75
Linseed oil[8]	0	17.5
High oleic sunflower oil[9]	0	17.5
Antioxidant mix[10]	0.4	0.4

[1]Vitamin-free micropulverised (ICN Biomedical Ltd., High Wycombe, UK).
[2]Passeli WA4 (Avebe (U.K.) Ltd., Ulceby, South Humberside, UK.
[3]LT94, Low temperature fish meal (Ewos Ltd., Bathgate, UK).
[4]Supplied (per kg diet): KH_2PO_4, 22 g; $FeSO_4 \cdot 7H_2O$, 1.0 g; $ZnSO4 \cdot 7H_2O$, 0.13 g; $MnSO_4 \cdot 4H_2O$, 52.8 mg; $CuSO_4 \cdot 5H_2O$, 12 mg; $CoSO_4 \cdot 7H_2O$, 2 mg.
[5]Supplied (mg/kg diet): ascorbic acid, 1000; myo-inositol, 400; nicotinic acid, 150; calcium pantothenate, 44; all-rac-α-tocopheryl acetate, 40; riboflavin, 20; pyridoxine hydrochloride, 12; menadione, 10; thiamine hydrochloride, 10; retinyl acetate, 7;3; folic acid, 5; biotin, 1; cholecalciferol, 0.06; cyanocobalamin, 0.02.
[6]Northern hemisphere fish oil (FOSOL, Seven Seas Ltd., Hull, UK).
[7]Tesco, Cheshunt, UK.
[8]ICN Biomedical Ltd., High Wycombe, UK.
[9]Croda, Hull, UK.
[10]Dissolved in propylene glycol and contained (g/l): butylated hydroxy anisole, 60; propyl gallate, 60; citric acid, 40. All the other ingredients were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, UK.

were 10 weeks old (approximately 4–5 g) at the initiation of the dietary trial and were fed a standard salmonid starter diet containing 54% protein and 15% oil prior to initiation of the dietary trial (Fry 02, pellet size, 1.0–1.5 mm; Trouw Aquaculture Ltd., Scotland). Tilapia were stocked initially at a density of 45 fish per tank in duplicate 20 l circular aquaria (0.5 × 0.5 m) supplied with recirculating, filtered (mechanical and biofilter) freshwater at a flow rate of 3 l × min^{-1} with a system replacement rate of approximately 0.4 l × min^{-1}, representing approximately $^1/_3$ of the total system volume per day. Water temperature was maintained at a constant 27 ± 1 °C.

The experimental diets were prepared in the Institute of Aquaculture. The diets contained 50% protein and 11% lipid and their formulation is shown in Table 1. The dry ingredients were combined and mixed before the addition of the oils and antioxidants and mixing continued for 5 min. Water was added to 30% of the dry weight to enable pelleting. The pellets were air dried for 48 h before storage at −20 °C until use. Pellets of 1.0–1.5 mm were prepared and fed directly to tilapia. For zebrafish, sieved fines of up to 0.5 mm were used. The fatty acid compositions of the diets are shown in Table 2. All diets were formulated to satisfy the nutritional requirements of freshwater fish (U.S. National Research Council 1993).

Lipid extraction and quantitation

For zebrafish, three whole fish per dietary group were analysed for lipid and fatty acid compositions at the end of the dietary trial. With tilapia, liver, brain and a fillet of white muscle were dissected from three fish per dietary group at the end of the trial. Total lipid was extracted from fish tissues and diet samples by homogenisation in ice-cold chloroform/methanol (2:1, v/v) by the method of Folch et al. (1957) and quantified gravimetrically.

Lipid class and fatty acid composition

Separation and quantification of lipid classes in fish body and tissue samples was performed by single-dimension double-development high-performance thin-layer chromatography (HPTLC) followed by scanning densitometry as described previously (Henderson and Tocher 1992). Fatty acid methyl esters (FAME) from total lipid were prepared by acid-catalyzed transmethylation (Christie 1989), and were extracted and purified by thin-layer chromatography (TLC) as described previously (Tocher and Harvie 1988). Analysis of FAME was performed by gas chromatography in a Fisons GC8000 gas chromatograph (Crawley, UK) equipped with a fused-silica capillary column (30 m × 0.32 mm i.d., CP Wax 52 CB, Chrompack, UK) using hydrogen as carrier gas. Temperature programming was from 50 to 150 °C at 35 °C/min and to 225 °C at 2.5 °C/min. Individual FAME were identified by comparison with known standards and published data (Ackman 1980; Bell et al. 1983).

Table 2. Lipid (oil) content (percentage of diet dry weight) and fatty acid composition (percentage of total fatty acids by weight) of experimental diets containing fish oil (FO) or vegetable oil (VO) and of commercial diets fed to the zebrafish (tetraflake) and tilapia (trout pellet) prior to the initiation of the dietary trials

	Commercial diets		Experimental diets	
Fatty acid	Tetraflake	Trout pellet	FO	VO
14:0	2.5	5.6	5.3	0.4
15:0	0.6	0.5	0.7	n.d.
16:0	17.1	14.7	15.9	9.3
17:0	0.5	0.4	0.5	0.1
18:0	6.7	2.3	3.2	2.6
20:0	2.8	0.2	0.2	0.3
Total saturated	30.3	23.6	25.8	12.7
16:1n-9	0.1	0.2	0.3	n.d.
16:1n-7	3.1	4.7	5.4	0.7
18:1n-9	16.6	14.0	15.0	64.9
18:1n-7	2.5	2.5	3.2	0.4
20:1n-9	2.5	9.5	9.4	0.9
22:1n-11	2.8	13.5	12.1	0.8
22:1n-9	2.1	0.3	1.0	n.d.
24:1n-9	0.5	0.9	1.1	n.d.
Total monoenes	30.6	45.6	47.5	67.7
18:2n-6	27.2	3.2	1.5	9.1
18:3n-6	n.d.	0.2	0.2	n.d.
20:2n-6	0.1	0.4	n.d.	n.d.
20:3n-6	n.d.	0.6	n.d.	n.d.
20:4n-6	0.6	0.5	0.6	n.d.
22:5n-6	n.d.	0.2	0.2	n.d.
Total n-6 PUFA	27.9	5.0	2.5	9.1
18:3n-3	3.5	1.4	1.1	8.7
18:4n-3	0.4	3.4	2.0	0.1
20:4n-3	0.2	0.9	0.6	n.d.
20:5n-3	2.4	6.7	5.1	0.4
22:5n-3	0.5	0.9	1.0	n.d.
22:6n-3	4.2	11.3	7.9	0.7
Total n-3 PUFA	11.3	24.5	17.7	9.9
Total PUFA	39.2	30.4	20.2	19.0
Lipid content	9.2	15.0	11.0	11.0

n.d., not detected; PUFA, polyunsaturated fatty acids.

Preparation of isolated hepatocytes

Fish were killed by a blow to the head and the livers dissected immediately. The liver was chopped finely with scissors and incubated with 15 ml of solution A (calcium and magnesium-free Hanks balanced salt solution (HBSS) + 10 mM HEPES buffer + 1 mM EDTA) containing 0.1% (w/v) collagenase in a 25 ml 'Reacti-flask' in a shaking water bath at 25 °C for 30 min. The digested liver was filtered through 100 μm nylon gauze and the cells collected by centrifugation at 600 × g for 3 min. The cell pellet was washed with 20 ml of solution A containing 1% w/v fatty acid-free bovine serum albumin (FAF-BSA) and re-centrifuged as above. The hepatocytes were resuspended in 5 ml of Medium 199 containing

10 mM HEPES, 2 mM glutamine, 100 U ml penicillin and 0.1 mg ml streptomycin. One hundred μl of cell suspension was mixed with 400 μl of Trypan Blue and hepatocytes were counted and their viability assessed using a haemocytometer. One hundred μl of the hepatocyte cell suspension was retained for protein determination.

Assay of hepatocyte fatty acyl desaturation/elongation activities

The hepatocyte suspension (5 ml) was dispensed into a 25 cm^2 tissue culture flask. The cells were incubated with 0.25 μCi of [1-^{14}C] 18:3n-3 (final fatty acid concentration, ~1 μM), added as a complex with FAF-BSA in medium 199 prepared as described previously (Ghioni et al. 1997). After addition of isotope the flasks were incubated at 25 °C for 2 h. After incubation, the cell layer was dislodged by gentle rocking, the cell suspension transferred to glass conical test tubes and the flasks washed with 1 ml of ice-cold HBSS containing 1% FAF-BSA. The cell suspensions were centrifuged at 600 *g* for 2 min, the supernatant discarded and the cell pellets washed with 5 ml of ice-cold HBSS/FAF-BSA. The supernatant was discarded and the tubes placed upside down on paper towels to blot for 15 sec before extraction of total lipid using ice-cold chloroform/methanol (2:1, v/v) containing 0.01% (w/v) butylated hydroxy toluene (BHT) essentially as described by Folch et al. (1957) and as described in detail previously (Tocher et al. 1988).

Total lipid was transmethylated and FAME prepared as described above. The methyl esters were redissolved in 100 μl hexane containing 0.01% BHT and applied as 2.5 cm streaks to TLC plates impregnated by spraying with 2 g silver nitrate in 20 ml acetonitrile and pre-activated at 110 °C for 30 min. Plates were fully developed in toluene/acetonitrile (95:5, v/v) (Wilson and Sargent 1992). Autoradiography was performed with Kodak MR2 film for 6 days at room temperature. Areas of silica containing individual PUFA were scraped into scintillation mini-vials containing 2.5 ml of scintillation fluid (Ecoscint A, National Diagnostics, Atlanta, Georgia) and radioactivity determined in a TRI-CARB 2000CA scintillation counter (United Technologies Packard, UK). Results were corrected for counting efficiency and quenching of ^{14}C under exactly these conditions.

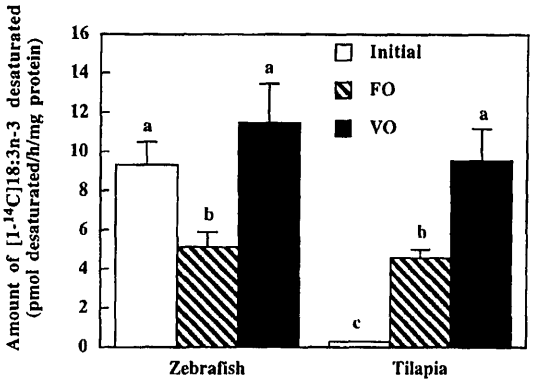

Figure 1. Effect of diet on total fatty acid desaturation activity in zebrafish (*Danio rerio*) and tilapia (*Oreochromis niloticus*) hepatocytes. Fish were fed for 10 weeks on diets containing either 11% fish oil (FO) or 11% vegetable oil (VO) after being initially reared on either a commercial flake diet (zebrafish) or a commercial trout pellet (tilapia) as described in Materials and methods. Columns for each species with different superscript letters are significantly different as determined by one-way ANOVA followed by Tukey's multiple range test.

Protein determination

Protein concentration in isolated hepatocyte suspensions was determined according to the method of Lowry et al. (1951) after incubation with 0.25 ml of 0.25% (w/v) SDS/1M NaOH for 45 min at 60 °C.

Materials

[1-^{14}C]18:3n-3 (50–55 mCi mmol) was obtained from NEN (DuPont (U.K.) Ltd., Stevenage, UK). HBSS, Medium 199, HEPES buffer, glutamine, penicillin, streptomycin, collagenase (type IV), FAF-BSA, BHT and silver nitrate were obtained from Sigma Chemical Co. (Poole, U.K.). TLC plates (20 cm × 20 cm × 0.25 mm) and HPTLC plates (10 cm × 10 cm × 0.15 mm), precoated with silica gel 60 (without fluorescent indicator) were obtained from Merck (Darmstadt, Germany). All solvents were HPLC grade and were obtained from Fisher Scientific UK, Loughborough, England.

Statistical analysis

Data are presented as means ± SD (n = 3). The significance of dietary effects on total fatty acid desaturation in hepatocytes was determined by one-way analysis of variance (ANOVA) followed where pertinent by Tukey's multiple range test. The significance of effects on individual products of hepatocyte fatty

Table 3. Lipid content (percentage of wet weight) and lipid class composition (percentage of total lipid) of zebrafish (whole fish) fed diets containing fish oil (FO) or vegetable oil (VO)

Parameter	FO	VO
Lipid content	10.2 ± 0.5	10.2 ± 2.7
Lipid class		
Phosphatidylcholine	7.6 ± 1.8	10.4 ± 0.3
Phosphatidylethanolamine	4.6 ± 0.9	6.2 ± 0.6
Phosphatidylserine	0.8 ± 0.1	1.4 ± 0.3*
Phosphatidylinositol	0.8 ± 0.2	1.5 ± 0.1*
Phosphatidic acid/cardiolipin	0.8 ± 0.2	0.9 ± 0.2
Shingomyelin	0.8 ± 0.1	1.4 ± 0.6
Total polar lipids	15.4 ± 3.0	21.9 ± 1.7*
Total neutral lipids	84.6 ± 3.0	78.1 ± 1.7*
Triacylglycerol	71.1 ± 5.2	60.0 ± 1.5*
Cholesterol	8.0 ± 1.3	13.0 ± 0.9*
Free fatty acids	2.6 ± 0.6	3.2 ± 0.7
Steryl esters	3.0 ± 1.1	1.9 ± 1.2

Results are means ± SD (n = 3). Differences between mean values for fish fed the VO diet compared to fish fed the FO diet were determined by the Student's t-test and are significantly different where indicated (* $P < 0.05$).

acid desaturation and whole fish or tissue lipid and fatty acid compositions due to diet were determined by the Student's t-test. Differences were regarded as significant when $P < 0.05$ (Zar 1984).

Results

Effects of diet on hepatocyte fatty acid desaturation/elongation activities

The total desaturation/elongation of [1-^{14}C]18:3n-3 in isolated hepatocytes was significantly higher in both zebrafish and tilapia fed the VO diet compared to fish fed the FO diet (Figure 1). Initially, the total fatty acid desaturation activity in hepatocytes from zebrafish being fed the commercial flake diet was high and not significantly different to the activity in fish fed the VO diet. In contrast, the total fatty acid desaturation activity in hepatocytes from tilapia being fed the commercial trout diet at the initiation of the dietary trial was very low and significantly lower than in fish at the end of the trial fed either the VO or FO diets (Figure 1). The main products of desaturation of [1-^{14}C]18:3n-3 in zebrafish hepatocytes at the initiation of the dietary trial were 18:4n-3 followed by 20:5n-3 (Figure 2). The primary effect of feeding

Table 4. Fatty acid composition (percentage of total fatty acids by weight) of carcasses of zebrafish fed the diets containing fish oil (FO) or vegetable oil (VO)

Fatty acid	FO	VO
14:0	1.7 ± 0.2	0.7 ± 0.1*
15:0	0.4 ± 0.0	0.3 ± 0.0
16:0	23.1 ± 0.2	21.9 ± 1.7
17:0	tr	tr
18:0	3.2 ± 0.1	3.2 ± 0.1
20:0	tr	tr
Total saturated	28.4 ± 0.1	26.1 ± 1.7
16:1n-9	1.4 ± 0.3	2.9 ± 0.4*
16:1n-7	4.1 ± 0.3	3.2 ± 0.4*
18:1n-9	37.4 ± 0.6	47.8 ± 0.5*
18:1n-7	2.5 ± 0.2	2.6 ± 0.2
20:1n-11	0.6 ± 0.1	0.3 ± 0.0*
20:1n-9	2.2 ± 0.1	0.7 ± 0.1*
20:1n-7	0.1 ± 0.0	0.0 ± 0.0
22:1	1.9 ± 0.1	0.3 ± 0.0*
24:1n-9	0.2 ± 0.0	0.1 ± 0.0
Total monoenes	50.3 ± 0.5	57.8 ± 0.9*
18:2n-6	4.5 ± 0.5	4.9 ± 0.5
18:3n-6	0.2 ± 0.0	0.2 ± 0.0
20:2n-6	0.5 ± 0.1	0.9 ± 0.2*
20:3n-6	0.4 ± 0.0	0.6 ± 0.0*
20:4n-6	1.3 ± 0.2	2.2 ± 0.4*
22:4n-6	0.2 ± 0.0	0.4 ± 0.1*
22:5n-6	0.2 ± 0.0	0.3 ± 0.0*
Total n-6 PUFA	7.3 ± 0.6	9.5 ± 0.9*
18:3n-3	1.4 ± 0.2	2.0 ± 0.2*
18:4n-3	0.7 ± 0.1	2.0 ± 0.0*
20:3n-3	0.1 ± 0.0	0.0 ± 0.0
20:4n-3	0.4 ± 0.0	0.1 ± 0.0*
20:5n-3	2.8 ± 0.2	0.4 ± 0.2*
22:5n-3	0.9 ± 0.0	0.2 ± 0.1*
22:6n-3	7.8 ± 0.8	3.7 ± 1.2*
Total n-3 PUFA	14.0 ± 0.9	6.6 ± 1.8*
Total PUFA	21.3 ± 0.4	16.1 ± 2.5*

Results are means ± SD (n = 3). Differences between mean values for fish fed the VO diet compared to fish fed the FO diet were determined by the Student's t-test and are significantly different where indicated (*, $P < 0.05$). PUFA, polyunsaturated fatty acids; tr, traced value (<0.05%).

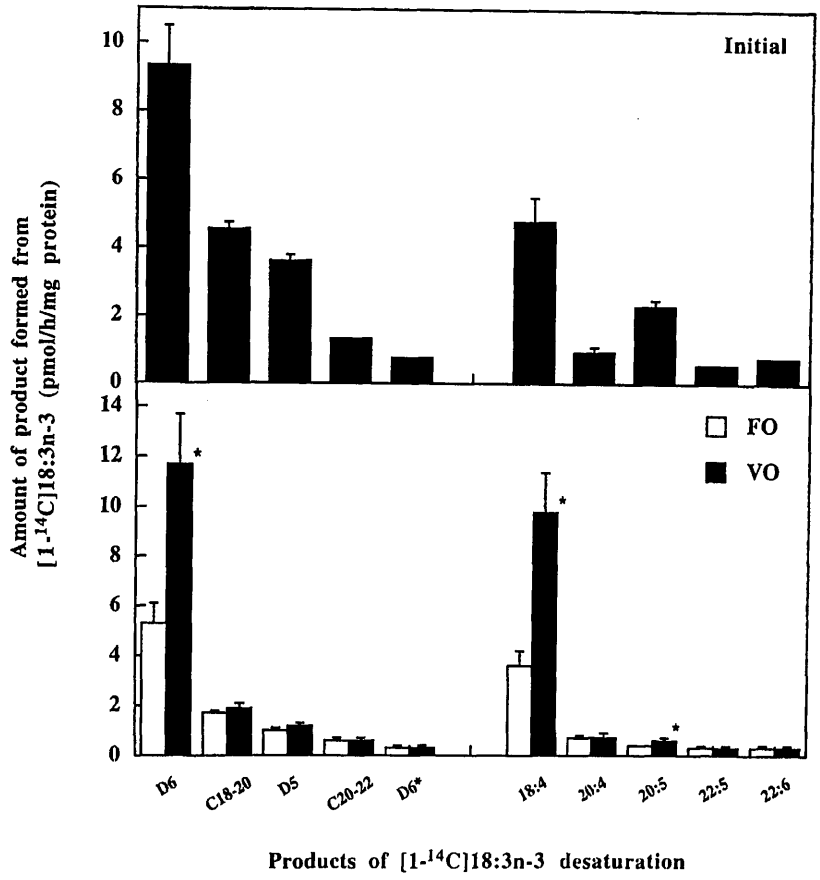

Figure 2. Effect of diet on the apparent activities of the fatty acid desaturation and elongation pathway, and on the individual fatty acid products of the desaturation/elongation pathway in zebrafish (*Danio rerio*) hepatocytes. Fish were fed for 10 weeks on diets containing either 11% fish oil (FO) or 11% vegetable oil (VO) after being initially reared on a commercial flake diet as described in Materials and methods. Differences between mean values for fish fed the VO diet compared to fish fed the FO diet were determined by the Student's *t*-test and are significantly different where indicated (*, $P < 0.05$).

the experimental diets was that the amount of 18:4n-3 was very significantly lower in fish fed the FO diet, with the amount of 20:5n-3 also significantly reduced compared to that in fish fed the VO diet (Figure 2). Summing the products of the various enzymic steps in the desaturation/elongation pathway indicated that the major difference between zebrafish fed the FO and VO diets was significantly reduced Δ6 fatty acid desaturase activity in fish fed the FO diet (Figure 2). The predominant product of desaturation of [1-^{14}C]18:3n-3 in tilapia hepatocytes at the initiation of the dietary trial was 20:5n-3 although the data are not shown due to the very low level of activity. In tilapia at the end of the dietary trial, all the individual products of desaturation were increased in fish fed the VO diet compared to fish fed the FO diet (Figure 3). The greatest increases were in the amounts of 20:5n-3 and 22:6n-3 produced, whereas the increase in 20:4n-3 was not significant (Figure 3). Summing the products of the various enzymic steps in the desaturation/elongation pathway in tilapia indicated that the products of all the steps in the pathway were significantly increased in fish fed the VO diet compared to those in fish fed the FO diet (Figure 3).

Effects of diet on lipid and fatty acid compositions of zebrafish

There was no apparent effect of diet on the lipid content at the end of the trial in zebrafish (whole fish) (Table 3). However, fish fed the FO diet had significantly higher percentages of triacylglycerol and total

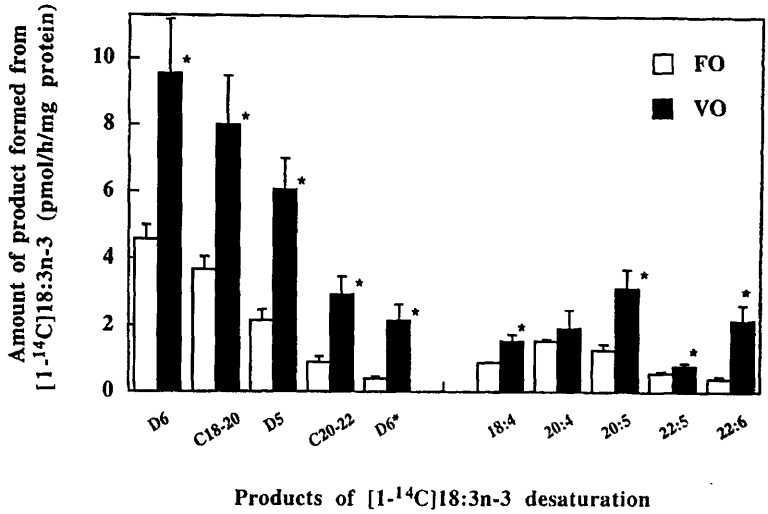

Figure 3. Effect of diet on the apparent activities of the fatty acid desaturation and elongation pathway, and on the individual fatty acid products of the desaturation/elongation pathway in tilapia (*Oreochromis niloticus*) hepatocytes. Fish were fed for 10 weeks on diets containing either 11% fish oil (FO) or 11% vegetable oil (VO) after being initially reared on a commercial trout pellet diet containing 20% fish oil as described in Materials and methods. Differences between mean values for fish fed the VO diet compared to fish fed the FO diet were determined by the Student's *t*-test and are significantly different where indicated (*, $P < 0.05$).

neutral lipid than fish fed the VO diet. Whole bodies of zebrafish fed the FO diet showed higher proportions of total n-3PUFA, due to increased percentages of 20:5n-3 and 22:6n-3, and total PUFA, whereas fish fed the VO diet had higher proportions of 18:3n-3, 20:4n-6 and total n-6PUFA (Table 4). The zebrafish fed the FO diet also showed higher proportions of 20:1 and 22:1 but lower total monoenes due to lower 18:1n-9 than fish fed the VO diet. There were no major significant differences in whole body saturated fatty acids between zebrafish fed the FO and VO diets.

Effects of diet on lipid and fatty acid compositions of tilapia tissues

The lipid content of tilapia tissues was similar in fish fed the FO and VO diets (Table 5). In contrast to zebrafish, tissues of tilapia fed the VO diet showed increased proportions of triacylglycerol compared to fish fed the FO diet although there was some variability in tissue triacylglycerol levels as shown by the high deviations which made the increase non-significant in muscle (Table 5). However, in muscle and brain, the percentages of all the other lipid classes were generally reduced in tilapia fed the VO diet indicating that the primary effect of the VO diet was increased triacylglycerol deposition which resulted in the relative amounts of all other lipid classes decreasing. Liver was slightly different in that there were also quite large decreases in the percentages of free fatty acids and sterol esters in fish fed the VO diet and so, in consequence, the proportions of polar lipids were generally increased (Table 5).

The major differences in fatty acid compositions of tilapia fed the two experimental diets were observed in all tissues. Thus, tissues of fish fed the VO diet were characterised by having higher proportions of monoenes, due primarily to greatly increased percentages of 18:1n-9, n-9 PUFA and 18:3n-3, and greatly reduced proportions of all other n-3 PUFA, especially 20:5n-3 and 22:6n-3, compared to fish fed the FO diet (Table 6). Other dietary differences varied between tissues, with the brain being least affected. In fish fed the VO diet, liver and muscle had much lower proportions of 20:1 and 22:1, but brain showed no differences in these fatty acids. Saturated fatty acids and 18:0 were increased in livers of fish fed the VO diet, whereas in brain, saturated fatty acids and 18:0 were decreased, and muscle showed no differences in saturated fatty acids (Table 6). The percentage of 18:2n-6 was increased in muscle and decreased in liver in fish fed the VO diet, but was unaffected by diet in brain.

Table 5. Lipid content (percentage of wet weight) and lipid class composition (percentage of total lipid by weight) of tissues from tilapia (*Oreochromis nilotica*) fed diets containing fish oil (FO) or vegetable oil (VO)

	Liver		Muscle		Brain	
	FO	VO	FO	VO	FO	VO
Lipid content	16.3 ± 3.3	15.4 ± 2.0	1.3 ± 0.2	1.2 ± 0.3	10.0 ± 2.0	12.4 ± 3.8
Lipid class composition						
PC	6.3 ± 1.3	9.0 ± 0.6*	22.8 ± 2.4	19.0 ± 3.1	11.2 ± 0.4	8.3 ± 1.5*
PE	2.7 ± 0.6	4.4 ± 0.3*	11.2 ± 1.6	10.5 ± 2.0	13.4 ± 1.3	9.7 ± 1.4*
PS	0.9 ± 0.3	1.2 ± 0.1	3.1 ± 0.1	1.9 ± 0.4*	5.6 ± 0.3	3.7 ± 0.7*
PI	1.1 ± 0.3	1.4 ± 0.2	4.2 ± 0.2	3.3 ± 0.6	2.0 ± 0.2	1.2 ± 0.3*
PG/CL	0.8 ± 0.2	1.3 ± 0.1*	1.7 ± 0.6	1.6 ± 0.5	0.5 ± 0.2	0.3 ± 0.1
Sphingomyelin	1.3 ± 0.3	1.5 ± 0.1	3.0 ± 0.3	2.1 ± 0.3*	1.4 ± 0.0	0.9 ± 0.2*
Cerebrosides	n.d.	n.d.	n.d.	n.d.	5.3 ± 0.4	3.4 ± 0.6*
Sulphatides	n.d.	n.d.	n.d.	n.d.	1.7 ± 0.4	0.9 ± 0.3
Total polar	13.1 ± 3.0	18.8 ± 1.1*	45.9 ± 4.9	38.4 ± 6.6	41.1 ± 3.0	28.6 ± 5.0*
Total neutral	86.9 ± 3.0	81.2 ± 1.1*	54.1 ± 4.9	61.6 ± 6.6	58.9 ± 3.0	71.4 ± 5.0*
Cholesterol	8.6 ± 0.6	7.7 ± 0.6	13.4 ± 1.0	11.6 ± 1.6	17.8 ± 0.9	16.5 ± 0.9
Triacylglycerol	54.3 ± 3.2	64.6 ± 1.8*	35.0 ± 5.8	47.3 ± 8.2	37.2 ± 4.2	53.7 ± 6.0*
Free fatty acids	11.6 ± 1.1	1.9 ± 0.8*	2.9 ± 0.6	0.5 ± 0.2*	2.0 ± 0.4	0.4 ± 0.2*
Sterol	12.3 ± 0.9	7.1 ± 0.7*	2.7 ± 1.0	2.1 ± 0.6	1.9 ± 0.2	0.8 ± 0.3*

Results are means ± SD ($n = 3$). Differences between mean values for fish fed the VO diet compared to fish fed the FO diet were determined by the Student's *t*-test and are significantly different where indicated (*, $P < 0.05$. CL, cardiolipin n.d., not detected; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Discussion

The warmwater zebrafish and tilapia had all the enzymic activities necessary to produce DHA, similar to the situation in salmonids from temperate or cold waters, including Atlantic salmon (Bell et al. 1997; Tocher et al. 1997, 2000), rainbow trout (Buzzi et al. 1996), and Arctic char (Olsen and Ringo 1992). Zebrafish are small and low in the food chain, and so are predominantly planktonivorous. Tilapia are much larger fish, but they also naturally feed on zooplankton, phytoplankton, insects and bottom organisms and so are not normally piscivorous (although they can be cannibalistic under certain conditions). Consistent with their non-piscivorous feeding habits, both zebrafish and tilapia displayed hepatocyte fatty acid desaturation and elongation activities adhering to the so-called 'freshwater' pattern. Previously, an *in vivo* study looking at the conversion of intraperitoneally-injected radiolabelled 18:2n-6 and 18:3n-3 had concluded that tilapia were capable of desaturating these fatty acids (Olsen et al. 1990).

Nutritional regulation of hepatocyte fatty acid desaturation was clearly observed in both tropical species, with the total desaturation activity being approximately 2-fold greater in fish fed the VO diet compared to fish fed the FO diet. The vegetable oil blend used here was olive/linseed/high oleic sunflower oils (approx. 4:1:1, by vol.) which gave an 18:3n-3/18:2n-6/n-3HUFA ratio of 9:9:1. In a previous study on salmon parr approaching smoltification, a 1:1 blend of rapeseed/linseed oil that gave a ratio of 18:3n-3/18:2n-6/n-3HUFA of 5:5:1, increased hepatocyte total desaturase activity over that obtained with a diet containing fish oil by just over 2-fold at the time of peak activity (Bell et al. 1997). In a similar trial with salmon parr, diets containing rapeseed oil (18:3n-3/18:2n-6/n-3HUFA of 2:4:1) and linseed oil (18:3n-3/18:2n-6/n-3HUFA of 8:4:1) resulted in total desaturase activities 2.4- and 1.7-fold higher, respectively, relative to that in fish fed a diet containing fish oil (Tocher et al. 2000). In another study where comparisons are possible, the total desaturase activities in hepatocytes from salmon smolts fed diets containing sunflower oil and olive oil containing predominantly 18:2n-6 as the C_{18} PUFA were approximately 2-fold greater than the activity obtained with fish fed diets containing fish oil (Tocher et al. 1997). Although not directly comparable, similar (2- to 3-fold) increases in desaturase activity were observed with trout fed olive

Table 6. Fatty acid composition (percentage of total fatty acids by weight) of tissues from tilapia (*Oreochromis nilotica*) fed diets containing fish oil (FO) or vegetable oil (VO)

	Liver		Muscle		Brain	
	FO	VO	FO	VO	FO	VO
14:0	3.2 ± 0.3	2.5 ± 0.3*	2.0 ± 0.3	1.5 ± 0.3	2.0 ± 0.3	2.3 ± 0.5
15:0	0.4 ± 0.0	0.1 ± 0.0*	0.4 ± 0.0	0.2 ± 0.0*	0.3 ± 0.0	0.2 ± 0.0
16:0	15.0 ± 0.8	20.6 ± 0.3*	18.0 ± 0.6	18.7 ± 1.3	17.6 ± 0.3	16.1 ± 1.0
18:0	3.8 ± 0.4	6.3 ± 0.5*	5.8 ± 0.4	6.4 ± 0.7	10.0 ± 0.9	5.6 ± 1.7*
Total saturated	22.4 ± 1.2	29.5 ± 0.4*	26.3 ± 0.7	26.8 ± 1.8	29.9 ± 0.8	24.2 ± 2.1*
16:1n-9	0.3 ± 0.0	1.1 ± 0.1*	0.3 ± 0.1	0.6 ± 0.1*	0.4 ± 0.0	0.6 ± 0.1
16:1n-7	4.6 ± 0.3	4.4 ± 0.6	2.7 ± 0.5	2.3 ± 0.2	3.5 ± 0.3	3.7 ± 0.6
18:1n-9	18.2 ± 1.5	49.9 ± 1.3*	13.1 ± 1.0	34.7 ± 2.3*	19.6 ± 0.4	38.8 ± 2.6*
18:1n-7	3.3 ± 0.2	2.6 ± 0.1*	3.5 ± 0.1	3.0 ± 0.3	2.4 ± 0.1	2.5 ± 0.2
20:1n-11	2.4 ± 0.1	0.2 ± 0.0*	0.9 ± 0.0	0.3 ± 0.0*	0.7 ± 0.1	0.4 ± 0.1*
20:1n-9	8.4 ± 0.4	2.1 ± 0.1*	5.3 ± 0.5	2.9 ± 0.3*	3.5 ± 0.6	3.5 ± 0.7
20:1n-7	0.5 ± 0.0	0.2 ± 0.0*	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
22:1	8.1 ± 0.6	0.5 ± 0.1*	4.2 ± 0.6	1.3 ± 0.2*	3.6 ± 0.6	2.5 ± 0.7
24:1n-9	0.8 ± 0.0	0.1 ± 0.0*	0.9 ± 0.0	0.4 ± 0.1*	1.1 ± 0.0	0.5 ± 0.1*
Total monoenes	46.6 ± 1.6	61.1 ± 2.1*	31.1 ± 2.4	45.9 ± 1.9*	34.9 ± 1.8	52.9 ± 2.9*
18:2n-9	n.d	0.7 ± 0.1*	n.d	0.5 ± 0.1*	n.d	0.5 ± 0.1*
20:2n-9	n.d	0.3 ± 0.1*	n.d	0.2 ± 0.1*	n.d	0.2 ± 0.0*
20:3n-9	n.d	n.d	n.d	n.d	n.d	n.d
Total n-9 PUFA	n.d	1.0 ± 0.2*	n.d	0.7 ± 0.2*	n.d	0.7 ± 0.1*
18:2n-6	3.6 ± 0.3	2.4 ± 0.3*	3.2 ± 0.2	4.2 ± 0.2*	2.6 ± 0.4	3.7 ± 0.7
18:3n-6	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
20:2n-6	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.0	0.9 ± 0.1*	0.3 ± 0.0	0.4 ± 0.0
20:3n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.6 ± 0.0*	0.2 ± 0.0	0.2 ± 0.0
20:4n-6	0.7 ± 0.2	0.5 ± 0.1	1.8 ± 0.2	1.6 ± 0.1	1.2 ± 0.2	0.8 ± 0.2
22:4n-6	0.2 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.4 ± 0.0*	0.1 ± 0.1	0.2 ± 0.0
22:5n-6	0.2 ± 0.0	0.2 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
Total n-6 PUFA	5.7 ± 0.5	4.1 ± 0.7*	6.6 ± 0.2	8.6 ± 0.2*	4.7 ± 0.4	5.7 ± 0.7
18:3n-3	0.9 ± 0.1	1.2 ± 0.3	0.6 ± 0.1	1.8 ± 0.2*	0.6 ± 0.1	1.9 ± 0.3*
18:4n-3	0.6 ± 0.0	0.1 ± 0.0*	0.6 ± 0.1	0.3 ± 0.0*	0.7 ± 0.1	0.5 ± 0.2
20:3n-3	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
20:4n-3	0.9 ± 0.1	0.1 ± 0.0*	0.6 ± 0.0	0.2 ± 0.0*	0.5 ± 0.1	0.3 ± 0.1
20:5n-3	1.8 ± 0.2	0.1 ± 0.0*	4.2 ± 0.4	1.1 ± 0.1*	2.2 ± 0.2	1.1 ± 0.4*
22:4n-3	0.2 ± 0.0	0.0 ± 0.0*	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	6.0 ± 1.0	0.4 ± 0.1*	5.7 ± 0.1	2.2 ± 0.2*	3.2 ± 0.3	1.9 ± 0.7*
22:6n-3	14.6 ± 1.7	2.2 ± 0.6*	24.0 ± 1.6	11.0 ± 1.3*	23.2 ± 2.3	8.7 ± 2.1*
Total n-3 PUFA	25.3 ± 2.3	4.3 ± 1.1*	36.0 ± 1.9	16.7 ± 1.2*	30.5 ± 1.7	14.7 ± 2.7*
Total PUFA	31.0 ± 2.3	9.4 ± 1.9*	42.7 ± 1.8	26.1 ± 1.1*	35.2 ± 1.6	21.1 ± 3.2*
Total DMA	n.d	n.d	n.d	1.3 ± 1.1	n.d	1.7 ± 1.6

Results are means ± SD ($n = 3$). Differences between mean values for fish fed the VO diet compared to fish fed the FO diet were determined by the Student's t-test and are significantly different where indicated (*, $P < 0.05$). DMA, dimethylacetals; n.d., not detected; PUFA, polyunsaturated fatty acids.

oil (Buzzi et al. 1996) and Arctic char fed purified C_{18} PUFA (Olsen and Ringo 1992). Previously, Olsen et al. (1990) had shown that the conversion of inraperitoneally injected 18:3n-3 was increased in tilapia fed a diet containing only linoleic acid. Therefore, the effects of replacing fish oil with vegetable oil in the diets of the tropical species, tilapia and zebrafish, were similar to those observed in salmonids. The effects were also similar in all species despite differences in dietary oil contents and the type of plant oil, with, in particular, not a great deal of difference between oils rich in 18:3n-3 or 18:2n-6. The primary factor in the increased desaturase active is almost certainly the reduction of product inhibition by the considerable lowering of n-3HUFA in diets containing vegetable oil, rather than provision of more substrate PUFA for the first and, reportedly, rate-limiting enzyme in the pathway, $\Delta 6$ desaturase, although this may also contribute to the overall result.

The zebrafish prior to the trial showed high fatty acid desaturation activity due to the fact that the commercial flake diet was essentially a VO-type diet. The fatty acid composition of the diet showed that it was formulated using an 18:2n-6-rich oil (probably corn oil) and fish products, which were responsible for the EPA and DHA content. However, the production of EPA and DHA from [1-^{14}C]18:3n-3 in hepatocytes was higher in fish when fed the commercial flake diet than in fish fed the VO diet, despite the fact that the percentages of EPA and DHA were higher in the commercial flake diet than in the VO diet. Even considering the slightly higher lipid content of the VO diet the absolute amounts of EPA and DHA delivered by the flake diet would be higher than the VO diet. The flake diet was characterised by very much higher 18:2n-6, and lower 18:3n-3, than the VO diet (18:2n-6/18:3n-3 ratios of 7 and 1, respectively) and it is possible that this difference in the relative amounts of the C_{18} PUFA, the direct substrates for $\Delta 6$ desaturase, were responsible for the effects observed, although the mechanism is not clear. Perhaps consistent with this was the result with salmon described above where a diet containing rapeseed oil gave a larger increase in desaturase activity than a diet containing linseed oil (18:2n-6/18:3n-3 ratios of 2 and 0.5, respectively) (Tocher et al. 2000).

In contrast, the desaturase activity in tilapia at the initiation of the experiment was very low due to the fact that the fish had been reared on trout/salmonid pellets containing a northern hemisphere fish oil (as evidenced by the high 20:1 and 22:1 contents) (Ackman 1980). The very low activity was the combined result of the high proportions of EPA and DHA, and the fact that the commercial pelleted trout diet contained 15% lipid. Therefore, the absolute amount of EPA and DHA in the diet was higher than in the experimental FO diet which was 11% lipid.

Whole zebrafish, and tilapia tissues, from fish fed the VO diet were characterised by decreased proportions of EPA, DHA, total n-3 PUFA compared to fish fed the FO diet. Fish fed the VO diet also showed generally increased proportions of 18:3n-3, 18:2n-6 and total n-6PUFA, and also increased total monoenes due to increased percentages of 18:1n-9, whereas fish fed the FO diet were characterized by high proportions of 20:1 and 22:1, monoenes characteristic of northern hemisphere fish oils. Therefore, these differences reflect the dietary fatty acid input, despite the fact that the fatty acid desaturase activities measured in hepatocytes were significantly higher in both zebrafish and tilapia fed the VO diet compared to the FO diet. In addition, the intermediates of 18:3n-3 desaturation and elongation, which are not normally in high concentration, such as 18:4n-3 and 20:4n-3, were not increased in whole zebrafish or tissues of tilapia fed the VO diet. This may indicate that desaturation of dietary 18:3n-3 was proceeding to EPA and DHA, but that the increased activity was insufficient to compensate for the lack of EPA and DHA in the VO diet and so levels of tissue EPA and DHA were significantly reduced in VO-fed fish. This has also been observed in salmon and trout, where the increased activity of the hepatocyte fatty acid desaturation pathway did not fully compensate for the lack of EPA and DHA in the vegetable oil diets, and so levels of n-3HUFA are reduced, and those of C_{18} PUFA increased, in fish fed the diets containing vegetable oils (Buzzi et al. 1996; Bell et al. 1997; Tocher et al. 1997, 2000).

In conclusion, this study has shown that hepatic fatty acid desaturation/elongation was nutritionally regulated in two warm water fish, zebrafish and tilapia, and that activities were higher in fish fed dietary vegetable oil compared to those in fish fed fish oil. However, the fatty acid compositional data indicated that despite increased activity, desaturation of 18:3n-3 was insufficient to maintain tissue proportions of EPA and DHA in fish fed vegetable oil at the same level as in fish fed fish oil. These results indicate that manipulation of tilapia diets in commercial culture in response to the declining global fish oil market could have important consequences for fish fatty acid composition and the health of consumers. Both zebrafish and tilapia

are the subject of active genome mapping projects, and although the zebrafish genome is probably the most fully characterised of fish species, both could be useful model species in studies of lipid and fatty acid metabolism at molecular biological and genetic levels.

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