Fatty Acid Metabolism in Isolated Enterocytes from Salmonid Fish

Thesis submitted for the degree of
Doctor of Philosophy

by

Jorge Fonseca Madrigal
B.A., M.Sc.

Institute of Aquaculture
University of Stirling
Stirling, Scotland
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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DECLARATION

I hereby declare that this thesis has been composed entirely by myself and has not been submitted in any previous application for a degree.

The work of which it is a record has been carried out by myself. The nature and extent of any work carried out by, or in conjunction with, others has been specifically acknowledged by reference.

Jorge Fonseca-Madrigal

ABSTRACT

Considering the results found, through *in vivo* experimentation, that intestine of trout and specifically pyloric caeca was an important site of metabolic activity (Bell et al., 2003c), it was contemplated to begin with a research project to demonstrate and establish the rate in which n-3 HUFA are synthesised by intestinal cells using *in vitro* assays. The aim of the research work was to determine the importance of intestine and caecal enterocytes in fatty acid metabolism in salmonids, focusing on their potential role in HUFA synthesis and the effects of replacement of fish oil (FO) with vegetable oils (VO) in the diets. The results of the investigation gave answer to the most important questions about intestinal metabolism of fatty acids and brought suggestions for future research work. A simple method for the isolation of viable enterocytes from salmonids suitable for use in both laboratory and fish farm conditions was established, and a method to analyse desaturation/elongation and β-oxidation activities in a single combined assay was developed. Pyloric caeca enterocytes were found to be more active than other intestinal tissues tested in terms of HUFA synthesis capacity and they were used for the evaluation of the following dietary trials. The performance of the HUFA synthesis assay in the enterocyte preparations was determined under a variety of parameters to standardize an assay protocol for use in all comparative studies. Regarding the effect of replacing FO with VO in diets for salmonids on fatty acid metabolism in enterocytes it was found that VO (a blend) can be good substitute for FO and can be use by the salmon industry without compromising the standard of quality of the product. Caecal enterocytes were not more active than hepatocytes and, irrespective of the diet, the primary fate of the substrate was β-oxidation rather than HUFA synthesis. HUFA synthesis in enterocytes and hepatocytes, increased in fish fed VO compared to fish fed FO and fish fed VO increased fatty acid oxidation activity in enterocytes whereas there was no dietary effect in hepatocytes.

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CHAPTER ONE

1. GENERAL INTRODUCTION

1.1. Fatty acid metabolism

1.1.1. Fatty acid nomenclature

A fatty acid is an organic acid containing a hydrocarbon chain with a terminal carboxylic acid group and a methyl group at the opposite end. If the hydrocarbon chain contains only single carbon to carbon bonds, it is termed saturated, as all the carbons in the chain have the maximum number of atoms of hydrogen attached. However, the hydrocarbon chain can contain carbon to carbon double bonds, and these carbons are no longer fully saturated with hydrogen atoms and the chains are referred to as unsaturated. For this reason, the enzymes that insert the double bonds into the hydrocarbon chains are termed desaturases. Therefore, fatty acids differ from each other primarily in hydrocarbon chain length and in the number and position of their unsaturated bonds. Based on the aforementioned characteristics, fatty acids can be grouped in families according to the number of double bonds in the molecule; as described above fatty acids with no double bonds are known as saturated fatty acids; those with one double bond are called monounsaturated; and those with two or more double bonds are called polyunsaturated fatty acids (PUFA). Another term in common usage in the aquaculture field is HUFA, an abbreviation of highly unsaturated fatty acids. This is often used without proper definition but it has been defined as polyunsaturated fatty acids having carbon chain lengths of $\geq C_{20}$ and with ≥ 3 double bonds (Sargent *et al.*, 2002).

There are two main nomenclatures systems used to identify individual fatty acids, both based of the chain length, degree of unsaturation (number of double bonds)

and the position of the double bonds in the carbon chain. Chain length and number of unsaturated bonds are shown as numbers separated by a colon in both systems, with the number of carbons in the chain presented before the colon and number of double bonds presented after the colon. For example, 20:4 represents a fatty acid with a chain length of 20 carbons with four double bonds. The two nomenclatures differ on how they depict the positions of the double bonds. With the Δ or delta nomenclature, the carbons in the chain are counted from the carboxyl group and so the numbers after the Δ indicate the positions of each double bond in the chain counted from the carboxyl carbon which is numbered one. Thus, 20:4 $^{\Delta 5, 8, 11, 14}$ (or arachidonic acid) is 20 carbons long with four double bonds situated at carbons 5, 8, 11 and 14 from the carboxyl end. This nomenclature has been popular in chemistry as it details the positions of all bonds and has been the system used traditionally to name the desaturase enzymes (Fig. 1.1).

Over the years, an alternative nomenclature has proved popular in biology and nutrition. The n- nomenclature counts the carbons in the chain from the methyl end and hence has also been termed the omega or "n" system, as the last carbon is the ω carbon. In this nomenclature, the number after the n (or ω) indicates the position of the first double bond in the chain counted from the methyl end of the carbon chain (methyl carbon is one). Although only the position of the first double bond in the chain is defined, in PUFA in biological systems (particularly vertebrates), the double bonds are generally interrupted by a single methylene (CH_2) group to generate "methylene – interrupted *cis* diene" structures. Therefore, by specifying the position of the first double bond the entire structure can be defined. Thus, the example used above (arachidonic acid) is depicted as 20:4n-6 (or 20:4ω6) indicating that the first double bond is situated at carbon six from the methyl end. More examples of the most

important fatty acids with its common name and the n- and Δ-designation are given in Table 11.

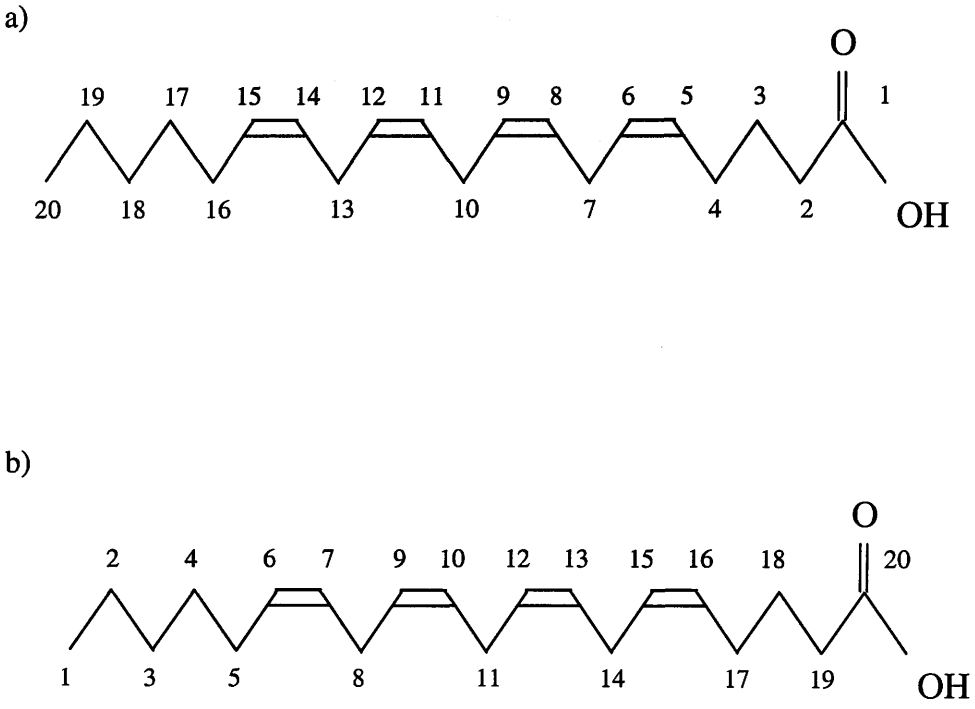


Figure 1.1. Arachidonic acid (20:4 $^{\Delta 5, 8, 11, 14}$) as an example of the Δ nomenclature (a); this designation counts the carbons in the chain starting from the carboxyl end of the acyl chain. (b) Arachidonic acid (20:4n-6) as an example of the n-nomenclature which counts the carbons in the chain starting from the methyl end of the acyl chain.

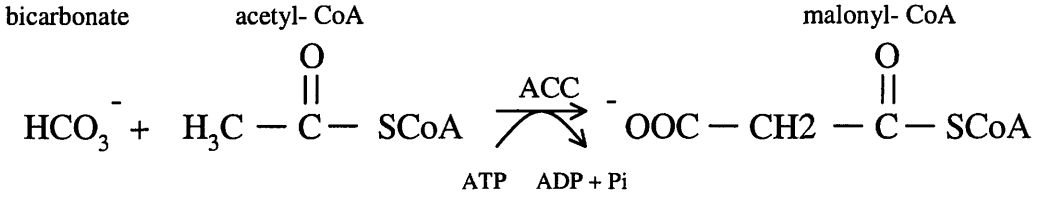
Table 1.1. Structure and nomenclature of the major long chain fatty acids.

n-designation	Δ-designation	Systematic name	Common name
saturated			
12:0	12:0	dodecanoic	lauric acid
14:0	14:0	tetradecanoic	myristic acid
16:0	16:0	hexadecanoic	palmitic acid
18:0	18:0	octadecanoic	stearic acid
20:0	20:0	eicosanoic	arachidic acid
unsaturated			
16:1n-7	$16:1^{\Delta 9}$	9-hexadecenoic	palmitoleic acid
18:1n-9	$18:1^{\Delta 9}$	9-octadecenoic	oleic acid
18:2n-6	$18:2^{\Delta 9,12}$	9,12- octodecadienoic	linoleic acid
18:3n-3	$18:3^{\Delta 9,12,15}$	9,12,15-octadecatrienoic	α-linolenic acid
18:3n-6	$18:3^{\Delta 6,9,12}$	6,9,12-octadecatrienoic	γ-linolenic acid
20:4n-6	$20:4^{\Delta 5,8,11,14}$	5,8,11,14-eicosatetraenoic	arachidonic acid (ARA)
20:5n-3	$20:5^{\Delta 5,8,11,14,17}$	5,8,11,14,17-eicosapentaenoic	EPA
22:6n-3	$22:6^{\Delta 4,7,10,13,16,19}$	4,7,10,13,16,19-docosahexaenoic	DHA

1.1.2. Fatty acid biosynthesis

Fatty acid biosynthesis in fish is similar to that in mammals with acetyl-CoA, the ultimate carbon source, being formed in mitochondria from the oxidative decarboxylation of pyruvate when the source is carbohydrates, or alternatively the oxidative degradation of some amino acids if the source is proteins. In animals, the catalytic components required for the entire fatty acid biosynthetic pathway are integrated into two multifunctional polypeptides, acetyl-CoA carboxylase (ACC) and

fatty acid synthetase (FAS) (Wakil *et al.*, 1983). The pathway for fatty acid synthesis occurs in the cytoplasm and involves the oxidation of NADPH. Fatty acid synthesis utilizes an activated two carbon intermediate, acetyl-CoA, although it exists temporarily bound to the enzyme complex as malonyl-CoA. The synthesis of malonyl-CoA is the first committed step of fatty acid synthesis and the enzyme that catalyses this reaction, ACC, is the major site of regulation of fatty acid synthesis. Malonyl-CoA formation is described as in the following equation:



The rate of fatty acid synthesis is controlled by the equilibrium between monomeric ACC and polymeric ACC and the activity of ACC requires polymerisation. This conformational change is enhanced by citrate and inhibited by long-chain fatty acids. ACC is also controlled through hormone-mediated phosphorylation (King, 2005). Once malonyl-CoA has been generated from acetyl-CoA by the action of the rather complex acetyl-CoA carboxylase reaction, the ensuing reactions of fatty acid synthesis occur in a sequence of six successive steps catalysed by the six enzymes of the fatty-acid synthetase system. The seventh protein of this system, which has no enzymatic activity itself, is acyl carrier protein (ACP), to which the growing fatty acid chain is covalently attached and serves as an anchor to which the acyl intermediates are

esterified (Lehninger, 1975). The acetyl-CoA and malonyl-CoA are transferred to ACP by the action of acetyl-CoA transacylase and malonyl-CoA transacylase, respectively. The attachment of these carbon atoms to ACP allows them to enter the fatty acid synthesis cycle (King, 2005).

The synthesis of fatty acids from acetyl-CoA and malonyl-CoA is carried out by FAS. The active enzyme is a dimer of identical subunits. All of the reactions of fatty acid synthesis are carried out by the multiple enzymatic activities of FAS in a cyclic process. Long-chain fatty acid synthesis involves 4 enzymatic activities. These are, β-keto-ACP synthase, β-keto-ACP reductase, 3-OH acyl-ACP dehydratase and enoyl-CoA reductase. The two reduction reactions require NADPH oxidation to $NADP^+$. The saturated acyl chain product of one cycle becomes the primer substrate for the following cycle, so that two saturated carbon atoms are added to the primer with each turn of the cycle (Rangan and Smith, 2002). The overall reaction catalysed by the animal FAS can be summarized by the equation:

$$\text{Acetyl-CoA} + 7\text{Malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \longrightarrow \text{Palmitic acid} + 7\text{CO}_2 + 8\text{CoA} + 14\text{NADP}^+ + 6\text{H}_2\text{O}$$

The eukaryotic FASs synthesize predominantly the 16-carbon saturated product with smaller amounts of 14- and 18-carbon products. The main products of FAS, 16:0 (palmitic acid) and 18:0 (stearic acid), can be biosynthesised *de novo* by all known organisms, including fish (Sargent *et al.*, 1989). After these fatty acids are released from the enzyme they can then undergo separate elongation and/or unsaturation to yield other fatty acid molecules.

1.1.3. Fatty acid elongation

De novo synthesis of fatty acids produces mainly 16-carbon palmitic acid, with minor amounts of 18-carbon stearic acid. Most eukaryotic cells have the capacity for 2-carbon chain elongation of both endogenously synthesized and dietary fatty acids. Liver, brain and other tissues are capable of elongation of fatty acids mainly in the endoplasmic reticulum and in less magnitude in mitochondria. Peroxisomes also contain an acetyl-CoA dependent elongation system (Cook and McMaster, 2002). Specific roles for elongation in peroxisomes have not been defined but this organelle may produce the very long chain saturated and polyenoic fatty acids of 24-36 carbons.

When long chain fatty acids are not supplied adequately in the diet, chain elongation in the endoplasmic reticulum is the most significant source of acyl chains greater than 16 carbons for membrane phospholipids during growth and maturation. 18- to 24-carbon polyunsaturates are necessary for neural growth and myelination, regardless of dietary fluctuations. Nevertheless, many major fatty acyl chains are longer than 16 or 18 carbons, constituting more than half of the total acyl chains of many tissues.

Fatty acyl chain elongation associated with the endoplasmic reticulum is highly active. Fatty acids must be activated to CoA derivatives; the CoA moiety provides energy for attachment of the donor group to a growing fatty acyl chain. Several fatty acyl-CoA synthetases with specificities for chain length and degree of unsaturation have been purified and their cDNAs cloned (Oh *et al.*, 1997). Describing their precise subcellular locations is a necessary step in determining links to the synthesis of particular lipids (Cook and McMaster 2002).

Chain elongation of a long chain fatty acid, previously activated by CoA, is essentially performed by a multi-enzyme complex basically summarized in a four-component reaction. The initial step is a condensation of fatty acyl-CoA and malonyl-CoA to form β-ketoacyl-CoA, and is the step which determines fatty acyl specificity and results in the addition of the 2-carbon moiety. The enzyme that controls this reaction in the elongation process is generally known as the "elongase". It is rate limiting, determines fatty acyl specificity and results in addition of the 2-carbon moiety. The second reaction is the reduction of β-ketoacyl-CoA, catalysed by β-ketoacyl-CoA reductase that utilizes NADPH (in preference to NADH) to form β-hydroxy acyl-CoA. The third reaction in chain elongation involves dehydration of β-hydroxy acyl-CoA (by β-hydroxy acyl-CoA dehydrase) to form enoyl-CoA. The final reaction is a second reduction of enoyl-CoA (catalysed by 2-*trans*-enoyl-CoA) that requires NADPH.

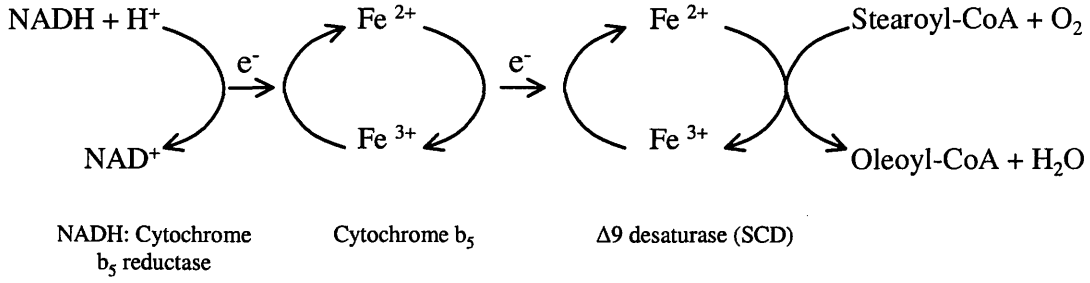
The variety of fatty acyl chains needed to meet the requirements of lipid storage, membrane synthesis and maintenance, and lipid regulation of cellular processes must be supplied by the diet or *de novo* fatty acid synthesis and chain elongation. Therefore, unsaturated fatty acids must be synthesized in cells, and supplemented by dietary essential fatty acids.

1.1.4. Fatty acid desaturation

Most cellular fatty acids are not saturated, but contain double bonds that are introduced by a family of enzymes called desaturases that belong to the class of nonheme-iron enzymes. Mammals have terminal desaturases of broad chain-length specificities, for example, the Δ9-, Δ6- and Δ5-fatty acetyl-CoA desaturases. These enzymes catalyse the desaturation of fatty acids in all animals, fish included. The

reaction takes place in the endoplasmic reticulum of cells of particular tissues via an aerobic process utilising CoA-linked substrates and requiring NADPH and O_2, catalysed by multicomponent systems comprising NADPH-cytochrome b_5 reductase, cytochrome b_5 and terminal desaturase enzymes (Brenner, 1974).

The first double bond is introduced into the saturated acyl chain at the Δ9 position by the enzyme stearoyl-CoA desaturase (SCD) or Δ9 desaturase. The equation of the desaturation process of Δ9 desaturase, including the electron transport is:



All organisms including fish are capable of desaturating 16:0 and 18:0 to yield, respectively, 16:1Δ9 = 16:1n-7 (palmitoleic acid) and 18:1Δ9 = 18:1n-9 (oleic acid). The Δ9 desaturase is the predominant, if not exclusive, desaturation enzyme for saturated acids and is rate-limiting in the formation of 18:1n-9. In the process, one molecule of molecular oxygen (O_2) is used as an acceptor for two pairs of electrons, one pair derived from palmitoyl-CoA or stearyl-CoA substrate and the other from NADPH, which is a required coreductant in the reaction. Thus, the main products of SCD are 18:1n-9 and/or 16:1n-7 so that substrates for further desaturation contain either a Δ9 double bond or one derived from the Δ9 position by chain elongation.

This first desaturation reaction is of particular physiological importance in that the monounsaturated products formed (16:1n-7 and 18:1n-9) have markedly lower melting points (phase transition temperatures) than their saturated precursors (16:0 and 18:0) (Tocher, 2003). Hence Δ9 fatty acid desaturase provides a means of regulating the viscosity of cell membranes by altering the phase transition temperatures of the fatty acids in their constituent phosphoglycerides.

Animal systems cannot introduce double bonds between the Δ9 position and the methyl end of the carbon chain. Thus animal desaturases are all members of the family of desaturases termed "front-end" desaturases that can introduce double bonds between the Δ9 position and the carboxyl end of the acyl chain, never in the methyl side. Consequently, double bonds are at the Δ9, Δ6, Δ5 and Δ4 positions of the chain as a result of desaturation in animals. Plants, on the other hand, introduce second and third double bonds between the existing double bond at the Δ9, and the terminal methyl group (Δ12 and Δ15 positions). Some invertebrates and insects can also desaturate at Δ5, Δ6, Δ9, Δ12 and Δ15 positions (Fig. 1.2). The insertion of the last, Δ4 position, double bond in 22:6n-3 does not occur through direct Δ4 desaturation of its immediate precursor 22:5n-3. Rather, 22:5n-3, is chain elongated to 24:5n-3 which is then converted by Δ6 desaturation to 24:6n-3 which is then converted, by a chain shortening reaction in the peroxisomes, to 22:6n-3. Whereas 22:6n-3 is the main end product of further desaturation and elongation of 18:3n-3, 20:4n-6 is generally the main end product of desaturation and elongation of 18:2n-6. However, 20:4n-6 can be further desaturated and elongated to 22:5n-6 to some extent, with insertion of the last Δ4 double bond being achieved in the same manner as for 22:6n-3 (Tocher, 2003) (Fig. 1.2).

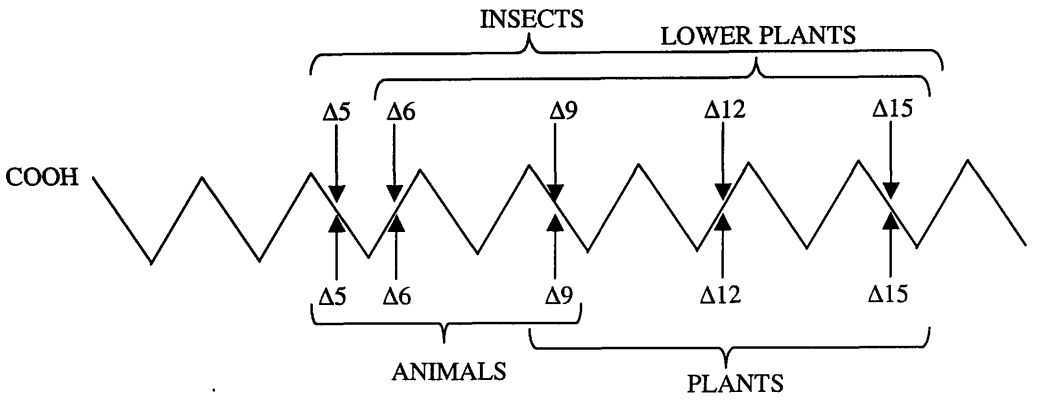


Figure 1.2. Positions of fatty acyl chain desaturation by enzymes of animals, plants, insects and lower plants. Note: chain elongations occur between successive Δ6 and Δ5 desaturations.

Interestingly, humans and other mammals only have a limited spectrum of desaturases that are required for the formation of these particular double bonds in unsaturated fatty acids. Because they lack the Δ12 and Δ15 desaturases, no vertebrate species can produce PUFA *de novo* and so all have an absolute dietary requirement for certain PUFA. If a dietary deficiency occurs, the animal stops growing and reproducing, it develops various pathologies and it eventually dies (Castell *et al.*, 1972; Watanabe, 1982; Sargent *et al.*, 2002). Thus, animals are completely dependent on plants (or insects) for providing double bonds in the Δ12 and Δ15 positions of the two major precursors of the n-6 and n-3 fatty acids. When the Δ12- and Δ15-bonded acyl chains reach animal tissues they are converted into fatty acids containing 3-6 double bonds. Such vegetal produced acyl chains, specifically linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) are termed essential fatty acids (EFA) and the biologically

active forms of EFA are generally their C_{20} and C_{22} metabolites, 20:4n-6 + 22:5n-6 and 20:5n-3 + 22:6n-3 respectively.

As explain previously, cellular processes of animals cannot be satisfied by an appropriate *de novo* production or provision of fatty acids and chain elongation alone. To achieve this, the suitable essential fatty acids must be provided in the diet, and supplemented with further unsaturation of fatty acids in cells. The degree to which an animal can perform these conversions is dependent upon the relative activities of fatty acid elongases and desaturases in their tissues, and these activities in turn are dependent on the extent to which the species can or cannot readily obtain the end product 20:4n-6, 20:5n-3 and 22:6n-3 fatty acids preformed from their natural diets (Tocher, 2003). For instance, an extreme carnivore such as the cat, which can obtain abundant preformed 20:4n-6, 20:5n-3 and 22:6n-3 from its natural prey, appears to lack, or express very low Δ6 and, possibly, Δ5 desaturases (Rivers *et al.*, 1975). The tissues of both freshwater and marine fish are generally very rich in C_{20} and C_{22} fatty acids, especially 20:5n-3 and 22:6n-3, and so the origins of these fatty acids are of particular interest. The pathway of biosynthesis of 20:5n-3 and 22:6n-3 from 18:3n-3 is present in salmonids, and probably in other freshwater fish (Tocher, 2003). The pathway is complex but is now reasonably well understood and appears to be the same, at least qualitatively, in rainbow trout (Buzzi *et al.*, 1996, 1997) as in rats (Voss *et al.*, 1991) (Fig. 1.3).

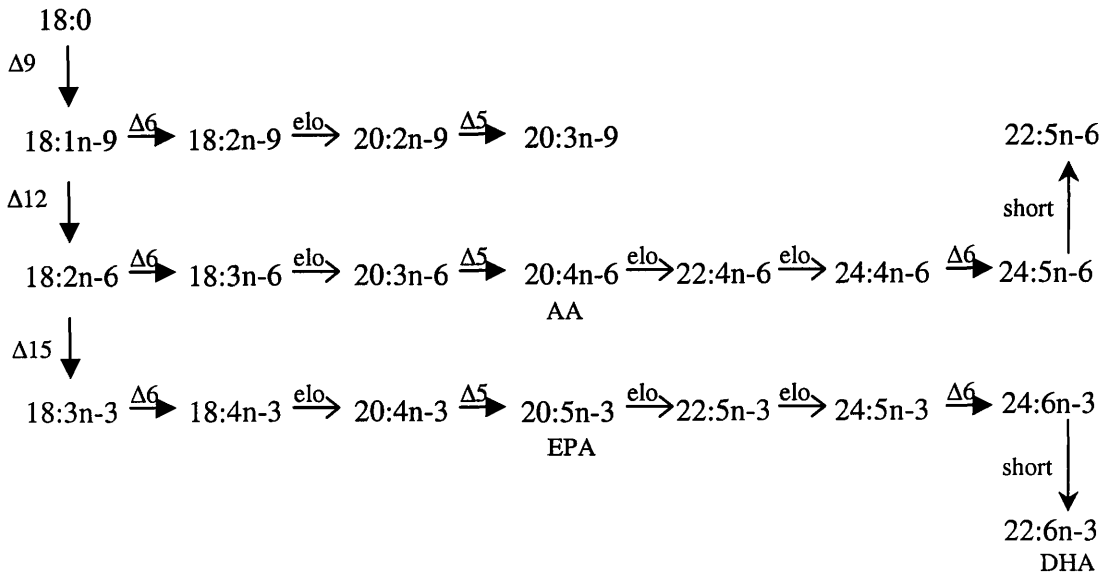


Figure 1.3. Major pathways of fatty acyl desaturation and chain elongation in animal tissues. Δ5, Δ6, Δ9, Δ12, Δ15 = desaturases; elo = elongase; short = chain shortening.

Relationships among fatty acids in the metabolic pathways can be evaluated by considering groups or families of fatty acids based on the parent unsaturated acid in the sequence. The predominant fatty acid families are: n-6 acids derived from 18:2n-6, n-3 acids derived from 18:3n-3, n-9 acids derived from 18:1n-9, and n-7 acids derived from 16:1n-7.

18:2n-6 has a crucial role in the metabolism of fatty acids in general because it is precursor of the most important fatty acid of this family, arachidonic acid, 20:4n-6. 18:2n-6 is not prominent in marine microalgae but it can be abundant in freshwater microalgae (Ahlgren *et al.*, 1992). 18:2n-6 is abundant in the seed oils of plants and also freshwater insects can have 18:2n-6 together with 18:3n-3 frequently as the major PUFA (Stanley-Samuelson *et al.*, 1988; Ogg *et al.*, 1993). It has been shown that insect

larvae (bloodworms) used commercially as fish feed have 18:2n-6 as their major PUFA (Henderson *et al.*, 1996). Bell *et al.* (1994) showed that other freshwater invertebrates contained 18:2n-6 together with 18:3n-3 and 20:5n-3 as their principal PUFA. As said previously, arachidonic acid, 20:4n-6, is the most biologically important member of the n-6 family. It is an abundant polyenoic acyl chain found in most animal tissues. 20:4n-6 can be derived from 18:2n-6 by the alternating sequence of Δ6 desaturation, chain elongation of the 18:3n-6 intermediate, and Δ5 desaturation of 20:3n-6 (Fig 1.3). Δ6 desaturation is considered rate-limiting in most situations. 20:4n-6 is a component of phospholipids contributing to the structural integrity of membranes and is the primary precursor of important biological signalling molecules (e.g. prostaglandins and leukotrienes) with a variety of biological activities (Cook and McMaster, 2002). Frequently, 20:4n-6 is referred to as an essential fatty acid as there is an absolute requirement for it; however, 18:2n-6 can be converted adequately to 20:4n-6 by most mammalian cell types. In some tissues, 22:4n-6 and 22:5n-6 are quantitatively significant. Exogenous 22:4n-6 also is a substrate for "retroconversion". This process of partial degradation involves loss of either a 2-carbon fragment or a double bond and 2 or 4 carbons by β-oxidation. In general, retroconversion only significantly occurs with fatty acids of 20 carbons or greater.

The most abundant n-3 acyl chains are 20:5n-3 and 22:6n-3. These fatty acids are esterified to phospholipids in high concentrations in cerebral cortex, retina, testes, muscle and liver. Many animal tissues convert dietary 18:3n-3 to 20:5n-3 and 22:6n-3 by desaturation and elongation combined with peroxisomal chain shortening (Fig. 1.3). Freshwater microalgae unlike marine microalgae generally have 18:3n-3 rather than 20:5n-3 and 22:6n-3 as their principal PUFA (Ahlgren *et al.*, 1992). The major PUFA in the green leaves of terrestrial and freshwater plants is 18:3n-3 and in freshwater

insects 18:3n-3 along with 18:2n-6 are the most frequent PUFA (Stanley-Samuelson *et al.*, 1988; Ogg *et al.*, 1993). Also, it has been shown that other organisms with relevant content of members of this family of fatty acids are the freshwater invertebrates which contain mainly 18:3n-3, and 20:5n-3 together with 18:2n-6 as their principal PUFA (Bell *et al.*, 1994). It has been investigated extensively the fact that consumption of fish oil products, abundant in 20:5n-3 and 22:6n-3, has beneficial effects upon health of animals and humans (Fernandes and Venkatraman, 1993; Peet *et al.*, 1996; Stevens *et al.*, 1996). The production of 20:5n-3 and 22:6n-3 from 18:3n-3 is carried out by the enzymes in phytoplankton that are consumed by fish, or in fish themselves. Competition among fatty acids of the n-3 and n-6 families for desaturation and chain elongation enzymes is extensively documented from a variety of *in vivo* and *in vitro* experiments. 18:3n-3 is a better substrate for Δ6 desaturase than is 18:2n-6; accordingly, abundance of 18:3n-3 can affectively decrease formation of 20:4n-6 (Cook and McMaster, 2002).

In the n-9 family the prominent acyl chain is 18:1n-9. Generally, competition from 18:2n-6 and 18:3n-3 for Δ6 desaturase prevents formation and accumulation of more unsaturated n-9 acids. However, in animals on a diet deficient in EFA, competition by 18:2n-6 or 18:3n-3 is removed and 18:1n-9 is utilized as a substrate for the rate limiting Δ6 desaturase. Further chain elongation of 18:2n-9 to 20:2n-9 and Δ5 desaturation results in accumulation of 20:3n-9 or Mead acid. While 20:3n-9 may partially substitute for some physical functions within membranes, it is not a precursor of prostaglandins and cannot alleviate the signs of EFA deficiency. Since deficiency of essential fatty acids markedly reduces 20:4n-6 while increasing 20:3n-9, a ratio of triene to tetraene (20:4n-6/20:3n-9) of less than 0.2 in tissues and serum usually indicates EFA deficiency. However, use of this ratio has limitations as inhibition of Δ6 desaturase will

reduce formation of both 20:3n-9 and 20:4n-6 and can result in a deficiency state without significantly altering the ratio. The total amount of n-6 or n-3 acids may be a better reflection of essential fatty acid deficiency (Cook and McMaster, 2002).

The primary n-7 acid in membranes and circulating lipids is 16:1n-7. Likewise, all organisms including fish are capable of desaturating 16:0 by a microsomal Δ-9 fatty acid desaturase to yield 16:1n-7. As many analyses do not distinguish specific 18:1 isomers, the contribution of 18:1n-7 to the 18:1 fraction is seldom appreciated even though in developing brain 18:1n-7 comprises 25% of total 18-carbon monoenes. Potentially, C20n-7 PUFA could be formed from 16:1n-7; however, high levels of PUFA derived from 16:1n-7 are not detected even on a fat free diet, although increased levels of 16:1n-7 frequently accompany deficiency of EFA (Cook and McMaster, 2002).

1.1.5. Fatty acid esterification

All fatty acids, whether endogenously synthesized or those derived from the diet, can either be esterified into cellular lipids or β-oxidised and are potential substrates for esterification enzymes. When provided in excess, they are incorporated into newly synthesized triacylglycerol (TAG) and stored as lipid droplets in cells. Very little of the fatty acid in cells is used to esterify cholesterol and form cholesterol ester, the storage form of cholesterol in the cells (Buzzi, 1996). In general, the fatty acids that enter the cells are preferentially esterified into phospholipids (PL) or TAG. Cellular TAG is the most important storage form of fatty acids because it is the most energy rich molecule per gram.

Recent studies with salmon hepatocytes have shown that pathways of lipid metabolism enclose specificity towards fatty acids at almost every level (Stubhaug *et al.*, 2005). The previous statement is based on studies with salmon hepatocytes that incorporated ~85% of both, 18:2n-6 and 18:3n-3 into TAG, with the remainder being incorporated into PL (Ruyter and Thomassen, 1999; Stubhaug *et al.*, 2005). The same trend was observed for other fatty acids, but for 16:0 and 22:6n-3 a more even distribution between TAG and PL was observed (Stubhaug *et al.*, 2005). It has also been reported that there is a higher degree of esterification of 18:3n-3 and 20:5n-3 than 18:2n-6 (Ruyter *et al.*, 2003).

The immediate fatty acid substrate for esterification into phospholipids, triacylglycerols and cholesterol esters is fatty acyl-CoA. The formation of fatty acyl-CoA is catalysed by acyl-CoA synthetases present in the mitochondrial matrix of heart, kidney and skeletal muscle. In liver, the synthetase is only found in the cytosol. Long chain acyl-CoA synthetase activities are also found in the endoplasmic reticular membranes, in the outer mitochondria membrane and in peroxisomes (Buzzi, 1996). Unsaturated fatty acids are activated at higher rates than saturated acids. In intact cells, the activity of synthetases is regulated both by substrate (free fatty acids and CoA) availability, and by product inhibition by acyl-CoA (Gurr and Harwood, 1991).

Acyltransferase enzymes are responsible for the incorporation of fatty acyl-CoA into phospholipids and into triacylglycerols. Studies with rainbow trout hepatocytes provided evidence that exogenous C_{18} fatty acids are initially incorporated into triacylglycerols and retained for subsequent utilisation as desaturation or elongation substrates (Sellner and Hazel, 1982a; 1982b). The newly desaturated and elongated fatty acids, particularly the Δ6- and Δ5-desaturase products, are preferentially esterified

into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Sellner and Hazel, 1982a; 1982b).

1.1.6. Fatty acid oxidation

Lipids and fatty acids are major energy sources in many species of fish (Sargent *et al.*, 2002). Energy is obtained from fatty acids primarily through the process of β-oxidation. Whereas the biosynthesis of fatty acids occurs in the cytosol, the catabolism of fatty acids occurs in the cellular organelles, mitochondria (and peroxisomes) via a completely different set of enzymes. The process involves the sequential cleavage of two-carbon units, released as acetyl-CoA, through a cyclic series of reactions catalysed by several distinct enzyme activities rather than a multi-enzyme complex as in the anabolic pathway.

Activated fatty acids (CoA derivatives) are transported into the mitochondrion in the form of fatty acyl-carnitine esters formed through the action of carnitine acyltransferase, and are converted back into fatty acyl-CoA derivatives within the mitochondrial matrix prior to entering the β-oxidation cycle. The fatty acyl-CoAs then undergo a round of dehydrogenation, hydration, second dehydrogenation and cleavage steps producing a fatty chain two carbons shorter along with acetyl-CoA and NADH (Lehninger, 1975; Schulz, 2002). The acetyl-CoA can then be metabolised via the tricarboxylic cycle to produce more NADH. The NADH produced from the oxidation of fatty acids can then provide metabolic energy in the form of ATP through the process of oxidative phosphorylation (Lehninger, 1975; Schulz, 2002). Under certain conditions such as fasting, acetyl-CoA can be exported from the liver in the form of

ketone bodies, acetoacetate and 3-hydroxybutyrate, which are used by peripheral tissues as fuel through oxidation back to acetyl-CoA (Bartlett and Eaton, 2004).

The processes of mitochondrial β-oxidation and ketone body formation have all been established in fish although ketone bodies are probably only important energy in elasmobranches and not in teleost fish other than perhaps sturgeons (*Acipenser* sp.) (Henderson and Sargent, 1985b). Mitochondrial fatty acid oxidation is an important source of energy in several tissues in fish including liver, heart, red and white muscle, playing a significant role in overall fatty acid oxidation in Atlantic salmon (Froyland *et al.*, 1998, 2000). At a cellular level, peroxisomes are another site of β-oxidation in mammals where they are possibly specifically utilised in the initial chain shortening of very long chain, highly unsaturated or unusual fatty acids prior to conventional β-oxidation in the mitochondria. Relatively high levels of peroxisomal β-oxidation were observed in red muscle of Atlantic salmon (Froyland *et al.*, 2000). Peroxisomal β-oxidation may account for significant amounts (up to 30%) of total hepatic β-oxidation under certain conditions such as in Antarctic fish (Crockett and Sidell, 1993).

1.2. Intestine and intestinal metabolism

1.2.1. Anatomy of the digestive tract

The intestine of fish is the main digestive/absorptive organ. Overall, the gastrointestinal tract of fish has evolved to ensure optimum utilisation of dietary nutrients, which in many cases means efficient primary digestion and large intestinal absorptive surface area (Olsen and Ringo, 1997).

It is generally accepted that fishes can be characterised according to feeding habitats and diet into carnivores (eating fish and larger invertebrates), herbivores

(consuming mainly plant material), omnivores (mixed diet eaters) and detrivores (feeding largely on detritus). Some fish have a relative intestinal length (RIL = length of intestine/length of body) of less than one (less than the length of their body) while others have intestinal lengths of ten to twenty times their body lengths. It is known that the length of the intestine does not necessarily correlate with feeding habitats, but the highest RIL tends to occur in species that feed on detritus and microalgae, while the shortest is found more often in strict carnivores (Fig 1.4). It may be that increasing the intestinal length of, for example, herbivores versus carnivores is due to the requirement for a larger absorptive surface. Another point of view is that herbivorous (or detrivorous) fish consuming plant fibres and detritus rely on extended intestines in order to increase digestion and utilization efficiency (Olsen and Ringo, 1997).

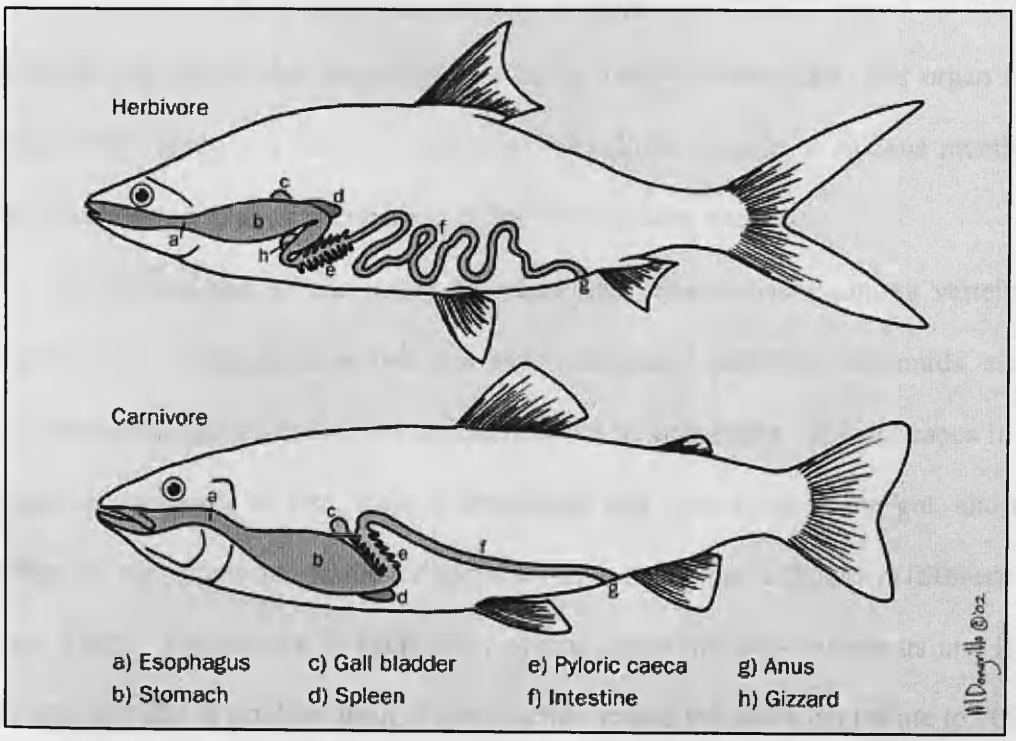


Figure 1.4. Representation of the digestive system of two fish with different feeding habitats (Myers *et al.*, 2005).

In comparison with the proportions of a mammal intestine, the foregut of fish is generally short, wide and straight. The oesophagus of freshwater fish is lined with a multiplayer of squamous epithelium containing a large number of mucous cells. However the oesophagus of some marine teleosts is lined with complex, highly vascularized mucosal folds of columnar epithelium and a few mucosal cells (Al-Hussaini, 1947; Stevens and Hume, 1995). Yamamoto and Hirano (1978) suggested that this might be a common feature in marine teleosts, associated with the osmotic regulatory function of their oesophagus (Fig. 1.4).

The stomach is defined as a portion of the digestive tube with a distinctive cell lining, and where acid is secreted, usually along with some digestive enzymes. Although absent in some fish species, most teleosts, including all salmonids have a discreet stomach. The stomach can display a range of different shapes in different families of fish, but in the particular case of the family Salmonidae, this organ has a siphon or "U" shape. A valve, or sphincter, of cellular muscle, a mucous membrane fold, or both are present at the junction of the stomach and intestine.

An arrangement of the upper intestinal tract, characteristic among vertebrates and present in a wide range of fish that have a stomach, including salmonids, are the blind-sacs at the pyloric end of the stomach called pyloric caeca. Pyloric caeca in fish fluctuate significantly in size, state of branching, and connection to the gut, and their number can vary from one single pyloric caecum to more than a thousand (Stevens and Hume, 1995). Particularly in salmonids, pyloric caeca are very numerous and it has been reported that in rainbow trout *Oncorhynchus mykiss* the caeca contribute to 70% of the postgastric surface area (Buddington and Diamond, 1986, 1987). It is believed that in fish like trout or salmon, with carnivorous/piscivorous habits, this development may

be a simple, more convenient arrangement for increasing the surface area without increasing the length of the intestine (Fig. 1.5).

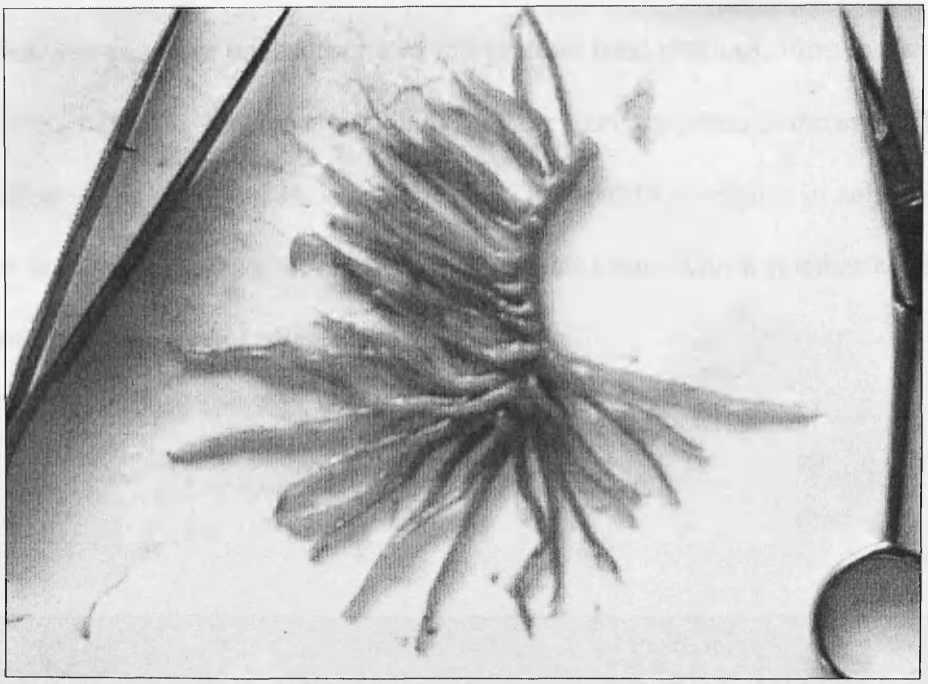


Figure 1.5. Pyloric caeca of rainbow trout (*Oncorhynchus mykiss*).

It appears that the hindgut has a special excretory, absorptive, and motor function in some species of fish. The hindgut of most fish cannot be distinguished from the midgut by the relative diameter or changes in epithelium although an ileorectal valve is present in many teleosts and in general the hindgut muscle is rarely increased in thickness. In the present work the midgut-hindgut section of the intestine is divided in three different segments and referred as the anterior intestine, posterior intestine and rectum. In vertebrates the anterior intestine is called the small intestine and the posterior intestine is the large intestine; in the present work the anterior gut was

considered as the region of the intestine in which pyloric caeca are attached (Fig. 1.6). Immediately after the last pyloric caeca is found, the digestive tube presents a clear tissue differentiation, this part, in the present work, was called posterior gut or posterior intestine (Fig. 1.6).

Faeces, composed of unabsorbed and indigestible food residues, form in the last part of the hindgut, where they are stored until they are excreted through the anus. This region is called the rectum and it presents a clear tissue differentiation in salmonids. This section is characterised by an elastic thin muscular tissue with a number of rings alongside the posterior gut and anus (Fig. 1.6).

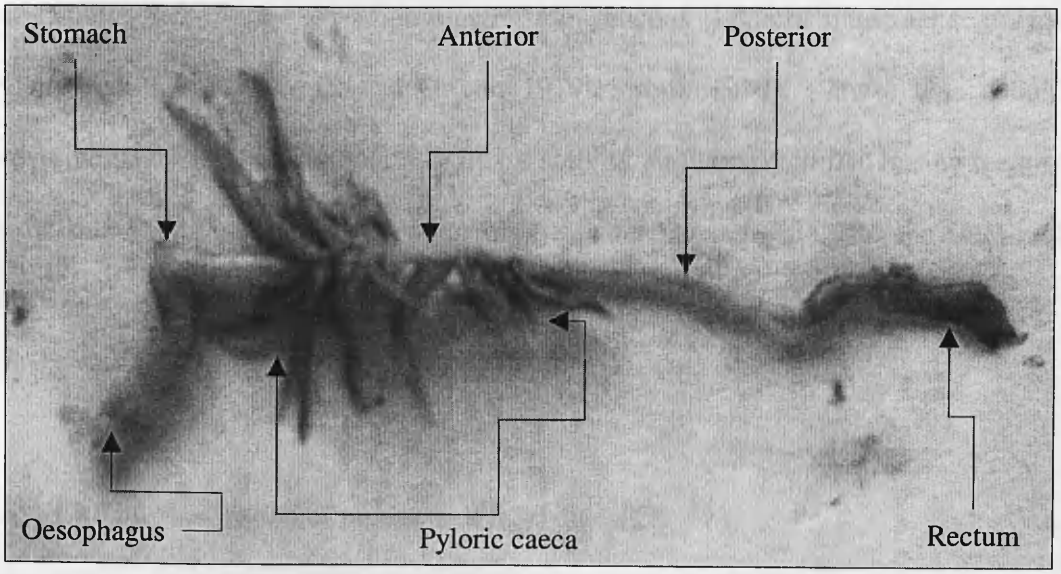


Fig. 1.6. Intestine of rainbow trout (*Oncorhynchus mykiss*) segmented into six different regions used and termed in the present work.

1.2.2. Digestion of lipids

The enzymatic degradation of dietary nutrients including lipid, known as digestion, is carried out in the intestine of fish in generally a similar manner to that in mammals and all vertebrates. It has been found that the lipolytic activity in fish is generally higher in the proximal part of the intestine and the pyloric caeca if present, but can extend into the lower parts of the intestine with the activity decreasing progressively (Buddington and Doroshov, 1986; Lie *et al.*, 1987; Bazaz and Keshavanath, 1993; Lied and Lambertsen, 1982; Ostos Garrido *et al.*, 1993).

The pancreas or hepatopancreas is generally assumed to be the major source of digestive lipase enzymes in fish as it is in mammals (Kapoor *et al.*, 1975; Fänge and Grove, 1979). Digestive lipases may also be secreted by the intestinal mucosa as several studies have found high lipase activity in mucous layers or intestinal segments of many fish species (Fänge and Grove, 1979; Smith, 1989). These data should however be interpreted with caution, as lipase activity particularly in fish during feeding may be due to the possible adsorption of the pancreatic enzyme into the intestinal mucosa (Olsen and Ringo, 1997; Tocher, 2003).

1.2.3. Absorption of products of lipid digestion

The mechanisms of absorption of the products of lipid digestion have not been extensively studied in fish but the basic physical processes including bile-enhanced emulsification and transport of the hydrolysed products are assumed to be generally similar to that in mammals (Tocher, 2003). Thus, the main hydrolytic products are solubilised or emulsified in bile salt micelles, followed by diffusion to the intestinal

mucosa where uptake into the enterocytes occurs, probably mainly by passive diffusion. Absorption generally proceeds at a much slower rate in fish compared to mammals, as a result of lower body temperature, which strongly influences the rate of nutrient digestibility (Kapoor *et al.*, 1975). Studies on absorption of free fatty acids using isolated enterocytes from rainbow trout showed that the uptake of 20:4n-6, 20:5n-3 and 22:6n-3 was lower than that of 16:0, 18:1n-9, 18:2n-6 and 18:3n-3, with most of the fatty acids recovered in triacylglycerols, although the recovery of HUFA and 16:0 in phosphoglycerides was higher than that of the other fatty acids (Perez *et al.*, 1999). As in mammals, lipid absorption in fish occurs predominantly in the proximal part of the intestine coinciding with the highest lipolytic activity. However, lipid can be absorbed along the entire length of the intestine, although, as with digestive function, in diminishing amounts.

1.2.4. Transport of lipids

Lipids are exported from the intestine in the form of lipoproteins. The re-esterification reactions occur primarily in the endoplasmic reticulum leading to the production of chylomicron - like and very low density lipoprotein (VLDL) - like particles in the lumen, as has been directly observed in freshwater species. In mammals, intestinal lipoproteins are transported from the intestine almost exclusively via the lymphatic system. Similarly, in fish the majority of the intestinal lipoproteins are transported via the lymphatic system before appearing in the circulatory system and being delivered to the liver (Sheridan *et al.*, 1985). However, a portion of intestinal lipoproteins may be transported directly to the liver via the portal system.

Intracellular transport of fatty acids in fish is essentially the same as that in higher vertebrates (Tocher, 2003). The intracellular transport of free fatty acids in mammals is facilitated by specific low molecular weight and highly conserved cytoplasmic proteins that bind both long-chain fatty acids and other hydrophobic ligands. These fatty acid binding proteins (FABPs) are tissue specific and those from liver, intestine, adipose tissue, brain and heart, have been extensively characterised in mammals (Veerkamp and Maatman, 1995).

1.2.5. Role of pyloric caeca in lipid digestion

There are several studies that have demonstrated the importance of pyloric caeca in lipid digestion and their richness in digestive enzymes (Greene, 1913; Ash, 1980; Buddington and Diamond, 1987; Olsen and Ringo, 1997). However, the basic cell structure and tissue architecture in pyloric caeca is generally identical to that in small intestine (Buddington and Diamond, 1987).

Therefore, it is widely accepted that the pyloric caeca have two primary functions, to increase retention time and so aid digestion and to provide an increased surface area and so aid absorption of digestion products, particularly in species that consume diets high in lipid (Patton *et al.*, 1975) and species such as salmonids including rainbow trout *Oncorhynchus mykiss* (Bergot *et al.*, 1975).

1.2.6. Role of pyloric caeca in fatty acid metabolism

The liver is generally thought to be the main region of PUFA biosynthesis in vertebrates, including fish (Tinoco, 1982; Henderson and Tocher, 1987). Hepatocytes

and liver subcellular fractions have traditionally been used to elucidate the metabolic pathways of desaturation and elongation of fatty acids (Voss *et al.*, 1991; Buzzi *et al.*, 1996). In contrast, there has been virtually no research in relation to the capacity of intestine as a metabolic organ involved in fatty acid conversions including desaturation and elongation. Recently though, interesting data were obtained from *in vivo* stable isotope studies utilizing deuterium-labelled 18:3n-3 described by Bell *et al.* (2003c), that suggested that pyloric caeca in rainbow trout are sites of significant quantities of newly synthesized 22:6n-3 and of oxidation of 18:3n-3. However, these studies can only give the perspective of the whole animal and it is not possible to unequivocally determine the source of labelled 22:6n-3 in caecal tissue, and whether it was synthesized *in situ* or was deposited there after synthesis in the liver. Therefore, *in vitro* studies with intestinal and caecal tissue were essential to determine the extent and nature of fatty acid metabolism in the tissue and discriminate that from metabolism in other tissues such as liver. It was for this reason that this project was initiated and the detailed study of the biochemical processes of fatty acyl desaturation/elongation and oxidation were investigated in caecal cells in salmonids.

1.3. Lipid and fatty acid nutrition in fish

1.3.1. Essential fatty acids in fish

As alluded to earlier, dietary lipids and their constituent fatty acids are a major source of energy in all fish, especially carnivorous species, which includes most marine fish where carbohydrate plays a minor role as an energy source due to its low abundance in natural diets. However, all vertebrates, fish included, have an absolute dietary requirement for certain PUFA. If a dietary deficiency occurs, the animal stops

growing and reproducing, it develops various pathologies and it eventually dies (Castell *et al.*, 1972; Watanabe, 1982; Sargent *et al.*, 2002). The PUFA in question are termed "essential fatty acids" (EFA) and they include members of both the n-6 and n-3 series typified by linoleic acid, 18:2n-6, and α-linolenic acid, 18:3n-3. Requirements for PUFA cannot be met by *de novo* metabolic processes in most animals, including all vertebrates and fish, as they lack the Δ12 and Δ15 fatty acid desaturases responsible for converting monounsaturated fatty acids, such as 18:1n-9, to PUFA, specifically 18:2n-6 and 18:3n-3, the ultimate precursors of the n-6 and n-3 series of PUFA, respectively (Cook and McMaster, 2002). However, the C_{18} PUFA do not have any direct functional roles in themselves in fish, and rather serve only as precursors of the functionally bioactive HUFA, AA, EPA and DHA (Tocher, 2003). Some fish species, including many freshwater species and salmonids including rainbow trout and Atlantic salmon (*Salmo salar*), can convert C_{18} PUFA to the C_{20} and C_{22} HUFA (Buzzi *et al.*, 1996, 1997; Tocher *et al.*, 1997). In species that cannot perform these conversions, and this includes most marine and carnivorous/piscivorous fish, the C_{20} and C_{22} HUFA themselves are dietary EFA and their C_{18} homologues do not satisfy EFA requirements. In species that can perform the conversions, C_{18}, C_{20} and C_{22} HUFA can all be termed EFA although the C_{20} and C_{22} HUFA are often more effective nutritionally than their C_{18} counterparts (Sargent *et al*, 2002). The extent to which the foregoing statements apply quantitatively to a given fish species varies widely. Therefore, a vital area in lipid nutrition in fish is the provision of sufficient amounts of the correct EFA to satisfy the requirements for normal growth and development, requirements that can vary quantitatively during the life of the fish.

1.3.2. EFA in human nutrition

Humans like all animal organisms, are not capable to synthesise 18:2n-6 and 18:3n-3 *de novo*, thus these fatty acids are considered as essential dietary nutrients for humans (Innis, 1991, 1993; Sinclair *et al.*, 2002). It has been mention that it is possible for humans to synthesize 18:2n-6 and 18:3n-3 by chain elongation of 16:2n-6 and 16:3n-3 found in green leafy vegetables contained in human diet, and even has been propose to replace the term EFA and non-EFA by dispensable, conditionally dispensable, and indispensable fatty acids (Cunnane, 1996); however under the consideration that the amount of FA synthesised would be limited (Cunnane *et al.*, 1995; Cunnane, 1996) in the present work 18:2n-6 and 18:3n-3 will be referred as EFA.

18:2n-6 has been shown to be important for growth, reproduction, and skin function in mammals (Burr, 1942; Holman, 1968; Hansen and Jensen, 1985). 18:3n-3 is important for growth (Burr, 1942) and skin function (Fu and Sinclair, 2000), and it has been demonstrated to be the preferred substrate for β-oxidation (DeLany *et al.*, 2000) and for carbon recycling in the brain and other tissues (Cunnane *et al.*, 1999). In addition, 18:2n-6 and 18:3n-3 are important in human nutrition as precursors of the more biologically active 20:4n-6, 20:5n-3 and 22:6n-3 (Nickaman *et al.*, 1967; Emken *et al.*, 1994). 20:4n-6 and 22:6n-3 are found in high concentrations in membrane structural lipids, particularly excitable membranes of the central nervous system, in the brain and the retina (Neuringer *et al.*, 1988; Connor *et al.*, 1992; Lauritzen *et al.*, 2001). Delayed development of visual acuity has been shown in pre-term and term infants when fed formulas lacking these FA (Uauy *et al.*, 2003). Moreover, a number of studies have also shown a link between these FA and psychomotor development and cognitive development (Uauy *et al.* 2003). In several animal studies, reductions of 22:6n-3 in the

brain were also associated with altered learning behaviour and impaired visual function (Connor *et al.* 1992; Umezawa *et al.*, 1999; Greiner *et al.*, 1999). There is also evidence that many mental disorders in human such as schizophrenia and Alzheimer's disease are associated with depressed levels of 22:6n-3 and 20:4n-6 in brain cells (Conquer and Holub, 1997).

In humans, 20:4n-6 and 20:5n-3 are direct precursors of eicosanoids that regulate many metabolic processes (Tapiero *et al.*, 2002). 20:4n-6-derived eicosanoids have general proinflammatory effects whereas 20:5n-3-derived eicosanoids are less potent and therefore reduce the inflammatory response. Because the proportion of eicosanoids produced from 20:4n-6 and 20:5n-3 modulates the inflammatory response, it is important to determine the 20:4n-6/20:5n-3 ratio in human diets. In view of this, dietary n-3 HUFA 20:5n-3 and 22:6n-3, have a central role in mediating many health disorders arising from increased eicosanoid production from 20:4n-6.

The intake of n-3 PUFA, specifically 20:5n-3 and 22:6n-3 that are found in high concentrations in fish and seafoods, has been considered to be beneficial in human health (Dyerberg *et al.*, 1975, 1978; Stansby 1990; Kelly 1991). Studies with human populations which are considered as fish consumers have demonstrated that these people were apparently protected from cardiovascular disease due to their high fish intake (Keys, 1970; Dyerberg *et al.*, 1975; Bang and Dyerberg, 1980). The importance of the n-3 highly unsaturated fatty acids (HUFA) in human nutrition has led to considerable research effort in the intervening years. There is now considerable evidence from clinical and epidemiological research that increased consumption of n-3 HUFA are efficient in the prevention or attenuation of many inflammatory conditions that are prevalent in the developed world, including rheumatoid arthritis, atopic illness, psoriasis, multiple sclerosis, bronchial asthma, type I diabetes, inflammatory bowel

disease and various neurological conditions (Leaf and Weber, 1988; Harris, 1989; Kinsella *et al.*, 1990; Calder, 1997; de Deckere *et al.*, 1998; Simopoulos, 1999a; Connor, 2000).

The importance of HUFA, specifically 20:4n-6 and 22:6n-3 was recently highlighted in the evolution of human brain field as it is thought that *Homo sapiens* is unlikely to have evolved a large, complex, metabolically expensive brain in a terrestrial environment which did not provide abundant dietary long chain PUFA; it is however, predictable that exploitation of the littoral marine and lacustrine food chains which provide consistently greater amounts of pre-formed long chain PUFA than the terrestrial food chain, would have provided the advantage in multigenerational brain development which would have made possible the advent of *H. sapiens* (Broadhurst *et al.*, 2002). A number of anthropological and nutritional studies indicate that during the last 150 years major changes have taken place in human diets in the type and amount of fat, due to changes in food technology and production (Simopoulos, 1999b). Modern developed societies are characterized by having an increase in energy intake, saturated fat and n-6 PUFA and a decrease in n-3 PUFA. It is estimated that the present so-called "western" diet is providing a n-6/n-3 PUFA ratio of around 15:1 compared to a value of about 1-5:1 that is considered to be optimum (Simopoulos, 1999b). These changes in the fat intake patterns have been linked with the occurrence of many health disorders common in the western/industrialised world such as coronary heart disease and various types of cancer. However, these types of disorders, once considered a problem primarily of the western world, are rapidly becoming a major public health problem in many parts of Asia (Janus *et al.*, 1996; Okuyama *et al.*, 1997; Bulliyya, 2000). The major advances in technology and economic growth in this region have brought increased urbanization and

lifestyle changes that are associated with an increased intake of saturated fat and n-6 PUFA that in turn are major risk factors for the health disorders aforementioned.

Fish and marine mammals are by far the richest source of n-3 PUFA in nature. Since it has been shown that fish have beneficial effects on several health disorders and human intake of n-6 PUFA is often excessive and the n-6/n-3 PUFA ratio is imbalanced, it is medically recommended to increase the intake of n-3 PUFA, and particularly 20:5n-3 and 22:6n-3, and decrease the intake of n-6 PUFA in our diet (Okuyama *et al.*, 1997; de Deckere *et al.*, 1998; British Nutrition Foundation, 1999; Sinclair *et al.*, 2002). As fish farming is becoming a major contributor in world fish supplies, the Aquaculture sector must ensure that the resultant end product is providing these essential nutrients for the consumer.

1.3.3. EFA and aquaculture

Fish meal and fish oil derived from industrial feed-grade fisheries, e.g. capelin, herring, sand eel, mackerel, anchovy and sardine fisheries, have traditionally been the standard ingredients of bulk feeds for intensively farmed fish, particularly salmonids and marine fish. The requirements of marine fish for 20:5n-3 and 22:6n-3 make fish oil the only commercially available source of these fatty acids, essential in marine fish feeds (Sargent *et al.*, 2002). World fisheries though are being maximally exploited, many would argue overexploited, but what everyone agrees is that fish oil production has, at best, reached its sustainable limit. Therefore, the current average yield of fish oil from industrial fisheries, circa 1.4 million tonnes in 2000 (Sargent and Tacon, 1999), is very unlikely to be significantly exceeded in future. Unpredictable climatic conditions, for example El Niño, can severely affect fish oil production in some years as it did in

1998, when world fish oil supplies decreased to just 0.8 million tonnes (FAO, 2000). Fish farming consumed a total of 800,000 tonnes of fish oil in 2000, which accounted for 57% of total world supply that year, with farmed salmon and trout consuming over 60% of that total (Sargent and Tacon, 1999). Global aquaculture has grown at 11.6% per annum compound growth since 1984 (Tacon, 1996) and is continuing to grow at a similar rate. As a result, if current usage continues at the same high rate, it is estimated that 98% of the total world supply of fish oil will be required for aquafeeds by 2010 (Barlow, 2000). Obviously this problem will be greatly exacerbated in the event of an El Niño. In addition, there is growing pressure from various environmental groups to decrease the level of exploitation on finite marine resources and by increasing consumer perception that levels of pollutants such as dioxins, PCBs and flame retardants in fish oils have now reached unacceptable levels. For these reasons, finding alternatives to fish oils in farmed fish feeds is becoming an increasingly urgent issue (Sargent *et al.*, 2002).

As noted earlier, many freshwater fish can convert 18:3n-3 to 20:5n-3 and 22:6n-3 and can, therefore, be grown on diets containing 18:3n-3, at least in principle. Such fish include salmonids, most notably rainbow trout and Atlantic salmon (Buzzi *et al.*, 1996, 1997). The solution to replacing fish oils in aquaculture diets requires retaining as far as possible the health promoting properties of the end product for the consumer, which means retaining as far as possible the current high levels of 20:5n-3 and 22:6n-3 in farmed fish (Sargent and Tacon, 1999). Sargent *et al.* (2002) made important observations related to the consideration of replacements for fish oils in aquaculture feeds. Briefly, they noted the following five points.

1) Levels of 20:5n-3 and 22:6n-3 in current farmed fish feeds are well in excess of the minimum n-3 EFA requirements of the fish. A more judicious use of fish oil can allow a greater tonnage of farmed fish to be produced than is currently the case. However, distributing the available fish oil over greater quantities of fish does not increase the total input of 20:5n-3 and 22:6n-3 in the human diet.

2) Minimise the oxidation by fish of fatty acids that are particularly valuable in human nutrition (*i.e.* 20:5n-3 and 22:6n-3) by using vegetable oils in the diets containing fatty acids that appeared to be easily oxidised (*i.e.* 18:1n-9 and 18:2n-6 and 18:3n-3).

3) Due to the deleterious effects of dietary ratios of n-6:n-3 PUFA in man (currently averaging 15:1 in the western diet, ranging between 10:1 and 25:1), care should be exercised in substituting fish oils with vegetable oils rich in 18:2n-6. More research was suggested by Sargent *et al.* (2002), to evaluate the effect of this fatty acid on the farmed fish especially in terms of their response to stress and disease.

4) More research is needed to evaluate the extent to which 18:3n-3 – rich oils, specifically linseed oil, can successfully substitute for fish oils, especially in the salmonids and freshwater fish in general which are capable of converting this fatty acid to 20:5n-3 and 22:6n-3. It was suggested there was a possibility of selecting strains of fish with high activities in converting 18:3n-3 to 20:5n-3 and 22:6n-3, even in the presence of significant amounts of fish oil in the fish's diet. Perhaps further in the future is the possibility of maximally activating the genes determining conversion of 18:3n-3 to 22:6n-3 in marine fish.

5) Replacement of "lighter" oils containing C_{22} and C_{20} fatty acids with C_{18} fatty acids, whether monounsaturated fatty acids or PUFA, may not always result in good retention of the oil within adipocytes. This is important for the processing conditions of

the product involving low temperature storage and/or smoking. The successful development of alternatives to fish oil in aquafeeds requires much research to be done if projected targets for aquaculture expansion are to be met.

Clearly, there are many important issues to address in considering the replacement of dietary fish oils with vegetable oils in aquaculture feeds. As the first tissue to come into contact with dietary components, it may be expected that dietary vegetable oils will impact upon the metabolism of intestinal tissue. This is especially important in relation to the particular pathways of fatty acid metabolism that the present project is focusing on, namely HUFA synthesis from C_{18} PUFA. Therefore, in addition to investigating and characterizing HUFA synthesis and β-oxidation in pyloric caeca, this project also aimed to determine the specific effects on these pathways of feeding vegetable oils as replacements for fish oil.

1.4. General objectives

The overall aim of this research work was to determine the importance of intestine and caecal enterocytes in fatty acid metabolism in salmonids, focusing on their potential role in n-3 HUFA synthesis and the effects of replacement of fish oil with vegetable oils in the diets. Specifically the project had five primary objectives:

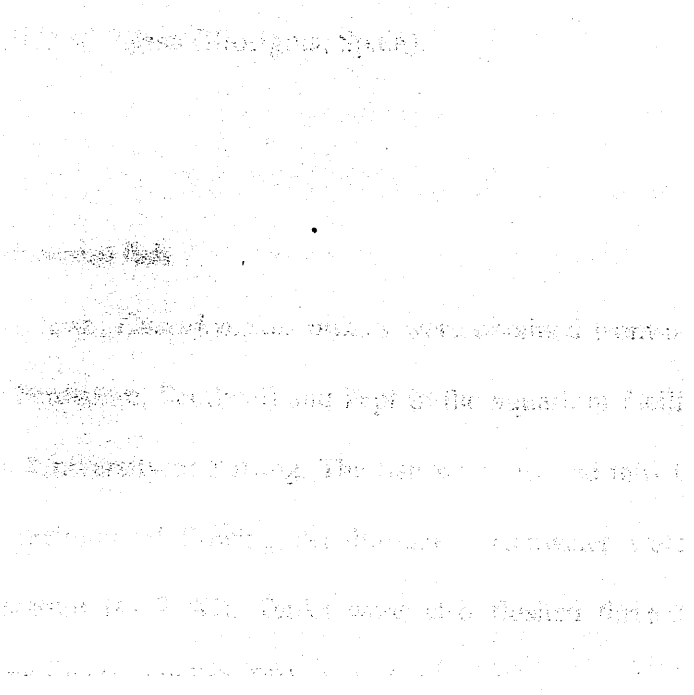
1) To develop and establish a simple method for the isolation of viable enterocytes from salmonids suitable for use in both laboratory and fish farm conditions.

2) To develop and characterize the assay of the fatty acid desaturation/elongation pathway in salmonid enterocyte suspensions.

3) To develop a combined assay for fatty acid desaturation/elongation and fatty acid β-oxidation in salmonid enterocytes suspensions.

4) To assess the relative importance of intestine in HUFA synthesis by comparison with hepatocytes, and to establish the specific areas of intestine most important in HUFA synthesis in salmonids.

5) To determine the effects of replacement of fish oil with vegetable oils in the diets of salmonids on fatty acid metabolism in enterocytes including effects of different vegetable oils and blends, dietary oil content, and interaction with seasonal/ environmental factors during an entire Atlantic salmon production (life) cycle.



CHAPTER TWO

2. GENERAL METHODS AND MATERIALS

2.1. Materials.

[1-^{14}C]18:3n-3 (50-55 mCi/mmol), was obtained from NEN Brand Radiochemicals (Perkin Elmer Life and Analytrical Sciences (U.K.) Ltd., Beaconsfield, Buckinghamshire, U.K.). HBSS, Medium 199, HEPES buffer, glutamine, collagenase (type IV), FAF-BSA, BHT, silver nitrate, perchloric acid and Trypan blue were obtained from Sigma Chemical Co. (Poole, U.K.). Thin-layer chromatography (TLC) plates, 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 U.K., Loughborough, England. Lactate dehydrogenase (LDH) activity kit was obtained from Sigma (Biosigma, Spain).

2.2. Experimental fish

Rainbow trout, *Oncorhynchus mykiss* were obtained from a local fish hatchery (Almondbank, Perthshire, Scotland) and kept in the aquarium facilities of the Institute of Aquaculture, University of Stirling. The fish were stocked into 100 L circular tanks supplied with recirculated flowing dechlorinated freshwater water at 1 L/min and ambient temperature (4-17 °C). Tanks were also flushed three times per week to maintain adequate water quality. Fish were fed two times per day with a commercial trout diet (Skretting, U.K.).

For characterisation of the enterocyte suspensions, some cell viability tests were carried out at the Universidad de La Laguna, Tenerife, Spain. Rainbow trout for these experiments were obtain from a local fish hatchery (Piscifactoría Aguamansa) and kept in the aquarium facilities of the University. The fish were stocked into 100 L circular tanks supplied with recirculated flowing chilled (15 °C) freshwater.

The specific conditions of experimental fish for the dietary trials with Atlantic salmon (*Salmo salar* L.) used in chapter 4, 6 and 7 are described respectively in the appropiate chapter.

2.3. Preparation of enterocytes and hepatocytes from salmonids.

Enterocytes were isolated by a modification of the method described previously for isolation of hepatocytes (Bell *et al.*, 1997, 2001a, 2002; Tocher *et al.*, 1997, 2000). This technique for isolating hepatocytes was specifically developed for use in large-scale cage feeding trials with Atlantic salmon (*Salmo salar* L.) in seawater, and so was optimised for use "in the field" at isolated fish farms often without any significant laboratory facilities. This isolation methodology was investigated in the laboratory at the Institute of Aquaculture, University of Stirling, to assess its suitability for also obtaining viable preparations of intestinal cells (enterocyte-enriched) using rainbow trout for the initial cell preparation, characterisation and viability trials.

The basic methodology adopted for the preparation of isolated caecal enterocyte-rich cell suspensions used for both the characterisation of the metabolic assays using rainbow trout in the laboratory, and the salmon cage feeding trials in the field is described below.

Fish were anaesthetised in MS-222 or benzocaine (depending on site), and the required number of fish (depending on the experiment) were killed by a blow to the head. A ventral incision was made with scissors and the gut rapidly removed. The pyloric caeca were identified after removing some accumulated fat and an appropriate number of them (3-10 depending on the size of the fish) were dissected out and placed on clean paper towel. The caeca were cleaned of adhering adipose tissue, and lumenal contents gently squeezed out of the caeca by holding the blind end and gently running the spatula up the caeca towards the open end and the contents rinsed away with calcium and magnesium-free Hanks balanced salt solution (HBSS) + 10 mM HEPES + 1 mM EDTA (solution A) (pH=7.4). The caeca were chopped finely with scissors in a 100 ml plastic beaker and then placed in a 25 ml "Reacti-flask" with 20 ml of solution A containing 0.1 % (w/v) collagenase. Reacti-flasks were then placed in a shaking water bath at 20 °C for 45 min to facilitate cell dissociation. The digested intestinal tissue was then filtered using 100 μm nylon gauze and retained in a plastic beaker, by washing through with ~40 ml of solution A containing 1 % w/v fatty acid-free bovine serum albumin (FAF-BSA) (HBSS/FAF-BSA) and the cells collected by centrifugation at 300 x g for 3 min. The washing was repeated with a further 20 ml of solution A without FAF-BSA and cells were collected again by centrifugation. Enterocytes were resuspended in either 6 ml (field experiments) or 5 ml (laboratory experiments) of Medium 199 containing 10 mM HEPES and 2 mM glutamine. Instead of Medium 199, Dulbecco´s modified Eagle medium (DMEM) was used in some viability test (oxygen consumption and lactate dehydrogenase, LDH activity) performed at the Universidad La Laguna, Tenerife, Spain.

With relatively minor modification, the above method was also used to isolate hepatocytes in order to carry out the comparative studies to determine the relative

importance of intestine in HUFA synthesis in salmonids. Briefly, after fish were sacrificed, the liver was dissected immediately and perfused via the hepatic vein with solution A to clear blood from the tissue. The liver was chopped finely in a plastic beaker and about 0.5 g of chopped liver was taken and incubated with 20 ml of solution A containing 0.1 % (w/v) collagenase as above. The digested liver tissue was filtered through 100 μm nylon gauze and the cells collected, washed and resuspended in medium as above.

100 µl aliquots of the hepatocyte and enterocyte enriched suspensions were retained in a microcentrifuge tube and immediately frozen at -20 °C, or in liquid nitrogen (dry shipper) in the case of the samples taken in the field, for protein determination.

2.4. Preparation of radiolabeled fatty acid substrate, [1-^{14}C]18:3n-3/BSA complex, for fatty acid metabolic assays.

The fatty acid chosen for the routine assay was 18:3n-3. Previous studies in the laboratory have determined this to be the best PUFA substrate for the HUFA synthesis pathway in fish systems (Bell *et al.*, 1997, 2001a, 2002; Tocher *et al.*, 1997, 2000, 2001). The fatty acid was added to the cell incubations as [1-^{14}C]18:3n-3 in a complex with FAF-BSA in phosphate buffered saline prepared as described previously by Ghioni *et al.*, 1997. Briefly, 25 µCi of the original batch (50-55 mCi/mmol) of [1-^{14}C]18:3n-3 in ethanol was transferred to a 2 ml reacti-vial containing a magnetic stirrer flea. The ethanol was evaporated under a stream of oxygen-free nitrogen (OFN) and 100 µl of 0.1 M KOH were added to the dry radiolabelled substrate, and the vial placed in a magnetic stirring heat block (without heating) and stirred at room temperature for 10 min. Five

ml of FAF-BSA solution (50 mg ml^{-1} of FAF-BSA in Medium 199) were added to the isotope/KOH solution and mixing on the heating block continued at room temperature for 45 min. Samples of the fatty acid substrate complex were taken to determine the precise amount of radioactivity. Ten µl samples were carefully pipetted into scintillation mini-vials (Meridian, 5 West Street Epsom, Surrey, U.K.), 2.5 ml of scintillation fluid (EcoScintillation Cocktail, Meridian, Surrey, U.K.) added and the vial mixed. Radioactivity was determined in a TRI-CARB 2000CA scintillation counter (United Technologies Packard, U.K.) and results were corrected by counting efficiency based on a quench curve using the Transformed Spectral Index Standard (tSIE) value (Fig. 2.1).

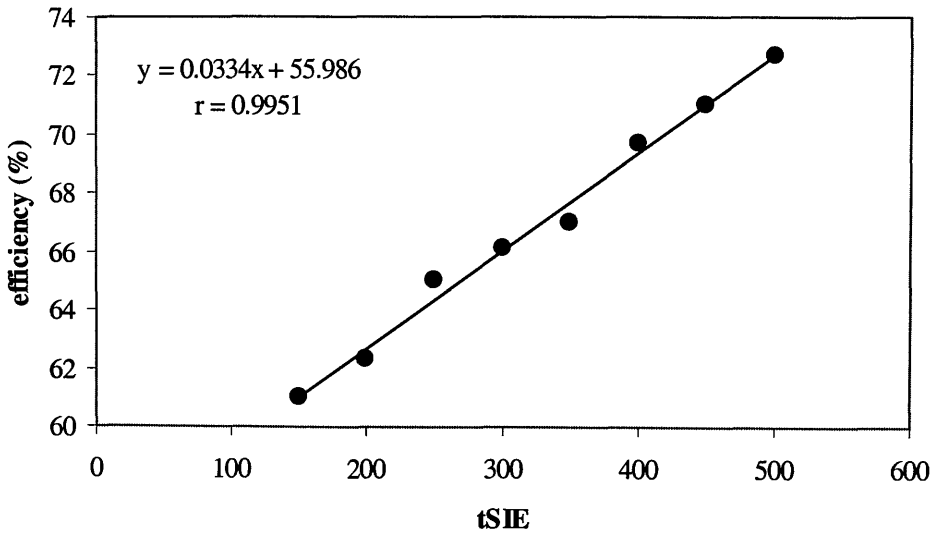


Figure 2.1 Transformed standard (tSIE) quench curve for [1-^{14}C]18:3n-3 to correct quench of radioactivity distributed in the HUFA synthesis and β-oxidation analysis. A correlation was made using the tSIE values and efficiency. The curve was fitted to the standard points and the DPM values calculated from the regression equation.

2.5. Incubation of enterocytes/hepatocytes preparation with radioactive substrate.

Each enterocyte (or hepatocyte) suspension was dispensed into 25 cm^2 polystyrene sterile tissue culture flasks (Falcon Labware, Beckton Dickenson, Europe, Meylan Cedex, France). Cells were incubated with 0.5 μCi (~2.5 μM) [1-^{14}C]18:3n-3 that translated to approximately 10 μl fatty acid substrate per 1 ml of suspension. After addition of isotope, the flasks were incubated at 20 °C for 2 hr. After incubation, the cell layer was dislodged by gentle rocking and the cell suspension transferred to glass conical test tubes using a plastic Pasteur pipette with relatively wide aperture. In studies where β-oxidation was also being determined, 1 ml of each suspension was then withdrawn into a 2 ml microcentrifuge tube for the β-oxidation assay as described in section 2.6. The cell suspensions remaining in the glass conical centrifuge tubes were used for the desaturation/elongation assay as described in section 2.5.

2.6. Determination of HUFA synthesis via assay of enterocyte (and hepatocyte) fatty acyl desaturation/elongation activities.

The cell suspensions were centrifuged at 500 x g for 2 min, the supernatant discarded and the enterocyte (or hepatocyte) pellets washed with 5 ml of ice-cold HBSS/FAF-BSA and recentrifuged. The supernatant was carefully discarded and total lipids of cells were extracted using ice-cold chloroform/methanol (2:1, v/v) containing 0.01% (w/v) BHT as described in detail in section 2.8. Total lipids were transmethylated and FAME prepared as described in section 2.9. The methyl esters were separated by argentation thin-layer chromatography (TLC) (Christie, 2003). TLC plates (20 x 20 cm x 0.25 mm) were impregnated with 2 g silver nitrate by dissolving in

20 ml acetonitrile and applying evenly with a syringe. Plates were air dried in the fume cupboard in the dark and then pre-activated at 110 °C for 30 min. FAME were redissolved in 100 µl isohexane containing 0.01 % BHT and applied as 2.5 cm streaks to the TLC plates and subsequently fully developed with toluene/acetonitrile (95:5, v/v) in a standard TLC tank placed in the dark (Wilson and Sargent, 1992). Plates were air dried in a fume cupboard in the dark and were marked with radioactive ink at three separate points at the sides and bottom of the plate. The plates were subjected to autoradiography at room temperature for between 4 and 7 days, as appropriate, using Kodak MR2 X-ray film (Kodak, Rochester, NY, USA). The radioactive ink points served as alignment references to the exact position in which the autoradiography film was placed over the plate.

With the developed film as reference, individual PUFA were identified and delimited with a soft pencil on the silica plate. Areas of silica corresponding to individual radioactive PUFA were scraped and deposited into scintillation mini-vials before adding 2.5 ml of scintillation fluid (as above), and radioactivity determined in a TRI-CARB 2000CA scintillation counter with results corrected for counting efficiency and quenching of ^{14}C.

2.7. Assay of fatty acyl oxidation activity in enterocyte (or hepatocyte) suspensions.

Fatty acid β-oxidation in isolated enterocytes and hepatocytes was estimated by determination of acid-soluble radioactivity as described previously (Frøyland *et al.*, 1996, 2000; Madsen *et al.*, 1998; Torstensen *et al.*, 2000). The assay is based on the concept that cells incorporate and activate [1-^{14}C]18:3n-3 by esterification with CoA to

form fatty acyl-CoA. The successive oxidative removal of acetyl-CoA units from the fatty acid is called β-oxidation. Acetyl-CoA is soluble in acid therefore this assay was developed to measure the amount of radioactivity in the acid soluble fraction of the cell suspension. The assay was initially developed for subcellular (homogenate) preparations (Willumsen *et al.*, 1993) but was modified for use with hepatocytes (Frøyland *et al.*, 1995, 2000; Ji *et al.*, 1996), and was further modified in this project. Preliminary experiments had shown that about 90 % of acid-soluble products were recovered in the medium but about 10 % remained in the cells. Therefore, the method was modified so that total oxidation products, whether released into the medium or still remaining in cells, could be measured. Briefly, after incubation of cell suspensions with [1-^{14}C]18:3n-3, 1 ml of enterocyte or hepatocyte suspension was transferred into a microcentrifuge tube and homogenised with a hand-held tissue disrupter (Ultra-Turrax with T8/S8N-5G probe, IKA-Werke GmbH & Co., Slaufen, Germany) to break cells and release oxidation products into the suspension, and then centrifuged at 10000 x g for 10 min to precipitate cell debri. Five hundred µl of supernatant was pipetted into another microcentrifuge tube and 100 µl of ice-cold 6 % aqueous FAF-BSA solution added to bind unmetabolised fatty acid substrate. After mixing thoroughly, the protein and bound unmetabolised fatty acid was precipitated by the addition of 1.0 ml of ice-cold 4 M perchloric acid ($HClO_4$) and the tubes centrifuged at 10000 x g for 10 min. Five hundred µl of the supernatant containing the acid-soluble fraction of β-oxidation (acetyl-CoA) was transferred to a scintillation vial, 4 ml of scintillant added and radioactivity determined as in section 2.3.

It important to consider that the radioactive substrate used in the present study were radiolabelled at the first carbon atom in the fatty acid chain from the carboxylic side ([1-^{14}C]). Hence, measurement of β-oxidation reflected only one cycle of β-

oxidation. Information beyond this initial cycle, such as whether the entire fatty acid chain was broken down could not be explored. The approach to this problem would be to use a uniformly labelled substrate ([U-^{14}C]) which will ensure the measurement of each cycle of β-oxidation that characterise this biochemical process. However, this substrate is not commercially available and therefore a 1-^{14}C substrate was used with the assumption that a fatty acid entering the β-oxidation cycle is completely oxidised.

2.8. Protein determination.

Protein concentration in isolated enterocyte/hepatocyte suspensions was determined according to the method of Lowry *et al.* (1951) after cell digestion. Briefly, 250 µl of 1 M NaOH / 0.25 % (w/v) sodium dodecyl sulphate (SDS) were added to 100 µl of defrosted suspension sample (see section 2.2) in microcentrifuge tubes, and the samples incubated at 60 °C for 45 min in a water bath. A standard curve of 0 – 100 µg protein was prepared in 12 disposable plastic test tubes with respectively 0, 20, 40, 60, 80 and 100 µl of bovine serum albumin (BSA) (1 mg/ml) in duplicates, volumes made up to 100 µl with distilled water. Duplicate samples of 50 and 100 µl of digested cell sample were transferred to disposable plastic test tubes with the final volume adjusted to 100 µl with distilled water in the case of 50 µl samples. One ml of 1 % (w/v) $CuSO_4$ and 1 ml of 2 % (w/v) of sodium potassium tartrate was made up to 100 ml with 2 % (w/v) Na_2CO_3 (or an appropriate volume at these ratios), and 2.5 ml of this solution was added to standards and samples. After 15 min incubation at room temperature, 250 µl of Folin-Ciocaulteau phenol reagent diluted 1:1 with water was added to the standard and samples. The absorbance at a wavelength 660 nm of standard and sample tubes was read on a spectrophotometer (Jenway 6405 UV/Vis) between 30 and 60 min later.

A standard curve was drawn and protein concentration of samples was calculated in mg/ml (Lowry *et al.*, 1951).

2.9. Total lipid extraction and quantification.

Total lipids of pyloric caeca, livers, enterocytes and hepatocyte suspensions, and diet samples were extracted by homogenisation in chloroform/methanol (2:1, v/v) containing 0.01 % (w/v) butylated hydroxytoluene (BHT) as antioxidant, basically according to the modified Folch *et al.* (1959) method as described by Christie (2003). Briefly, 1 g of tissue or diet was homogenised (Ultraturrax^{TM}) in quickfit boiling tubes (50 ml) with 16 ml of ice cold chloroform/methanol (2:1, v/v). After 1 h of their placement on ice, 4ml of aqueous potassium chloride (0.88% KCl, w/v) was added and mixed thoroughly by vortex mixer before allowed to settle to separate the organic phase on ice for 5 minutes. The tubes were then centrifuged (Jouan C312, France) at 400 g_{ave} (1500 rpm) for 2-3 minutes and the upper aqueous phase was drawn off by aspiration and discarded. The lower organic layer was transferred into a clean pre-weighed 10 ml test tube, through a pre-washed (with C:M 2:1) 9 cm filter paper (Whatman No.1). Solvent was evaporated under an oxygen-free nitrogen (OFN) stream on an N-evap (Organomation Associates, Inc. USA) and the "dry" sample desiccated under vacuum overnight. Subsequently, lipids were quantified gravimetrically by reweighing the test tube, and total lipid content determined per weight of material extracted. Lipids were redissolved in C:M (2:1) at a concentration of 10 mg/ml and transferred to small glass vials and stored under nitrogen or argon in a -20°C freezer.

2.10. Analysis of fatty acid composition.

The fatty acid composition of total lipids from diets and tissues were determined by capillary gas chromatography. Total lipids were subjected to acid-catalysed transesterification. The fatty acid methyl esters (FAME) were prepared by incubating the total amount of lipids overnight at 50 °C in the presence of 2 ml of 1 % (v/v) H_2SO_4 in methanol plus 1 ml toluene under nitrogen as described by Christie (2003). After incubation, FAME were extracted using isohexane/diethyl ether (1:1 v/v) and centrifugation at 350-400 g_{ave} (1500 rpm) for 2 min. The upper organic layer was transferred into a clean test tube. For a second extraction, 5 ml of isohexane/diethyl ether (1:1 v/v) with BHT (0.01 %) (w/v) was added to the original tube before mixing and centrifuging again. Once again the upper organic layer was transferred and mixed with the organic portion of the previous extraction and the aqueous layer discarded. Solvent was evaporated under a stream of OFN on an N-evap and FAME redissolved in 100 µl of isohexane. Methyl esters were applied (< 1.5mg) using microsyringes (Hamilton) onto 1 cm origin lines marked previously onto 20 x 20 cm TLC plates. FAME were purified by TLC using isohexane/diethyl ether/ acetic acid (85:15:1 v/v) as developing solvent as described previously (Tocher and Harvie, 1988). After the plate was chromatographed, it was removed from the tank and the solvent allowed to evaporate in the fume cupboard. When the plate was dry, it was sprayed with 1% (w/v) iodine in $CHCl_3$ to visualise the FAMEs. The outside lane of the plate was masked off with a blank glass plate so that only the very edge of the sample was exposed before being sprayed lightly with iodine. FAMEs chromatograph as a doublet, saturated and monounsaturated fatty acids form an upper band and polyunsaturated fatty acids a lower band. When the two FAME bands were visible they were marked carefully with pencil. The bands were scraped from the TLC plate into test tubes using a straight edged

scalpel or razor blade. FAME were eluted from the silica with 10 ml isohexane/diethyl ether (1:1, v/v), mixed on a vortex mixer, and centrifuged as described above, to precipitate the silica. The solvent was carefully removed into a clean 15 ml glass quickfit tube and evaporated under OFN and the samples transferred into 2 ml glass sample vials in 1 ml isohexane before the solvent was evaporated again under OFN. Finally, the recovered FAME were dissolved in an appropriate volume of isohexane containing 0.01% (w/v) BHT to make a 1-2 mg/ml solution of methyl esters. FAME were quantified by gas-liquid chromatography using a Fisons GC8600 gas chromatograph (Fisons Ltd., Crawley, U.K.) equipped with on-column injection and a 30 m x 0.32 mm i.d. wall-coated capillary column (CP wax 52CB; Chrompak Ltd., London, U.K). Hydrogen was used as carrier gas and temperature programming was from 50 °C to 180 °C at 40 °C/min and then to 225 °C at 2 °C/min. Individual methyl esters were identified by comparison to known standards and by reference to published data (Ackman, 1980).

CHAPTER THREE

3. DEVELOPMENT AND CHARACTERISATION OF ISOLATED ENTEROCYTE SUSPENSIONS, AND CHARACTERISATION OF HUFA SYNTHESIS ASSAY.

3.1. Introduction

3.1.1. Development and characterization of isolated enterocyte preparations.

There is a relationship between the diversity of functional demands placed on the intestine and the variation of structure. Correspondingly, of all vertebrate organ systems, the gastrointestinal tract and respiratory system are structurally the most variable. The expansive surface area of the intestine represents a selectively permeable barrier that allows nutrient absorption, but excludes most toxic substances and pathogenic organisms. Intestinal structure can respond rapidly and reversibly to changes in dietary inputs (Buddington and Diamond, 1986, 1987; Buddington *et al.*, 1997).

Enterocytes are the first cells to be in contact with ingested food. These cells perform a range of functions in digestion, but particularly are the primary absorptive cell taking up digested nutrients, which are then transported across the enterocyte membranes towards the underlying mucosal blood circuits. The roles of the digestive and absorptive functions of fish enterocytes have been studied previously (Collie, 1985; Olsen and Ringo, 1997, Røsjø *et al.*, 2000; Denstadli *et al.*, 2004), and are not the objective of this study. Rather the present study's primary objective is to identify and

characterize the intracellular metabolic role of fish intestinal cells, specifically enterocytes and particularly in relation to fatty acid metabolism, which has not been studied in detail before. In order to elucidate the range of intestinal fatty acid metabolic functions in fish, a suitable *in vitro* cell system is required. The cells from the mucosa of the small intestine of some mammals have been isolated to provide a model for the study of enterocyte morphology (Bjorkman *et al.*, 1986), metabolism (Hegazy *et al.*, 1983) and differentiation (Traber *et al.*, 1991) in addition to the investigation of membrane transport processes (Del Castillo and Whittembury, 1987). Therefore, a key requirement for the present work was the development of a suitable enterocyte preparation that allows the conditions to investigate and understand the intracellular intestinal fatty acid metabolism of fish, in this case, salmonids.

Cell cultures have been used extensively as model systems in metabolic studies in mammals, and culture of fish cells is long established and many cell lines are available. Cell culture has several advantages including control of the environment, such as conditions of culture (temperature, salinity etc.) and composition of medium (nutrients). It is also convenient for containment if potentially hazardous compounds are to be used such as radioisotopes or toxins. Cell lines have the advantage that they are immortal cells that attach, grow, divide and can be subcultured indefinitely, but their disadvantage is that they are usually de-differentiated cells, generally of fibroblast or epithelial morphology, and not reflecting any specific tissue. Primary cultures retain their differentiated phenotype for a period of days or weeks, and attach, grow and divide, but generally cannot be subcultured. Short-term "cultures" can be attached or in suspension but show no growth or division over the time course of the experiment, but again retain their differentiated phenotype.

Thus, the culture of cells under defined conditions *in vitro* is an extensively used technique because it provides cells that exhibit a wide range of metabolic or physiological functions that can be characteristic of the intact organ/tissue that they were prepared from, e.g. liver (Berry *et al.*, 1991). Although there are many descriptions of suitable techniques to isolate cells from a variety of organs/tissues, isolation of some cell types can be a difficult time-consuming and labour-intensive procedure. This project would require enterocytes to be isolated and experiments performed under difficult and restricted conditions at fish farms, often actually out on cages, and so the method adopted would have to be as simple and robust as possible.

Primary culture of salmonids cells, specifically trout hepatocytes, has been often used to investigate a wide range of metabolic processes of liver such as lipogenesis (Geelen, 1994; Segner *et al.*, 1994; Alvarez *et al.*, 2000), glucogenesis (Walton and Cowey, 1979; Knox *et al.*, 1980), hormonal responses (Segner *et al.*, 1994), and the study of toxicity and cancer research (Grieco *et al.*, 1978; Baksi and Frazier, 1990; Pesonen and Andersson, 1991; Sadar and Andersson, 2001). For some studies over a period of a few hours, like lipogenesis, hepatocyte suspensions provide a suitable system essentially because of their simplicity. An advantage of using cell suspensions is that they are convenient for sampling frequently from uniform population of cells. Furthermore, in the case of hepatocytes, suspension cultures provide a metabolic system that is thought to most resemble the intact liver (Berry *et al.*, 1991) and meaningful metabolic results are obtained over periods of up to 12 hours of incubation (Geelen, 1994; Segner *et al.*, 1994). Based on earlier work on fatty acid metabolism in salmonid hepatocytes, it was anticipated that incubation periods of between 1-3 hours were all that would be required for the study of fatty acid metabolism in enterocytes and so cell suspensions were adopted as the cell preparation of choice.

The establishment of an appropriate procedure of cell isolation and culture medium depends on the precise phenomena under investigation (Berry *et al.*, 1991). The present study required fresh cell suspensions for immediate use at the fish farm and so required an enterocyte isolation technique that would allow the preparation of at least a dozen separate cell suspensions (based on number of dietary treatments and tank replicates), and within a reasonable time frame.

A method for the sequential isolation of populations of viable enterocytes from the mammalian small intestine utilising enzymes and chelating agents was developed (Weiser, 1973), and then improved by the use of hyperosmolar, intracellular-like isolation media (Del Castillo, 1987). Recently, this method has been further modified to obtain enterocytes from the marine fish, the gilthead seabream *Sparus aurata* (Dópido *et al.*, 2004). The technique involves a carefully dissection of the fish to extract the whole gut or segments of it. Each segment is filled and incubated for 10 min with a hyperosmolar solution and clamped at both ends with forceps. Afterwards, the intestinal solution is discarded and intestinal sections are filled and incubated again for an additional 3 min with a disjunctive solution. During this time intestinal segments have to be gently palpated with the fingers. The luminal solution containing cells loosened from the epithelia is filtered through a mesh. Intestinal sections are then filled again with the disjunctive solution for 3 more minutes and the resultant cell suspension pooled together and centrifuged at 10 min. The resulting cell pellet obtained is resuspended in a Ringer-type solution with collagenase and incubated with shaking for 15 minutes. Then, the cell suspensions are filtered and centrifuged again for 10 min. The technique is a rather gentle method that is capable of providing relatively small amounts of good quality, high viability cell cultures/suspensions and can be adapted to a range of modifications. However, the technique is clearly highly labour-intensive, time-

consuming and does not have the possibility of producing the number of samples in a reasonable time scale required for the present research project. Furthermore, when working with fish under commercial conditions at fish farms far removed (over four hours) from a well-established laboratory, these techniques are especially difficult to perform.

Therefore, an evaluation of the essential characteristics required to develop a suitable isolation technique for the preparation of enterocytes suspensions was made, mainly taking into account the knowledge already documented in the literature for isolating hepatocytes. For instance, previous reports described that the most appropriate media for perfusing liver to obtain hepatocytes are those that mimic closely the plasma composition of the animal in question, so that the user should obtain the maximum yield of viable hepatocytes while minimizing biochemical changes in the cells and compromising the purity of the suspensions (Baksi and Frazier, 1990). The use of balanced salt solution buffered, with HEPES for instance, is known to be appropriate for fish systems where the pH of fish plasma *in vivo* is higher than in mammals (Moon *et al.*, 1985; Baksi and Frazier, 1990). Enzymatic disaggregation using microbial collagenase (Type IV) (*Clostridium* sp) has been shown to be the simplest and most efficient procedure to obtain isolated viable cells from intact livers of mammals (Berry *et al.*, 1991; Freshney, 1992) and fish (Moon *et al.*, 1985; Baksi and Frazier, 1990). In experiments using hepatocytes suspensions, where the incubation period does not extend beyond 24 hours, a simple culture medium such as Krebs-Henseleit physiological saline or Medium 199 with low amino acid and glucose contents is satisfactory to meet the cell's minimum requirements (Wolf and Quimby, 1969; Berry *et al.*, 1991).

Hepatocyte preparations, incorporating all the above techniques and so isolated using collagenase perfusion of intact livers and maintained in Medium 199 had been used previously within this laboratory (Tocher *et al.*, 1997, 2000). However the perfusion methods for the preparation of isolated hepatocytes are also prolonged, time-consuming, labour-intensive and complicated techniques, unable to be replicated at fish farms to produce sufficient numbers of replicates in a time-limited schedule. Therefore a much simpler method for the preparation of isolated hepatocytes, involving direct incubation of chopped tissue in a buffered, balanced salt solution containing collagenase, was developed specifically for use in the field such as at fish farms and on cages. Therefore, it was logical and sensible that the first method to be investigated for the preparation of isolated enterocytes was a modification of this hepatocyte procedure devised for field-work (Bell *et al.*, 1997; Tocher *et al.*, 1997). Although the field studies would be carried out with salmon, mainly in seawater, refurbishment of the seawater aquarium at the University of Stirling meant that salmon and seawater were not available at the start of this project. However, because of its similarity to Atlantic salmon and the amount of information available relative to fatty acid metabolic activity (mainly in hepatocytes), rainbow trout was selected for the development of the laboratory-based methodology and characterization experiments.

It is known that every region of the gut has a different digestive and absorptive capacity (Collie, 1985; Olsen and Ringo, 1997, Røsjø *et al.*, 2000; Denstadli *et al.*, 2004). Based on the results of *in vivo* stable isotope studies, Bell et al. (2003c), suggested that pyloric caeca was a region of substantial HUFA (specifically DHA) synthesis in rainbow trout. However, it was unclear the extent to which other areas of the intestinal tract could contribute to HUFA synthesis. Therefore, it was also a

prerequisite of the present study to investigate the HUFA synthetic capacity of different regions of the gut *in vitro*, to determine the region of choice for all comparative studies.

3.1.2. Characterisation of the HUFA synthesis (fatty acid desaturation/elongation) assay in rainbow trout enterocytes.

As well as developing a new technique for the preparation of salmonid enterocytes, and characterizing the isolated cell suspensions, it was also necessary to determine appropriate assay conditions and thus establish a standard protocol under which to perform all the comparative experiments. Obviously, as the salmonid enterocyte preparation adopted was completely new, this study was the first time that fatty acid metabolism was determined in these suspensions, and so it was necessary to test and observe the performance of cells under a range of conditions. This would provide the information required to make a judgment on the best conditions under which to perform the comparative studies from fish in feeding trials. Moreover, the assessment of changes of metabolic activity in enterocytes under different incubation characteristics plays an important role in attempting to "optimise" the assay conditions for the same purposes.

For the present study it was decided that it was important to determine the extent of possible competition between the HUFA synthesis (desaturation/elongation) and β-oxidation pathways for dietary 18:3n-3 absorbed by enterocytes. Thus, it was of interest to adopt an assay method in which it was possible to evaluate and compare simultaneously the desaturation/elongation activity with β-oxidation activity in cells in the same incubation and under precisely the same conditions. The analysis of β-oxidation has preferentially employed subcellular extracts such as cell homogenates or

mitochondrial preparations using fatty acyl-CoA substrates and supplemental cofactors (Frøyland *et al.*, 1998, 2000; Nanton, 2003). However, methodology for the analysis of β-oxidation in hepatocytes was developed in mammals (Frøyland *et al.*, 1995; Halvorsen *et al.*, 2001), and was soon modified and applied successfully to evaluate the oxidation capacity of hepatocytes from fish including salmonids (Frøyland *et al.*, 1998, 2000; Nanton, 2003). Thus methodology for the assay of β-oxidation in intact cells was available. However, it is not necessarily possible to optimise combined assays measuring activities in two pathways in the traditional sense. The pathway of primary interest in the project was HUFA synthesis and so these characterisation experiments focused on the effects of different assay conditions on that pathway.

A further complication is that HUFA synthesis in any organism, fish included, occurs as a biochemical pathway (Buzzi *et al.*, 1996, 1997; Sargent *et al.*, 2002; Tocher, 2003). That is, it is a series of discrete enzymatic steps arranged in a pathway. Thus, it is not possible to optimise an assay for a multi-step pathway like the desaturation/elongation pathway in the same way as assays involving a conventional single step enzymatic reaction. In this respect, the assay can be modified to suit the particular aims of the research work, but not, unfortunately, without compromising to some extent some other aspect of the assay. For example, the time of incubation of a cell suspension with a fatty acid substrate can be varied depending upon requirements. A short incubation time will give the most appropriate results if the influence of early steps in the pathway, such as Δ6 desaturase activity and the synthesis of its immediate products, is what the research is focusing on. Activity rates for the whole pathway will be relatively high but DHA synthesis will be relatively low. A longer time course should be chosen if significant production of DHA is the aim of the research work, but

activity rates for the whole pathway will be reduced due to the longer time-course. An intermediate incubation time will perhaps give the best indication of the whole pathway.

As described above, the methodology to be investigated for the isolation of enterocytes was, at least in the first instance, to be based on methods developed initially for the preparation of hepatocytes outwith the laboratory at fish farms. Hepatocytes prepared in this way have been used to successfully determine HUFA synthesis. Therefore, as with the cell preparation, it was logical, in the first instance, to test the assay in enterocytes under similar conditions to those used for the hepatocytes. Ideally, it was hoped that it would be possible to use near identical conditions to those used with the hepatocytes, as this would enable a meaningful comparison of activities in the two cell types. In order to use identical assay conditions in enterocytes to those used with hepatocytes, it was necessary to determine the effects of different assay conditions to enable a proper evaluation of any limitations that this may impose upon the assay in enterocytes. The primary assay conditions investigated included a time course of the incubation, protein and substrate concentrations, and incubation temperature. In addition to these external environmental factors that can influence the measured activity, the effects of the status of the fish were also examined, specifically the effects of feeding and starvation, and the size of fish was investigated as this had been shown to affect activities in the earlier *in vivo* experiments (Bell *et al.*, 2003c).

3.1.3. Aims.

In summary, the specific aims of this chapter were i) to develop and adopt an enterocyte isolation technique that could be used easily at fish farms in order to work with fresh cells from fish coming directly from the sea cages. Moreover, the application

of the technique should be relatively rapid to enable the preparation of at least 12 cells suspensions per day in order to assay the appropriate number of replicates of the different dietary treatments; ii) to determine the ideal intestinal tissue, primarily in terms of HUFA synthesis capacity, for the routine preparation of enterocytes and all comparative studies (gut regions); iii) to characterise the preferred isolated enterocyte preparation to establish the quality of the suspensions in terms of cell purity (percentage of enterocytes compared to other cell types), cell viability (percentage of living cells) and stability (viability and survival over experimental time course); iv) to determine how the performance of the HUFA synthesis assay in the preferred enterocyte preparation varies with a range of parameters and v) to decide upon and adopt a standardized assay protocol for use in all comparative studies that offers the best compromise for the simultaneous assay of HUFA synthesis and β-oxidation in the enterocytes, and the opportunity to compare meaningfully with data already existing and to be collected on fatty acid metabolism in hepatocytes.

3.2. Materials and methods.

3.2.1. Development and characterization of isolated enterocyte preparations.

Trypan Blue exclusion

In order to have reliable data of the viability of the cell suspensions, the dye exclusion method with Trypan Blue was carried out. The technique is based in the concept that viable cells do not take up Trypan Blue whereas damaged and non-viable cells are permeable to this dye and therefore turn a blue colour (Buzzi, 1996). After preparation of cells in Medium 199, 50 µl of the cell suspension was transferred into a 1

ml microcentrifuge tube containing 0.45 ml of 0.4 % (w/v) Trypan Blue in HBSS. The cell solution was gently but thoroughly mixed and allowed to stand for 10 min.

A small volume (~50 µl) of Trypan Blue-cell suspension was removed by automatic pipette (100 µl plastic tip) and transferred carefully by touching the edge of the cover-slip of a haemocytometer with the pipette tip allowing each chamber to fill by capillary action. Both viable (non-stained) and non-viable cells (stained in blue) were counted to determine the viability and total number of viable cells/ml suspension.

Viable cells/ml suspension = Average count per square * dilution factor * 10^4 (10^4 represent each square of the haemocytometer with the total volume of 0.1 mm^3 or 10^{-4} cm^2).

Cell viability (%) = [Total viable cells (unstained) ÷ total cells (Σ stained and non-stained)] * 100

To determine the time course of viability, cell suspensions were distributed into 25 cm^2 tissue culture flasks immediately after isolation and incubated at 20 °C during the course of the experiment. At the sampling times of 1, 3, 6 and 10 h after initial isolation, cells were dislodged from the tissue culture flasks by gentle rocking and removed into a test tube with a plastic pastette. Samples of cells were then taken for counting as described above.

Oxygen consumption

Oxygen consumption was measured on freshly isolated cells of rainbow trout in a closed system using a Clark electrode connected to a computerized data acquisition system and processed with a specialized software (RESPI software Jeulin SAV, France). Before determination of O_2 consumption of the enterocyte preparations, a sample of O_2-saturated DMEM (Dulbeco´s modified Eagle medium) was measured to obtain the maximum O_2 concentration value. The minimum value was obtained by adding 100 mg of sodium dithionite to the sample. During the whole procedure the sample was under magnetic agitation and hermetically closed to avoid any contact with air.

Once the system was calibrated, six enterocyte enriched preparation samples were measured. Approximately 350 $x10^6$ of isolated cells, resuspended in 3 ml of medium, were added to the open incubation chamber and maintained at 20 °C. After a 5 min stabilization period, O_2 concentrations were linear with time. The systems were then closed and O_2 concentrations recorded for 10 - 15 min. The rate of O_2 consumption (R_{Oxygen}) was computed as the slope of the linear relationship in the plots of [O_2] vs. time, and was expressed in nmol O_2/mg protein/hr.

Lactate dehydrogenase (LDH) activities

Lactate dehydrogenase (LDH) activities of isolated enterocytes were measured spectrophotometrically by means of enzymatic reactions coupled to NADH oxidation using a commercial assay kit (Sigma Kit DG1340-K).

Lactate dehydrogenase catalyses the interconversion of lactate and pyruvate. Procedures for determination of LDH activity are based on either the oxidation of lactate to pyruvate or on the reduction of pyruvate to lactate.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{L-Lactate} + \text{NAD}^+$$

The Sigma kit used to determine LDH activity in enterocytes of rainbow trout is an optimised method based on the following simple principle: During reduction of pyruvate an equimolar amount of NADH is oxidised to NAD. As NADH absorbs at 340 nm and NAD does not, the oxidation of NADH results in a decrease in the absorbance at 340 nm. The rate of decrease in absorbance at 340 nm is directly proportional to lactate dehydrogenase activity in the sample.

The LDH reagents contained in the kit used for this analysis had the following concentrations of active ingredients (Sigma Kit DG1340-K): LDH reagent A (buffer/coenzyme) = 0.194 mmol/L NADH and 54 mmol/L phosphate buffer (pH 7.5). LDH reagent B (pyruvate) = 16.2 mmol/L pyruvate and nonreactive stabilizers and fillers. A sample start reagent (SSR) is also prepared by adding 0.4 ml LDH reagent B to 10 ml of LDH reagent A.

For the LDH analysis procedure, 2.5 ml of SSR was added to a spectrophotometer cuvette before adding 0.1 ml of sample, mixed immediately by inversion and incubated for 30 seconds. Absorbance at 340 nm was recorded versus water as reference and a stopwatch was started at the same time. A record of absorbance at exactly 1, 2 and 3 min was performed following the initial absorbance

reading. The mean absorbance change per minute (A/min) was determined and the LDH activity was calculated by following the following equation:

$$\text{LDH activity (U/L)} = \frac{\Delta A \text{ per min} \times TV \times 1000}{6.22 \times LP \times SV}$$

Where:

ΔA per min = change in absorbance per minute at 340nm

TV = Total volume (ml)

SV = Sample volume (ml)

6.22 = Millimolar absorption (Extinction coefficient) of NADH at 340 nm

LP = Light path (1cm)

1000 = Conversion of units per ml to units per litre

So,

$$\text{LDH activity (U/L)} = \frac{\Delta A \text{ per minute} \times 2.6 \times 1000}{6.22 \times 1.0 \times 0.1}$$

$$\text{LDH activity (U/L)} = \Delta A \text{ per minute} \times 4180$$

Intracellular LDH activity assays were performed on the enterocyte cell pellet after homogenisation in 100 μl sucrose buffer (SB) containing 50 mM sucrose, 20 mM

Tris, 1 mM Na_2EDTA and 1 mM Phenylmethanesulfonyl fluoride Solution (PMSF) (pH = 7.4). Cell integrity using the LDH assay was determined by measuring extracellular and intracellular LDH activities. Extracellular LDH activity originates from damaged and non-viable cells and so the ratio of intracellular to extracellular activity is a measure of cell integrity of the suspension. Samples of 100 μl of enterocytes in DMEM suspensions in microcentrifuge tubes were centrifuged. The supernatant was removed and extracellular LDH activities were determined as described above.

3.2.2. Characterisation of the HUFA synthesis (fatty acid desaturation/elongation) assay in rainbow trout enterocytes.

a) Gut regions

In order to determine the relative activity of different regions of the intestinal tract of salmonids in terms of HUFA synthesis (desaturation/elongation) activity, the intestine of rainbow trout was divided into six different regions: oesophagus, stomach, pyloric caeca, anterior gut, posterior gut, rectum (Fig. 1.6). Sections of each of these regions were taken and cleaned of adhering adipose tissue and luminal contents, and isolated enterocytes prepared as described in section 2.2. The enterocytes suspensions from the aforementioned regions were assayed for HUFA synthesis activity by incubation with 50 µl [1-^{14}C]18:3n-3 for 2 hr as described in sections 2.4 and 2.5. The guts of three fish were used for each of these experiments that were performed three times on three different days in order to have replicates for statistical analysis (n = 9).

b) Time course

An experiment to assess the capacity of enterocytes to desaturate/elongate [1-^{14}C]18:3n-3 over a time course was prepared. Enterocytes from pyloric caeca from eight fish were prepared as described in section 2.2 and mixed into a single large pool. The cells were distributed in 5 ml aliquots into a series of 25 cm^2 tissue culture flasks and incubated with 50 µl [1-^{14}C]18:3n-3 as described in section 2.4 except that incubation time varied. Thus, replicate flasks of cells were harvested after 1, 2, 3, 6, 12 and 24 h and HUFA synthesis activity determined as described in section 2.5. Three replicates per treatment were performed in every experiment. The experiment was repeated twice on two different days in order to have a number of replicates for statistical analysis (n = 6).

c) Incubation temperature

Enterocytes from pyloric caeca from eight fish were prepared as described in section 2.2 and mixed into a single large pool. The cell suspension was then divided by 12 in order to have three replicates of four treatment (temperatures). The cells were distributed in 5 ml aliquots into a series of 25 cm^2 tissue culture flasks and incubated with 50 µl [1-^{14}C]18:3n-3 as described in section 2.4 except that incubation temperature varied. Temperature selection was made based on the available incubators in the Cell Culture Laboratory in the Institute of Aquaculture, seeking to have a wide range of temperatures in the 10 to 30 °C range. Thus, flasks were placed in cell culture incubators at 10, 15, 22 and 28 °C and incubation continued for 2 h after which the cells were harvested and HUFA synthesis determined as described in section 2.5. As

indicated above, three replicates per treatment were performed in every experiment. The experiment was repeated twice on two different days in order to have a number of replicates for statistical analysis (n = 6).

d) Protein concentration

With the aim of identifying how the rate of HUFA synthesis varied with different amounts of cells (as determined by protein content of assay) as in different enterocytes preparations, the following experiments were carried out. Enterocytes from pyloric caeca from 10 fish were prepared as described in section 2.2 and pooled into a single large "stock" of cells. Three dilutions (1/2, 1/4, 1/8) of the "stock" enterocyte enriched preparation were made to obtain four different concentrations of enterocytes (and protein). The cells from the stock and diluted preparations were distributed in 5 ml aliquots into a series of 25 cm^2 tissue culture flasks so that the protein contents in the flasks varied (45, 22.5, 11.3 and 5.6 mg per flask). The flasks were incubated with 50 µl [1-^{14}C]18:3n-3 for 2 h at 20 °C as described in section 2.4, and HUFA synthesis activity determined as described in section 2.5. Two replicates per treatment were performed in every experiment. The experiment was repeated twice on two different days in order to have a number of replicates for statistical analysis (n = 4).

e) Substrate concentration

To identify how HUFA synthesis activity varied with concentration of the fatty acid substrate, the following experiments were carried out. Batches of unlabelled

18:3n-3 fatty acid were prepared at three different concentrations (1.0 mM, 2.5 mM and 5 mM) and combined with the labelled [1-^{14}C]18:3n-3 as described in Table 3.1 to produce four different fatty acid substrates for addition to the cells that contained equal radioactivity (DPM) but at four fatty acid concentrations. Enterocytes from pyloric caeca from 8 fish were prepared as described in section 2.2 and mixed into a single large pool of cells. The enterocyte preparation was distributed in 5 ml aliquots into a series of 25 cm^2 tissue culture flasks and 50 µl of the [1-^{14}C]18:3n-3 substrates with the four different fatty acid concentrations (final fatty acid concentrations of 2, 22, 52 and 102 µM) added and incubation continued for 2 h at 20 °C, after which the cells were harvested and HUFA synthesis determined as described in section 2.5. Two replicates per treatment were performed in every experiment. The experiment was repeated three times on different days in order to have a number of replicates for statistical analysis (n = 6).

Table 3.1 Formulation and concentrations of fatty acid substrates used in the desaturation/elongation analysis.

N°	FAF-BSA + medium (µl)	Unlabelled substrate 18:3n-3 (µl)	Labelled substrate [1-^{14}C]18:3n-3 (µl)	Concentration of 18:3n-3 in assay (µM)
1	100	-	100	~ 2
2	-	100 of 1.0 mM	100	~ 22
3	-	100 of 2.5 mM	100	~ 52
4	-	100 of 5.0 mM	100	~ 102

FAF-BSA = Fatty acid-free bovine serum albumin.

f) Feeding regime

A group of rainbow trout were separated from the stock fish and placed in a separate tank. After acclimatization for a week, these fish were starved for 5 days while the stock fish continued to be fed on the commercial pelleted diet at the normal rate. After the starvation period, starved and fed fish were sacrificed and enterocytes from pyloric caeca from six fish per treatment were prepared as individual cell preparations as described in section 2.2. Six ml of cell suspension from each fish was placed in a 25 cm^2 tissue culture flasks and incubated with 60µl [1-^{14}C]18:3n-3 for 2 h at 20 °C and HUFA synthesis activity determined as described in sections 2.4 and 2.5. Six replicates per treatment were performed in the experiment (n = 6).

g) Simultaneous measurement of HUFA synthesis and β-oxidation in combined assay

In the previous experiment (subsection f), 1 ml of the enterocyte cell suspension after incubation with [1-^{14}C]18:3n-3 was used to estimate β-oxidation activity through determination of acid soluble products as described in section 2.6.

h) Comparison of HUFA synthesis and β-oxidation activities in enterocytes and hepatocytes

In the experiment described above in the two previous subsections (f and g), parallel incubations and assays were performed in isolated hepatocytes. Therefore, hepatocytes from starved and fed fish were prepared from six fish per treatment as

individual cell preparations as described in section 2.2. Six ml of hepatocyte cell suspension from each fish were placed in 25 cm^2 tissue culture flasks and incubated with 60 µl [1-^{14}C]18:3n-3 for 2 h at 20 °C and HUFA synthesis and β-oxidation activities determined as described in sections 2.4 and 2.6.

i) Age/Size of fish

A dozen rainbow trout of about 50 g and a dozen of 150 g were obtained from a local hatchery and transferred to the aquarium facilities of the Institute of Aquaculture. Fish were maintained under exactly the same conditions as described in section 2.1. After a 1 week acclimatization period, six fish per group were sacrificed and enterocytes from pyloric caeca prepared as individual cell preparations as described in section 2.2. The 5 ml cell preparation from each fish was placed in a 25 cm^2 tissue culture flask and incubated with 50 µl [1-^{14}C]18:3n-3 for 2 h at 20 °C and HUFA synthesis activity determined as described in sections 2.4 and 2.5. The experiment was designed to obtain an adequate number of replicas for statistical analysis (n = 6).

3.2.3. Statistical analysis

All results are means of the experimental replicates ± standard deviation (n as reported above). Significant of differences between means in the Trypan blue exclusions were determined by Kruskall-Wallis analysis and in the rest of the subsections the differences were determined by one-way ANOVA with Tukey post test ($P < 0.05$).

3.3. Results

The selection of the technique for the isolation of fish enterocytes was the basis of the rest of the research plan in the present work. In order to be able to carry out the assays required to elucidate the metabolic activity of enterocytes, the first technique tested was a modification of the method employed previously for hepatocyte isolation (Bell *et al.* 1997, 2001a, 2002; Tocher *et al.* 1997, 2000, 2001). This method, as described in detail in section 2.2, was successful; so the method was adopted as standard and all the characterization studies carried out in the present work were performed using the isolation methodology as described in section 2.2.

3.3.1. Development and characterization of isolated enterocyte preparations.

Trypan Blue exclusion

The results of the dye exclusion viability assay performed on pyloric caecal enterocytes over a 10 h time course showed that after 6 h of incubation, under the conditions mentioned above, the cell preparation still retained over 80 % viability (Figure 3.1). In contrast, less than 40 % of cells were viable after 8 h and < 20 % at the 10 h time point. It is particularly important to note that an average of around 90 % of cells remained viable after 2 h, the incubation period selected for the HUFA synthesis assay (Figure 3.1).

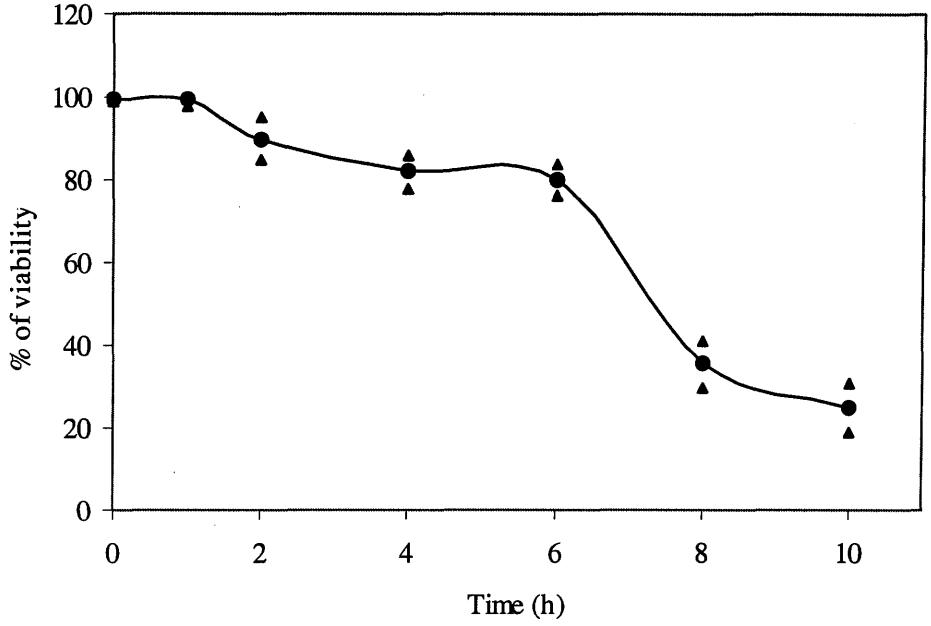


Figure 3.1. Average viability of *Oncorhynchus mykiss* pyloric caecal enterocytes during a 10 h time course incubation as determined by the Trypan blue exclusion test. Results are presented as percentage of cell viability. Circles represent the mean value. Small triangles represent maximum and minimum value of all samplings (n = 4).

In addition to assessing cell viability during the Trypan blue exclusion assay, the purity of the cell preparation was also determined by counting the number of non-enterocyte cells in the preparations. The data showed that the preparation was predominantly enterocytes and so, although there were secretory cells present, these constituted only 5-10 % of the total cell number and so the preparations can be accurately defined as "enterocyte-rich".

Oxygen consumption and LDH activities

The data from the Trypan blue exclusion assay were in good agreement with data obtained from other evaluations of cell viability used in characterising the cell

suspensions. The enterocyte enriched preparations submitted to the oxygen consumption assay showed a high oxygen intake of 313.8 nmol/mg protein/h after two hours of incubation (Table 3.2). The results of the intra- and extracellular lactate dehydrogenase (LDH) activity assay showed high levels of intracellular activity with less than 3 % of LDH activity found in the extracellular supernatant after 2 h of incubation of the cells. Thus, after the 2 h incubation period the ratio of intracellular/ extracellular LDH was over 45, which is in good agreement with the viability values aforementioned (Figure 3.1; Table 3.2).

Table 3.2. Viability tests performed on pyloric caecal enterocytes of *Oncorhynchus mykiss* after 2 h incubation.

Type of test	mean	std dev
Dye exclusion 2 h course (% Trypan blue exclusion)	90.0 (4)	± 3.0
Oxygen consumption (nmol/mg protein/h)	313.8 (6)	± 76.9
Intracellular LDH (mU/mg protein)	470.1 (5)	± 78.5
Extracellular LDH (mU/mg protein)	10.4 (5)	± 0.9
Intracellular LDH (%)	97.8	
Extracellular LDH (%)	2.2	
RLDH (intracellular/extracellular)	45.2	

Results are expressed as means and standard deviations. Numbers in parentheses indicate sample sizes. LDH: Lactate dehydrogenase. RLDH: intracellular to extracellular LDH activity ratio.

3.3.2. Characterisation of the desaturation/elongation activity assay in rainbow trout enterocytes.

a) Gut regions

There were significant differences between the different regions of the rainbow trout gut in their the capacity for HUFA synthesis as determined by their ability to desaturate/elongate [1-^{14}C]18:3n-3. HUFA synthesis in the two most anterior regions of the gut, oesophagus and stomach, was insignificant with little more than 0.05 pmol of [1-^{14}C]18:3n-3/h/mg protein desaturated, whereas the third region, pyloric caeca, was the most active of the entire intestine with 1.20 pmol [1-^{14}C]18:3n-3/h/mg protein being desaturated (Figure 3.2). Relative rates of HUFA synthesis activity after the pyloric caecal region decreased (from 1.20 in pyloric caeca to 0.20 pmol/h/mg protein in rectum) showing a proximal-distal gradient in the following regions of the whole intestine (Figure 3.2).

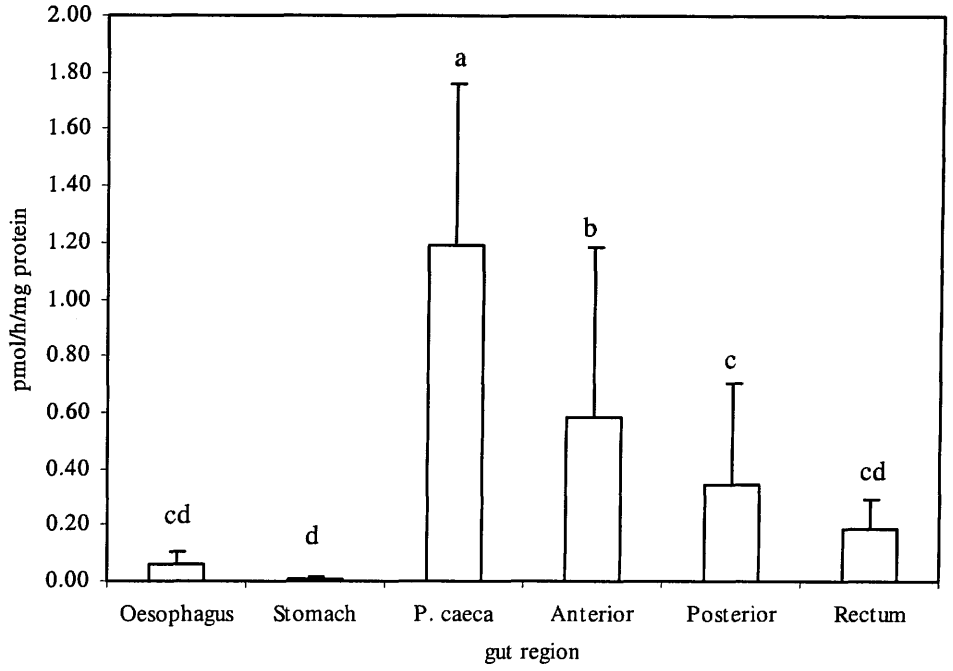


Figure 3.2. Total fatty acid desaturation/elongation activity in enterocytes of six different regions of *Oncorhynchus mykiss* gut. Results are means ± SD (n = 9) and represent the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Mean values with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post-test ($P < 0.05$).

b) Time course

The effects of time of incubation on HUFA synthesis in enterocytes from pyloric caeca showed there was a significant decrease in specific activity in incubations of 6 h or longer (Figure 3.3). The highest activities were observed after the 1, 2 and 3 h time point (3.33, 3.35 and 3.64 pmol/h/mg protein respectively) with no statistical differences between them. Based on the viability test it is possible to assume that the lower specific activities found at the 6, 12 and 24 h time points (0.98, 0.64 and 0.58

pmol/h/mg protein respectively) are caused by a decrease in cell survival (Figures 3.1 and 3.3).

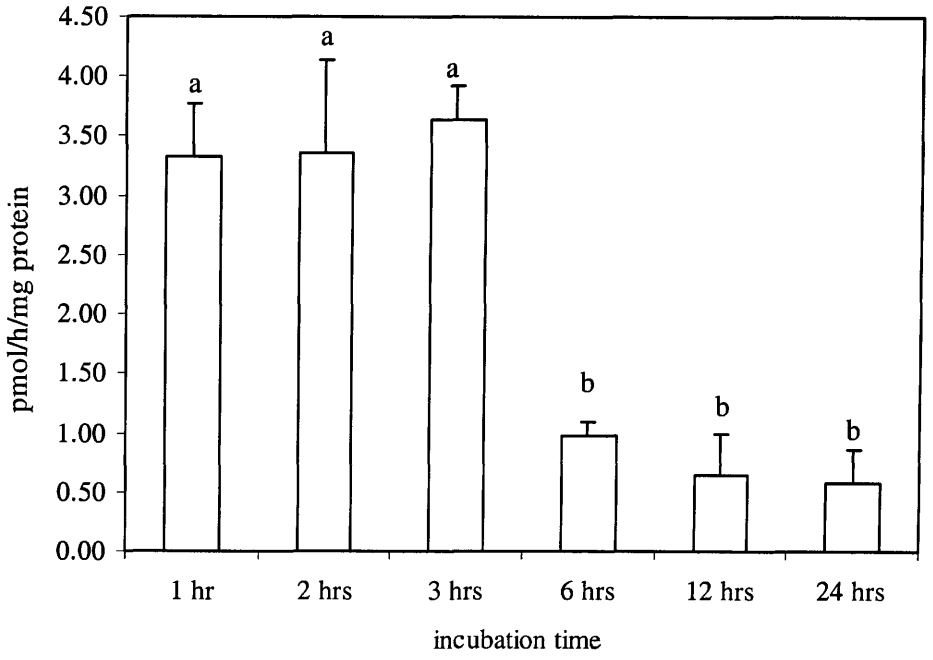


Figure 3.3. Total fatty acid desaturation/elongation activity in enterocytes of *Oncorhynchus mykiss* incubated at different times. Results are means ± SD (n = 6) and represent the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Mean values with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post-test ($P < 0.05$).

c) Temperatures

There were significant differences between HUFA synthesis activities of pyloric caecal enterocytes incubated at the four temperatures (Figure 3.4). The results show that there is a high negative correlation between temperature and the amount of [1-^{14}C]18:3n-3 metabolised (r = -0.998). The highest specific activity was obtained with cells incubated at the lowest temperature (10 °C) (2.61 pmol/h/mg protein); in contrast,

the lowest activity (0.82 pmol/h/mg protein) was obtained with cells incubated at the highest temperature tested (28 °C) (Figure 3.4).

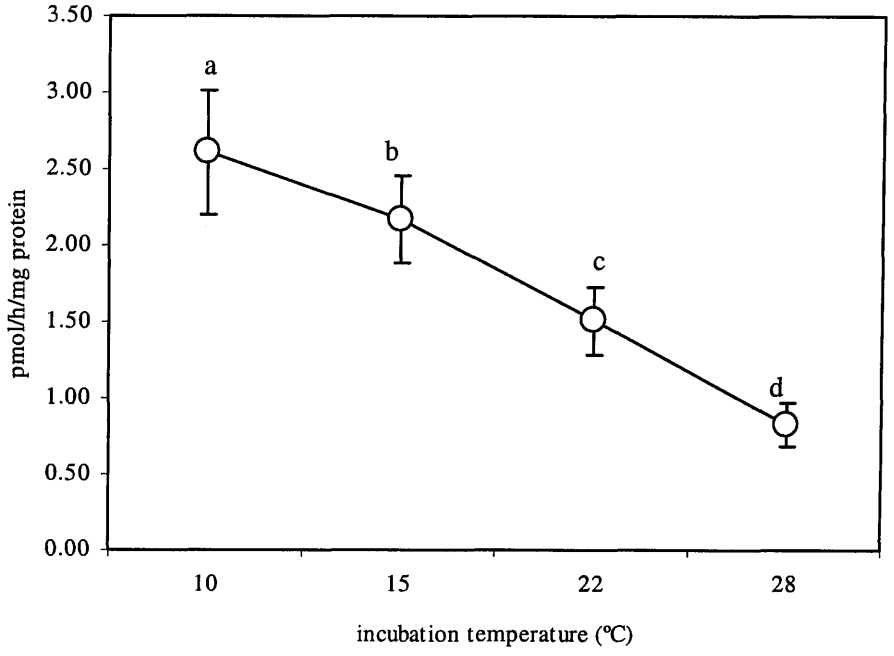


Figure 3.4. Total fatty acid desaturation/elongation activity in enterocytes of *Oncorhynchus mykiss* incubated at different temperatures. Results are means ± SD (n=3) and represent the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Mean values with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post-test ($P<0.05$).

d) Protein concentration

The specific activity of the HUFA synthesis pathway was higher in assays with lower protein concentration as an indicator of the importance of the number of cells in the assays (Figure 3.5). There were no significant differences in HUFA synthesis activity between the two treatments with the lower protein concentrations (11.3 and 5.6 mg protein). However there were significative differences between these assays and those with higher protein concentrations, with the treatment with the highest protein, 45

mg of protein per assay, having the lowest specific activity of all (1.78 pmol/h/mg protein) (Figure 3.5).

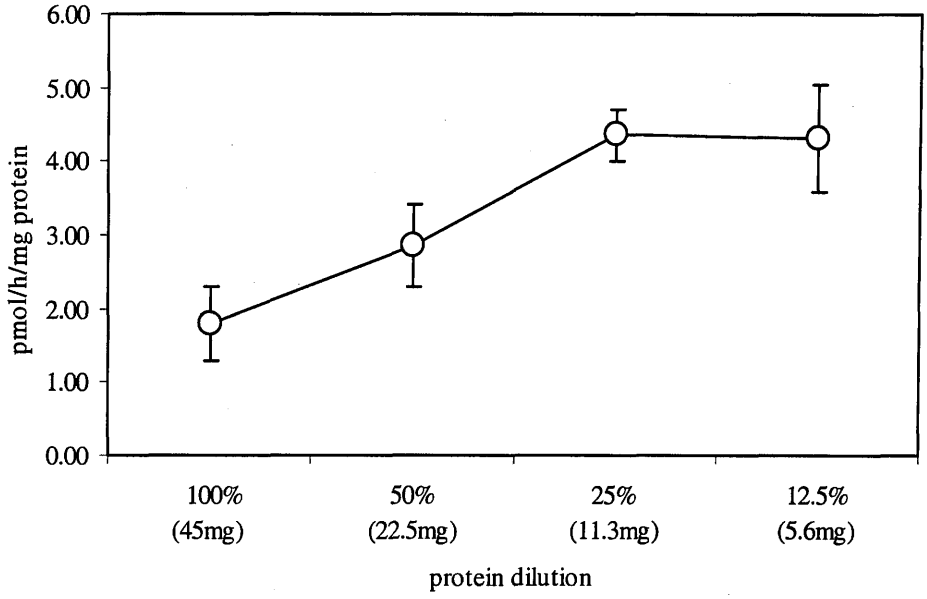


Figure 3.5. Total fatty acid desaturation/elongation activity in enterocytes of *Oncorhynchus mykiss* incubated at different protein concentrations. Results are means ± SD (n=3) and represent the rate of conversion (pmol/h/ mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Mean values with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post-test ($P<0.05$).

e) Substrate concentration

The specific activity of HUFA synthesis in enterocytes incubated with [1-^{14}C]18:3n-3 in substrates with different fatty acid concentrations showed quite high variation within treatments. There was no significant difference in activity in enterocytes incubated with fatty acid substrate at concentrations between ~22 and ~52 μM (2.46 and 2.25 pmol/h/mg protein, respectively), or between the lowest concentration, ~2μM, and the highest concentration ~102μM (1.82 and 2.05 pmol/h/mg

protein respectively). However, the activity was slightly, but significantly, higher in enterocytes incubated with the intermediate concentrations compared to the low and high concentrations (Figure 3.6). The individual products of desaturation/elongation of [1-^{14}C]18:3n-3 show that the steps required to form 20:5n-3, 22:5n-3 and 22:6n-3, occurred to a lesser degree than the first step of the pathway, the conversion to 18:4n-3. Ignoring 18:4n-3 in the desaturation/elongation pathway, the lowest concentration, ~2μM, gave the highest conversion to 20:5n-3, 22:5n-3 and 22:6n-3 (0.13, 0.04 and 0.08 pmol/h/mg protein, respectively) (Figure 3.7). The total desaturation activity ignoring 18:4n-3 step was also higher in cells incubated with the lower concentration of fatty acid in the substrate ~2 μM (0.26 pmol/h/mg protein) whereas the lowest activity was registered under the highest concentration ~102 μM (0.17 pmol/h/mg protein).

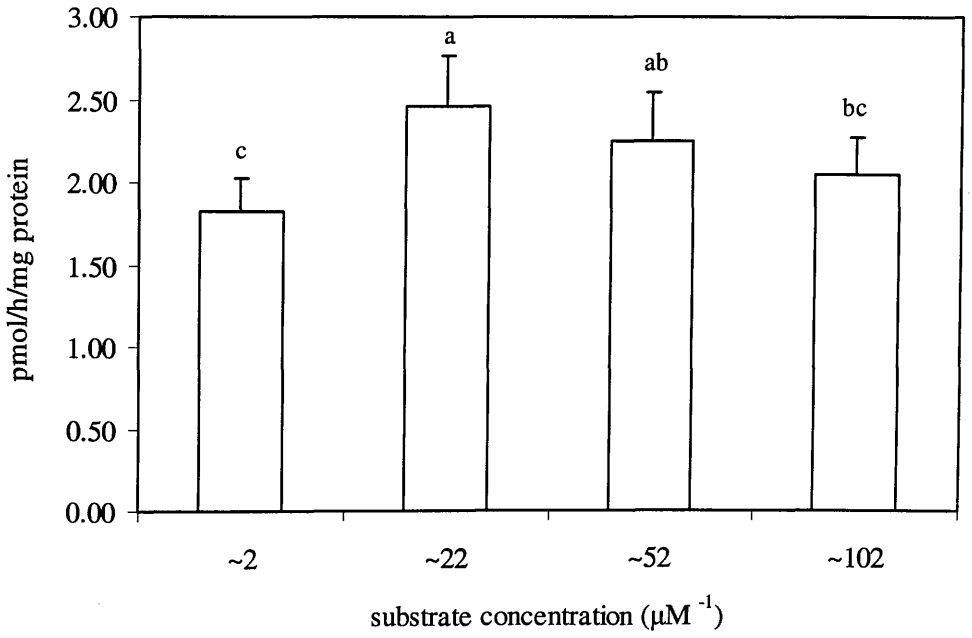


Figure 3.6. Total fatty acid desaturation/elongation activity in enterocytes of *Oncorhynchus mykiss* incubated with different fatty acid substrate concentrations. Results are means ± SD (n=9) and represent the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Mean values with different superscript letters between the same fatty acid in different treatments means significant differences determined by one-way ANOVA followed by the Tukey post-test ($P < 0.05$).

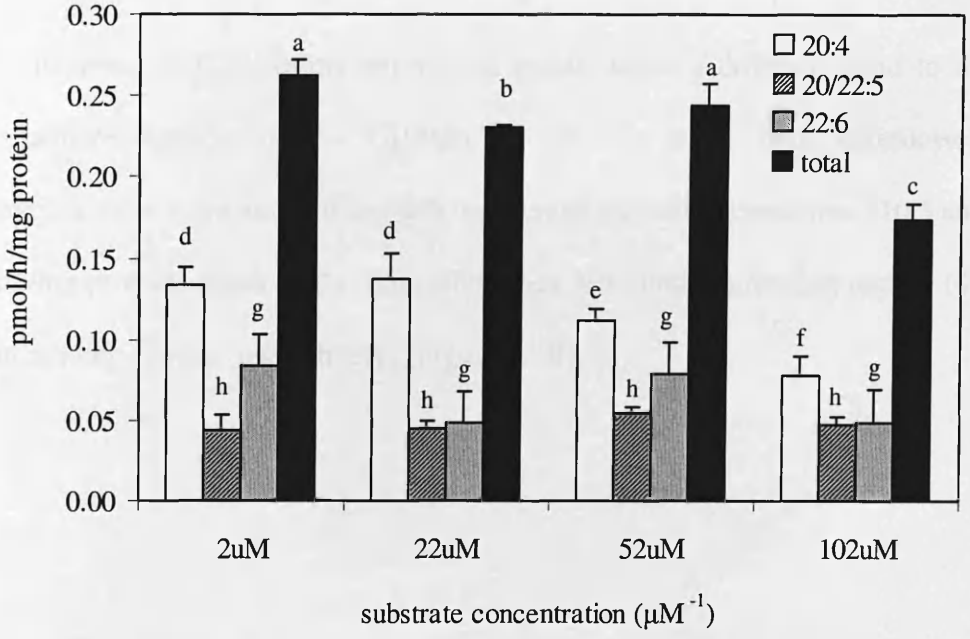
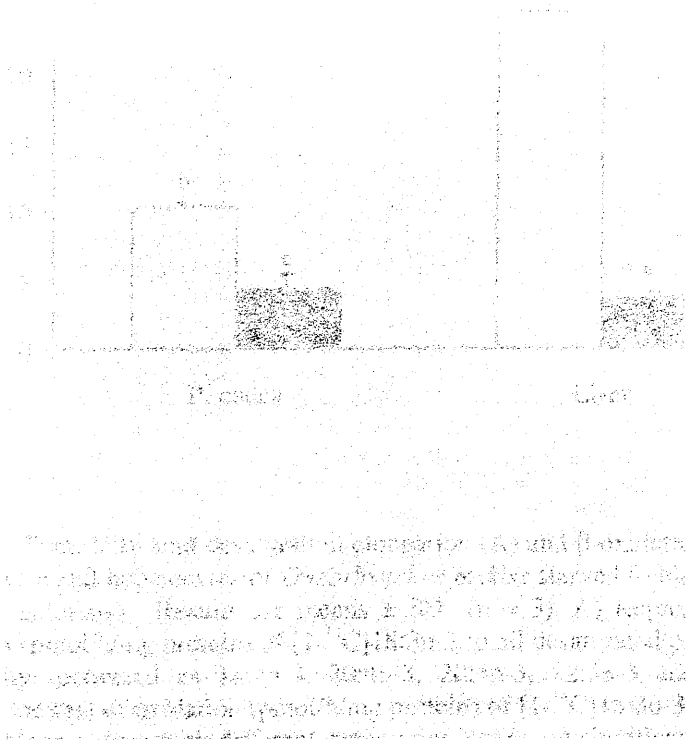


Figure 3.7. Individual products and total acyl desaturation/elongation activity in enterocytes of *Oncorhynchus mykiss* incubated with different fatty acid substrate concentrations. Results are means ± SD (n = 3) of one sampling point and represent the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3 and the sum of all products. Mean values with different superscript letters between the same fatty acid in different treatments means significant differences as determined by one-way ANOVA followed by the Tukey post-test ($P<0.05$).

f) Feeding regime, combined assay and comparison between enterocytes and hepatocytes

In pyloric caecal enterocytes, [1-^{14}C]18:3n-3 was desaturated/elongated to a greater extent when fish were under starvation conditions (4.5 pmol/h/mg protein) than under a feeding regime (2.6 pmol/h/mg protein). In contrast, hepatocyte preparations desaturated/elongated more [1-^{14}C]18:3n-3 when fish were under a feeding regime (1.8 pmol/h/mg protein), than under starvation conditions (0.9 pmol/h/mg protein) (Figure 3.8A).

In terms of β-oxidation activity the results show a different trend to that for desaturation/elongation of [1-^{14}C]18:3n-3. In this case, both enterocytes and hepatocytes were more active when fish were under starvation conditions (10.5 and 24.7 pmol/h/mg protein respectively) than when they were under a feeding regime (4.5 and 3.7 pmol/h/mg protein respectively) (Figure 3.8B).



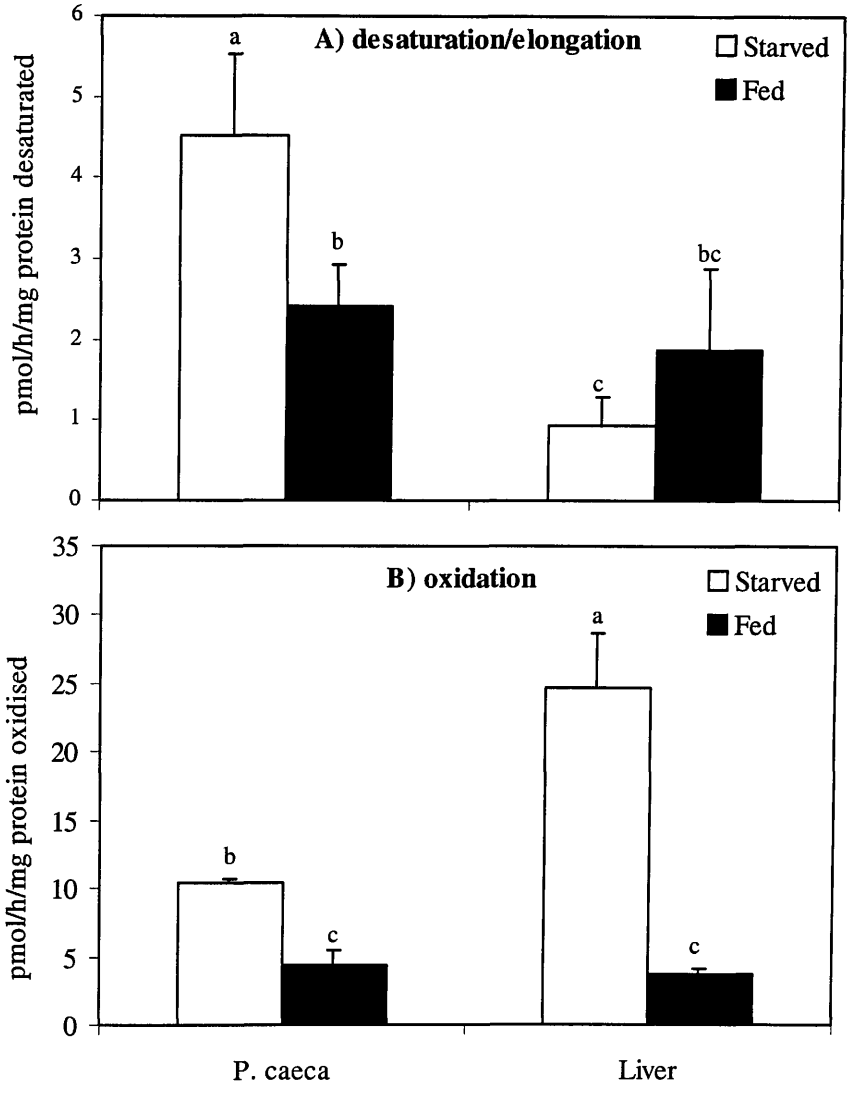


Figure 3.8. Total fatty acid desaturation/elongation (A) and β-oxidation (B) activities in enterocytes and hepatocytes of *Oncorhynchus mykiss* starved (white columns) and fed (black columns). Results are means ± SD (n = 3). A) Represent the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). B) Represent the rate of oxidation (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to acid-soluble products. Mean values with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post-test ($P < 0.05$).

g) Age/size of fish

The size of the fish reared under identical conditions was shown to have no effect on the desaturation/elongation activity of enterocytes. There were no statistical differences between the amount of HUFA synthetised by 50 g and 150 g fish (6.9 and 7.9 pmol/h/mg protein respectively) (Figure 3.9).

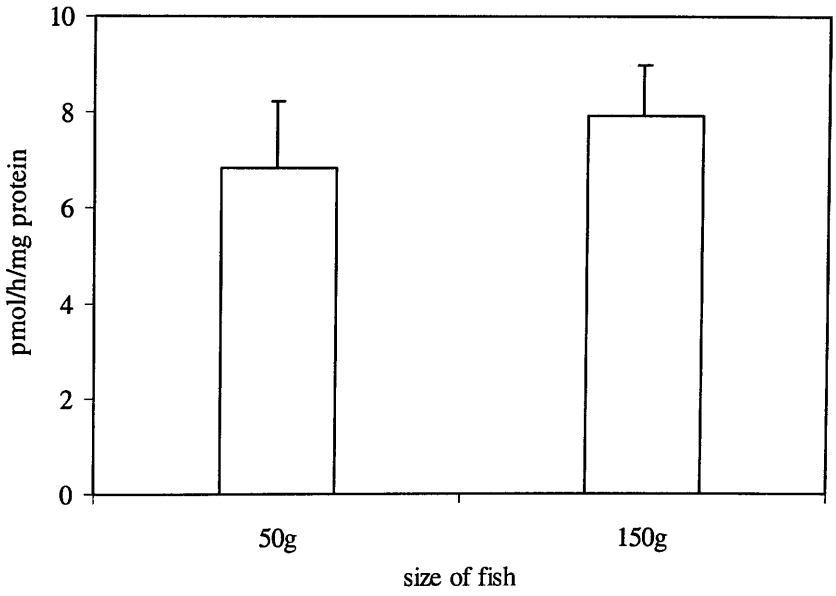


Figure 3.9 Total fatty acid desaturation/elongation activity in enterocytes of *Oncorhynchus mykiss*. Results are means ± SD (n = 3) and represent the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). There were no significant differences between means as determined by one-way ANOVA ($P < 0.05$).

3.4. Discussion

The overarching aim of this chapter was to develop and adopt an enterocyte isolation technique and HUFA metabolism assay that could both be used easily in the field at fish farms. The enterocytes isolation technique had to be relatively rapid to enable the preparation of at least a dozen cells suspensions per day in order to assay the appropriate number of replicates of the different dietary treatments. Techniques for the isolation of isolated enterocytes from mammals or fish were already reported in the literature but were all prolonged, time-consuming, labour-intensive and complicated (Weiser, 1973; Hegazy *et al.*, 1983; Del Castillo, 1987; Dópido *et al.*, 2004). A similar situation was encountered previously within the Nutrition Group when a simplified method for the preparation of isolated hepatocytes was required. The perfusion methods developed previously for the preparation of isolated hepatocytes were very successful, but were also prolonged, time-consuming, labour-intensive and complicated techniques that were unable to be replicated at fish farms to produce sufficient numbers of replicates in a time-limited schedule. Thus, a much simpler method for the preparation of isolated hepatocytes, involving direct incubation of chopped tissue in a buffered, balanced salt solution containing collagenase, was developed specifically for use in the field such as at fish farms and on cages (Bell *et al.*, 1997; Tocher *et al.*, 1997, 2000). It was explained in the introduction to the present chapter how it was logical that the first method to be investigated in order to find the most suitable technique for enterocyte isolation was a modification of this hepatocyte procedure devised for fieldwork (Bell *et al.*, 1997; Tocher *et al.*, 1997). The fact that this strategy immediately gave very satisfactory results was one of the great successes of this project

and had two very positive consequences. Firstly, it enabled an almost immediate start to the proper project without the need for time-consuming investigation of other possible isolation techniques; and secondly, that it enabled a more direct comparison to be made with hepatocytes, essentially prepared in exactly the same way, that could not have been done if different isolation techniques had to be developed and utilized. However, regardless of the success of the methodology, it was still necessary to perform some basic validation studies in order to characterise the nature of the isolated enterocytes preparation.

Observations made with the enterocyte preparations, obtained utilising this isolation technique, demonstrated that although there were secretory cells present, these constituted only < 10% of the total cell number. A roughly similar proportion of secretory cells was reported previously for enterocyte preparations of gilthead seabream (*Sparus aurata* L.) using a different isolation technique (< 5%) (Dopido *et al.*, 2004). This probably reflects the relative proportions of the two cell types in the intestinal tissue used rather than any significant enrichment. However, it clearly demonstrated that the rapid isolation technique resulted in a similar enterocyte-rich preparation to those achieved with the more laborious techniques, which is a factor that greatly facilitated the analysis of HUFA synthesis in enterocytes at fish farms.

The various cell viability tests performed on the caecal enterocyte preparations showed a very high degree of membrane integrity was maintained as indicated by the fact that approximately 90 % of cells excluded Trypan blue after two hours of incubation. Trypan blue exclusion by enterocytes was still high after six hours of incubation, and only significantly declined after this, which clearly showed that the fatty acid metabolism (HUFA synthesis and β-oxidation) assay, which was developed with a two hour incubation period was routinely performed with optimum cell viability

throughout that incubation period. It was possible to corroborate that enterocyte membrane integrity in the preparations was high because they also retained almost all of the lactate dehydrogenase (LDH) activity intracellularly, as judged from the intracellular/extracellular LDH ratio. The LDH ratio found in the present work indirectly suggested that more than 97 % of cells were alive and healthy after two hours of incubation.

In addition to the cell membrane integrity tests above, and based on the results of oxygen consumption rates, it can be assumed that the mitochondrial functional state of the isolated enterocytes was well-preserved during a two hour time course. Oxygen consumption rates were within the range of values published for isolated cardiomyocytes from sea raven and ocean pout (Legate *et al.*, 1998), and recently for seabream enterocytes (Dópido *et al.*, 2004). In the recent study characterizing seabream enterocytes, Dópido *et al.* (2004), performed a number of viability tests, including oxygen consumption and ATP concentrations, and emphasized that, whereas oxygen consumption rates were well below the values reported for intestinal epithelial cells isolated from endotherms (Brown and Sepúlveda, 1985; Del Castillo, 1987; Ferrer *et al.*, 1986), intracellular ATP concentrations were found to be markedly similar, reflecting the different metabolic efficiency between endo- and ectothermic animals. Unfortunately, cellular ATP concentrations in the isolated caecal enterocytes were not measured in the present study, but it is highly probable that the differences in oxygen consumption rates between those reported for mammals and the present study with rainbow trout were due to the higher metabolic efficiency related to ectothermic animals.

In summary, the viability tests performed to assess the cell isolation showed that the methodology used in the present work, which was based on the modified hepatocyte

isolation method, was suitable for the production of enterocyte-rich preparations that had good viability over a six-hour period and, in particular, reproducibly high and sustained viability in the initial two hours suggesting that it would be ideal during the time-course required for the HUFA synthesis assay.

In the second phase of this chapter the fatty acid metabolism assay was characterized, concentrating on the primary focus of this work, HUFA synthesis, to determine the appropriate conditions to be routinely applied and employed in the major feeding trials to be carried out at commercial fish farms investigating the effects of vegetable oil (VO) replacement in Atlantic salmon. However, in parallel with this aim, it was important to determine the relative importance of different regions of the intestinal tract and hence, following a logical order, the first aspect investigated was to determine the region of the fish gut with the highest activity in terms of HUFA synthesis. The results clearly demonstrated that pyloric caeca were the most active region of the intestinal tract, followed in order by anterior and posterior gut with the rectum having the least activity of all regions, revealing the existence of a proximal-distal gradient regarding fatty acyl desaturation/elongation activity of the intestinal tract. There are several previous studies that have shown the importance of pyloric caeca as a major digestive and enzymatic system in fish (Buddington and Diamond, 1986, 1987; Denstadli *et al.*, 2004; Dopido *et al.*, 2004), but there were not available reports in relation to caecal or intestinal HUFA synthesis. Thus, the first report suggesting that intestinal tissue (pyloric caeca) may possess significant and substantial fatty acyl desaturation/elongation (HUFA synthesis) activity in fish was an *in vivo* stable isotope study in rainbow trout (Bell *et al.*, 2003c). The results in the present study using an *in vitro* assay and enterocytes isolated from various regions of the intestine are in good agreement with those in that earlier report, including the fact that stomach had almost

no metabolic activity and that the posterior regions showed a considerably lower capacity for HUFA synthesis than pyloric caeca. The fact that caecal enterocytes were the most active cells of the entire intestine was vital information that played an important role in the development of the rest of the present project. As a result of this experiment, it was clear that pyloric caeca were the most important region of salmonid intestine in relation to HUFA synthesis and so it was decided that caecal enterocytes were adopted as the experimental tissue for the rest of the laboratory experiments and also in the large-scale feeding trials with salmon at commercial fish farms.

The specific assay conditions used in the above experiment on intestinal regions used a standard protocol adopted in recent years for investigating HUFA synthesis in isolated hepatocytes from fish (Bell *et al.*, 1997, 2001a, 2002; Tocher *et al.*, 1997, 2000, 2001). The desaturation/elongation (HUFA synthesis) assay adopted in these previous trials with hepatocytes was based on initial development work by Buzzi (1996) and Buzzi *et al.* (1996, 1997), in laboratory-based studies with rainbow trout. It was subsequently modified for large-scale salmon cage trials (see refs above). Therefore, after the successful preliminary trial of this protocol with isolated enterocytes in the intestinal region experiments, it was decided that a particularly important factor for the present research project was to continue to use precisely the same methodology with enterocytes, both in the preparation of the cell cultures (as described above) and also in the HUFA synthesis assay, as had been adopted and successfully used for hepatocytes. This, it was reasoned, would clearly allow the relative importance of enterocytes to be more accurately determined. However, although it was now decided that the standard protocol already developed for hepatocytes was going to be applied to the enterocytes, it was still necessary to determine the characteristics of the HUFA synthesis assay in enterocytes using this standard protocol.

Systematically the assay conditions were investigated. The incubation time for the assay was set at 2 hours as had been optimised for measuring the entire pathway from 18:3n-3 to 22:6n-3 in hepatocytes. In order to observe if the 2 h incubation used successfully with hepatocytes was also adequate for working with enterocyte preparations, a 24 hour time course experiment, measuring desaturation/elongation activity in caecal enterocytes, was performed. Interestingly, the results showed clear similarities with the viability tests aforementioned (Trypan blue exclusion, LDH activity and oxygen consumption). Therefore, it is likely that the decrease in fatty acyl desaturation/elongation activity after 3 hours of incubation was due to the deterioration in enterocytes viability. Although, the Trypan blue exclusion test results showed that cells remain viable after 6 hours of incubation, the remaining time course suggests that even at 6 hours the metabolic performance of the cells will have decreased and so they are unable to maintain maximal rates of fatty acid metabolism after this period. The reduced amounts of metabolic products found at the 12 and 24 h time points are interpreted as the remaining products of metabolic activity of the cells that were initially alive. It is worthy of mention that the figures are reduced due to the fact that the raw data are divided by the longer incubation time in order to present data of HUFA metabolism per hour. This, however can alter the final results of total activities due to the fact that the rates in which cells metabolise fatty acids are not exponential. However is was observed that the radioactivity contained in products of desaturation/elongation of the substrate sometimes was not enough to mark the autoradiography films when they are placed over a period of 8 days. In addition, it was decided to work with this time scale in order to obtain data that allowed comparisons with previous metabolic studies performed with hepatocytes which were incubated for 2 h (Bell *et al.*, 1997, 2001a, 2002; Tocher *et al.*, 2000, 2001). It should also be noted that the culture

medium and general incubation conditions used in the fatty acid metabolism assay were not specifically optimised for a long-term culture. For instance, no serum was included or additional oxygenation given. Nevertheless, despite the low activities found at the 6 hour incubation, the assay time course experiment demonstrated that the 2 hour incubation period adopted for routine use was optimal for this kind of experiment, particularly with the described assay medium and incubation conditions.

Another factor to be investigated was the temperature of incubation. In previous studies with hepatocytes, the incubations were maintained routinely at 20 °C (Bell *et al.*, 1997, 2001a, 2002; Tocher *et al.*, 2000, 2001). This temperature had been chosen as a compromise in order to work in fish farm conditions at all times of the year, including summer months, where a possibly more ideal lower temperature (around 15°C) is unable to be accurately maintained without cooling facilities, which were not available at the farms or able to be easily transported. In contrast, 20 °C was a temperature able to be maintained at all times in the year. It is well known that temperature has a direct effect on metabolic activities in almost all animals; more specifically, fatty acid desaturase activity is known to be modulated by water temperature in fish (Ninno *et al.*, 1974; De Torrengo and Brenner, 1976; Schuenke and Wodtke, 1983; Hagar and Hazel, 1985; Wodtke and Cossins, 1991). For instance, it has been shown that Δ6 and Δ5 desaturase activities of liver microsomes were 2- and 4-fold higher at 16 °C compared to 30 °C in *Pimelodus maculates* (Ninno *et al.*, 1974). Similarly, the activity of Δ6 desaturase was increased in liver microsomes from common carp (*Cyprinus carpio*) (Schuenke and Wodke, 1983) and rainbow trout (Hagar and Hazel, 1985) acclimated to 10 °C and 5 °C, respectively, compared to fish acclimated to 30 °C and 20 °C, respectively. DeTorrengo and Brenner (1976) showed that when fish acclimated to 28 °C were shifted to 18 °C, the activity of Δ6 desaturase activity was initially decreased

one day after transfer, probably due to a kinetic effect, but subsequently increased by one week after transfer, presumed to be due to induction of gene transcription. The results observed in the temperature profiling experiment were generally in agreement with the earlier literature reports. Although there are no published data regarding desaturases activities in enterocytes and the effect of temperature, the results obtained in the present chapter were generally predictable. It is clear that temperature affects desaturase activity in all type of cells, including enterocytes. Thus, in this case, the expected inverse relation between assay temperature and HUFA synthesis activity in trout enterocytes was corroborated. Therefore the temperature profiling confirmed that 20 °C may not be the absolute ideal temperature, but that enterocytes still expressed significant activity at that temperature and, as all experiments were to be carried out at that temperature, this would not affect a comparative study. In addition, it meant that all the data obtained were at the same temperature as all previous hepatocyte work.

Another important factor to be considered in the evaluation of the HUFA synthesis assay was the concentration of fatty acyl substrate added to the cell incubations. Following the culture conditions reported in previous hepatocyte work, the concentration of fatty acid substrate normally added is ~ 2 μM which is achieved by the addition of labelled fatty acid only, and thus a substrate with the highest possible specific activity. Incubating the enterocytes with higher concentrations of [1-^{14}C]18:3n-3 (achieved by diluting the labelled 18:3n-3 with unlabelled fatty acid) showed that in cultures incubated with concentrations of ~22 or 52 μM 18:3n-3, increased substrate concentration resulted in increased fatty acyl desaturation/elongation activity although the value for 52 μM was slightly lower than 22 μM. In contrast, a substrate concentration of 102 μM did not lead to increased activity and indeed the specific activity went down even further. This suggests that substrate saturation had been

achieved somewhere between 22 and 52 μM. Clearly though, when incubating with 2 μM 18:3n-3, the substrate saturation is not reached, and optimal activity over the pathway is not achieved. This was already known for hepatocytes (Buzzi, 1996). However, it must be remembered that this assay is determining activity over an entire metabolic pathway. Optimisation in strict enzymological assay terms, with a single enzyme activity, is not possible. This can be illustrated by Figure 3.7, which explains in more detail the increased activity observed with higher concentrations of [1-^{14}C]18:3n-3. Fatty acid concentrations of 22 and 52 μM indeed increased substrate availability and thus increased the desaturation of the absorbed 18:3n-3 fatty acid to the subsequent product 18:4n-3. In contrast, the following steps of desaturation and elongation, required to form 20:5n-3, 22:5n-3 and 22:6n-3, took place to a much lesser degree. Thus ignoring conversion to 18:4n-3, cell cultures incubated with the lowest concentration of 2μM actually provided the highest amount of 20:5n-3, 22:5n-3 and 22:6n-3 and the highest synthesis of the final product 22:6n-3. This suggests that a concentration of ~2μM [1-^{14}C]18:3n-3, although not optimal for showing conversion to 18:4n-3, is better for showing differences in production of EPA and DHA. Therefore, using undiluted labelled 18:3n-3 at ~2 μM for the HUFA synthesis assay was a sensible compromise to adopt, and was in agreement with previous studies carried out with hepatocytes under identical culture conditions.

The concentration of protein in the assay depends entirely on the amount of cells obtained after the isolation procedure. An excess or on the contrary, an insufficiency of cells in the preparations may have negative effects on the desaturation/elongation assay performed under otherwise identical culture conditions. Consequently, the analysis of the amount of protein in the cultures was imperative and has to be performed with special care. However, accurate analysis of the protein content prior to performing the

HUFA synthesis assay was impossible under fish farm conditions. Therefore, standardization of the amount of protein in each assay was achieved by taking the same initial amount of caecal tissue and treating identically throughout the isolation procedure, anticipating results in near identical enterocyte preparations. To assess the influence that any variation in protein content may have, it was considered necessary to evaluate the effect of protein concentration over the results obtained in the HUFA synthesis (desaturation/elongation activities) assay. Although there were some differences between results obtained in the four protein concentrations used in the protein evaluation assays, the activities observed were all within the range of values obtained in the other assay characterisation experiments in this section or in other published reports (Bell *et al.*, 1997; Tocher *et al.*, 1997, 2000). Therefore, some preliminary trials were carried out, processing different samples of caecal tissue to observe and calculate the sample size required to achieve the protein concentrations desired for the incubations. The results of this evaluation assay provided significant direction regarding the amount of protein to enable an optimal metabolic activity in enterocytes.

The primary aim of the large-scale trials at the fish farms was to compare the effects of diet between fish fed VO with those fed FO. In order to be able to detect differences between dietary treatments, HUFA synthesis assays were carried out with enterocytes isolated from fish in a normal feeding regime without fasting. Again this was also in order to be consistent with previous hepatocyte data, where all trials reported in the literature were performed with fed fish (Bell *et al.*, 1997, 2001, 2002; Tocher *et al.*, 2000, 2001). This was because it had been widely believed that desaturases may be down regulated by starvation in fish, as they were reported to be in mammals (Tocher, 2003). However, the data based in mammalian tissue, were

basically carried out on liver activities and nothing was known about the activities in intestine with regard to feeding regime. Therefore, it appeared prudent to investigate the effect of feeding and starvation on the HUFA synthesis activity in trout enterocytes. Furthermore, as fatty acid oxidation was also scheduled to be determined in a combined assay and as this was predicted to be affected by nutritional status, the possible effects of starvation in the metabolism of fatty acids in enterocytes, was measured in a combined assay in which hepatocyte metabolic activity was also measured as a reference of the fasting effects.

The results from this combined assay were noteworthy. It was interesting that pyloric caecal enterocytes desaturated/elongated [1-^{14}C]18:3n-3 to a greater extent when fish were under starvation conditions, whereas in contrast, hepatocytes from fed fish showed more HUFA synthesis activity. This is not in agreement with Buzzi's earlier study with trout hepatocytes (1996) where no differences of HUFA synthesis were found between fed or starved fish. It is possible that the different experimental conditions used for the purposes of the present work were a factor in the differences observed. However, the most interesting aspects of these data were the differences observed in HUFA synthesis between both enterocytes and hepatocytes from fed and starved fish. As mentioned previously, it had been suggested that desaturases may be regulated by nutritional status (fed / starved). This appears to be the case, and in hepatocytes desaturase activity decreased by half in fish sampled after starvation, but in enterocytes the opposite was true. Under starvation conditions, enterocytes, the first cells of the body in contact with food, appear to retain and indeed increase HUFA synthesis capacity, possibly in order to supply vital fatty acids to the other organs. In contrast, in terms of β-oxidation activity, the data were consistent with the predicted results in that both enterocytes and hepatocytes showed higher fatty acid oxidation

activities when fish were under fasting conditions. Based on the results it is therefore reasonable to affirm that catabolism of fatty acids is increased in both enterocytes and hepatocytes with an insufficient supply of nutrients. The results of this experiment did not alter the view that, in the present study, the experimental animals should be fed fish as they represent a more realistic situation of the dietary conditions of farmed fish, which are normally fed several times a day and never starved unless immediately prior to harvest.

In summary, there were a number of important objectives in these laboratory-based experiments developing and characterising the cell preparation and HUFA synthesis assay, and all of them were accomplished. It was possible to determine the ideal intestinal tissue, primarily in terms of HUFA synthesis capacity, for the routine preparation of enterocytes and all comparative studies. In addition, it was possible to characterise the isolated enterocyte preparation to establish the quality of the suspensions in terms of cell purity, cell viability and stability (viability and survival over experimental time course). Furthermore, it was possible to determine how the performance of the HUFA synthesis assay in the preferred enterocyte preparation varied with a range of parameters and to decide, adopt and confirm a standardized assay protocol for use in all comparative studies, that offers the best compromise for the simultaneous assay of HUFA synthesis and β-oxidation in enterocytes preparations, and the opportunity to compare meaningfully with data already existing and to be collected on fatty acid metabolism in hepatocytes. Therefore, besides the recommendation to work with fed fish, assay conditions were set at a 2 hour incubation (optimised for measuring entire pathway including DHA synthesis), 20 ^{o}C (optimised for fish farm conditions all year round), concentration of [1-^{14}C]18:3n-3 (2 μM) and between 6 and 12 mg of protein per assay.

CHAPTER FOUR

4. FISH OIL SUBSTITUTION WITH ALTERNATIVE VEGETABLE OILS/HIGH ENERGY DIETS IN ATLANTIC SALMON CULTURE (FOSIS).

4.1. Introduction

The salmon aquaculture industry has traditionally used fish meal and fish oil (FO), rich in the n-3 highly unsaturated fatty acids (HUFA), eicosapentaenoate (20:5n-3, EPA) and docosahexaenoate (22:6n-3, DHA), as protein and lipid sources for dry extruded feeds (Sargent and Tacon, 1999). This practice has been both scientifically sound, as salmonids are carnivorous/piscivorous in the wild, and commercially sound, as FO has been relatively cheap and readily available (Barlow, 2000). Moreover, the fatty acid composition of salmon and trout grown on diets containing FO are very similar to wild fish, being high in the n-3HUFA that are beneficial in the human diet (Ackman, 1980; Henderson and Tocher 1987; Bell *et al.*, 2001a, 2002). However over-exploitation of wild fisheries has meant that an increasing proportion of fish for human consumption is provided by aquaculture; consequently, demand for FO has been increasing with current estimates suggesting aquaculture feeds could consume over 90 % of world supplies by 2010 (Barlow, 2000). For aquaculture to continue to expand and supply more of the global demand for fish, alternatives to FO must be found. However, the only sustainable alternatives are vegetable oils, which are rich in C_{18} PUFA such as linoleate (18:2n-6) and α-linolenate (18:3n-3), but devoid of the n-3HUFA abundant in FO (Sargent *et al.*, 2002). Nevertheless, Atlantic salmon have

limited ability to convert 18:3n-3 and 18:2n-6, which are abundant in many vegetable oils, to their long-chain HUFA products which are essential physiological components of all cell membranes and organs (Tocher, 2003). For this reason, fish like Atlantic salmon are potential candidates for the replacement of dietary fish oil with the high quality n-3 and n-6 PUFA-rich vegetable oils (Sargent *et al.*, 2002).

In addition to supplying the necessary essential fatty acids (EFA) for formation of new cell membranes, lipids and their constituent fatty acids play a major role as sources of energy for metabolism and growth in fish (Tocher, 2003). Thus lipid and fatty acids can either be incorporated into cell membranes and thus the flesh of the fish, or they can be oxidised to provide energy, or lipid can be deposited in adipose tissue as an energy store (Tocher, 2003). Theoretically, the more energy supplied by dietary lipid, the less dietary protein will be used for energy, and so more protein can be "spared" for synthesis of new tissue/flesh (Wilson, 1989; Bell, 1998). This phenomenon has been exploited in aquaculture, and technical advances in extruded feed production have enabled the lipid or fat content of pelleted diets to increase greatly. However, although protein sparing by dietary lipid is well documented, the limits to its effectiveness have not been accurately defined for any fish species (Company *et al.*, 1999). Despite this, recent dietary formulations have tended to continue the upward trend in dietary lipid, particularly in the case of Atlantic salmon and diets containing up to 40 % lipid have been used (Tocher *et al.*, 2003a). In many cases this has successfully increased weight gains and reduced production times, but several studies have shown that the use of high-energy diets containing high percentages of oil can potentially increase the deposition of excess lipid in tissues, specifically flesh in the case of salmon (Sargent *et al.*, 2002), with detrimental consequences such as reduced pigment visualisation and reduced smoking performance, which can lead to processor or retailer

rejection and consumer dissatisfaction (Sheehan *et al.*, 1996; Johansen and Jobling, 1998). The replacement of fish oil with vegetable oils may exacerbate this problem through the deposition of triglycerides as a result of feeding high levels of fatty acids not readily utilised by salmon, particularly in seawater (Bell, 1998).

Several previous trials have investigated aspects of fish oil replacement in diets for Atlantic salmon. There have been trials investigating the replacement of fish oil with vegetable oil in the diets of salmon parr in freshwater (Bell *et al.*, 1997; Tocher *et al.*, 2000). These studies concluded that the inclusion of vegetable oils that mimic the fatty acid composition of freshwater invertebrates that make up the diet of wild salmon parr could be more beneficial in affecting the smoltification process than the marine fish oils that were favoured in parr diets by the aquaculture industry at the time. The abovementioned trials also provided basic scientific knowledge of the important changes in lipid metabolism that are involved in the highly complex physiological process of parr-smolt transformation. Other trials have looked at the effects of feeding vegetable oils on the ongrowing of smolts in seawater indicating, in general, that the substitution of fish oil by vegetable oils is feasible (Bell *et al.* 2001a, 2002; Rosenlund *et al.*, 2001; Torstensen *et al.*, 2000; Tocher *et al.*, 2002). A variety of different oils such as soybean (Hardy *et al.*, 1987; Lie *et al.*, 1993), sunflower (Bell *et al.*, 1991, 1993), borage (Tocher *et al.*, 1997), rapeseed (Tocher *et al.*, 2000; Bell *et al.*, 2001a), linseed (Tocher *et al.*, 2000, 2002) and palm oil (Torstensen *et al.*, 2000; Bell *et al.*, 2002) have been investigated, as well as oil blends (Bell *et al.*, 2003b; Rosenlund *et al.*, 2001; Jobling *et al.*, 2002a,b; Tocher *et al.*, 2003b) to determine the extent to which it is possible to substitute fish oil with seeds oils for salmon farming, and to observe the changes that the use of dietary vegetable oil cause in lipid metabolism.

The level of dietary lipid has effects on lipid metabolism in fish including modulation of lipogenesis. It has long been established that increased dietary lipid levels depress *de novo* fatty acid synthesis through inhibition of several enzymes involved in hepatic lipogenesis, including acetyl coenzyme A carboxylase, fatty acid synthetase and NADPH-generating enzymes such as glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Sargent *et al.*, 1989). The perceived view was that, in comparison to mammals where dietary lipid as low as 2.5 % can cause inhibition of lipogenesis, inhibition of lipogenesis in fish was only obvious with diets containing in excess of 10 % lipid (Sargent *et al.*, 1989). With almost all species a diet would not be considered "high-energy" with a lipid level lower than 10 %, but it can be safely assumed that current high-energy diets, utilizing well in excess of 30 % lipid, inhibit lipogenesis in salmon.

The effects of dietary oil level have been investigated for a number of years (see Cowey, 1993; Jobling, 2001) with more recent studies including levels of oil that are routine in the salmon farming industry today (Jobling *et al.*, 2002c). There has also been a trial looking at the effect of dietary oil level and vegetable oil replacement (Jobling *et al.*, 2002a,b). However, the above trials have been of relatively short duration and none of the previous trials have been run throughout the entire two year growth cycle of the salmon from first-feeding to harvest. Moreover, the capability of enterocytes to metabolise fatty acids has never been investigated in any dietary trials in Atlantic salmon.

The overarching hypothesis tested in the dietary trial described in this chapter is that salmon can be grown on diets containing vegetable oils at practical levels without deleterious effects on the fish itself or its value as an important nutritious food for human consumption. Therefore, the trial was designed to investigate the twin problems

of replacement of fish oil with alternative oils in combination with the use of high-energy (oil) diets in Atlantic salmon culture. Salmon were grown throughout the entire life cycle, from first feeding to harvest size, on diets in which fish oil was replaced by a vegetable oil blend at a high and low level of inclusion of dietary oil. Specifically, this chapter describes the effects of the experimental diets on tissue fatty acid compositions and aspects of fatty acid metabolism in caecal enterocytes including HUFA synthesis (fatty acyl desaturation/elongation) and β-oxidation. Therefore, at the end of trial, after feeding the vegetable oils for an entire two year growth cycle, the fatty acid compositions of intestinal tissue and liver were determined and, more importantly, the capacity of caecal enterocytes to contribute to HUFA synthesis was determined by measuring desaturation/elongation of [1-^{14}C]18:3n-3, and competition between this pathway and the fatty acid oxidation pathway determined.

4.2. Material and methods

4.2.1. Experimental fish and diets

In March 2000, Atlantic salmon fry (*Salmo salar* L.) were randomly distributed into 8 tanks (3 m x 3 m, depth 0.5 m) at a stocking level of 3000/tank, and weaned onto extruded feeds containing either fish oil (FO) or vegetable oil (VO), a 1:1 blend of rapeseed and linseed oils. Each oil was fed to duplicate tanks of fry/parr at either 14 % (L) or 25 % total oil (H), resulting in four dietary treatments in total, LFO (low fish oil), HFO (high fish oil), LVO (low vegetable oil) and HVO (high vegetable oil). Fish were fed the diets described above until seawater transfer in April 2001. At transfer, fish (average weight ~ 40 g) were stocked into 5 m x 5 m net pens at 600 fish/pen with a single pen per dietary treatment although fish from the duplicate freshwater tanks were

marked by fin clip to provide pseudo-replicates. The fish were fed the same diet in seawater as in freshwater although the dietary oil levels were increased to 17 % in the low oil diets (LFO and LVO) and 30 % (3 mm pellet) rising to 37 % (6 and 9 mm pellets) in the high oil diets (HFO and HVO) in the seawater phase. The diets aimed to be practical and therefore were formulated according to current practices in the salmon feed industry and were manufactured by the major salmon feed producers (BioMar Ltd., Grangemouth, Scotland; Ewos Ltd., Bathgate, Scotland; Nutreco, Stavangar, Norway). All diets were formulated to satisfy the nutritional requirements of salmonid fish (U.S. National Research Council 1993). Diet formulations and proximate compositions are given in Table 4.1 and fatty acid compositions in Table 4.2. Fish were ongrown until June 2002 at which time they had reached a marketable size. Final weights in Kg were 1.44 ± 0.37 (LFO), 1.88 ± 0.57 (HFO), 1.71 ± 0.36 (LVO) and 2.02 ± 0.57 (HVO) (all n = 80). Growth was significantly affected by both oil type and oil level in an independent manner, with higher growth in fish fed high oil level and vegetable oil (Tocher *et al.*, 2003a).

Table 4.1. Dietary formulations and proximate compositions

Component	LFO	LVO	HFO	HVO	LFO	LVO	HFO	HVO	LFO	LVO	HFO	HVO
Pellet size (mm)	2				3				6			
LT Fishmeal	50.0		66.0		65.0		50.0		35.2		43.5	
Wheat	15.0		13.0		14.0		12.0		22.0		13.5	
Soybean meal	20.0		0.0		10.0		11.5		12.0		6.5	
Other plant products[1]	5.5		0.0		4.8		2.5		11.8		7.2	
Premixes etc.[2]	2.0		2.0		1.0		1.0		1.0		1.0	
Fish oil[3]	7.5	-	19.0	-	10.0	-	25.4	-	14.6	-	29.2	-
Linseed oil	-	3.8	-	9.5	-	5.0	-	12.7	-	7.3	-	14.6
Rapeseed oil	-	3.8	-	9.5	-	5.0	-	12.7	-	7.3	-	14.6
Protein (%)	53.7	52.0	50.8	51.5	51.5	51.4	48.4	47.9	38.0	37.2	39.7	37.6
Fat (%)	14.1	13.6	26.3	24.4	16.2	17.9	28.9	28.8	16.9	16.9	36.9	36.5
Moisture (%)	4.4	5.7	3.9	4.8	8.0	7.7	3.2	3.1	7.9	8.7	1.8	5.8

[1]Rapeseed meal, wheat flour, wheat and corn glutens. [2]Vitamin and mineral pre-mixes, Finnstim, pigments according to feed company specifications. [3]Capelin or herring oils.

Table 4.2. Fatty acid compositions (percentage of weight) of diets used in freshwater (FW) and seawater (M6, 6 mm pellet and M9, 9 mm pellet).

	LFO			HFO			LVO			HVO		
	FW	M6	M9	FW	M6	M9	FW	M6	M9	FW	M6	M9
14:0	5.1	4.8	5.7	5.6	5.6	6.3	3.0	0.9	1.4	1.5	0.6	0.7
16:0	14.4	16.6	12.2	14.7	16.9	11.9	11.0	8.0	8.1	8.0	7.0	6.6
18:0	2.0	2.9	1.3	2.3	2.6	1.1	2.2	3.1	2.5	2.4	2.9	3.0
Total saturates[1]	22.6	25.7	20.1	23.6	26.5	20.2	17.0	12.7	13.1	12.7	11.3	11.0
16:1n-7[2]	4.3	5.5	7.6	4.9	6.3	8.5	2.2	1.1	1.7	1.2	0.7	0.8
18:1n-9	15.2	16.2	11.2	14.0	15.0	11.1	24.2	28.5	30.4	30.5	32.6	33.6
18:1n-7	2.5	2.1	2.9	2.5	2.4	3.2	2.9	1.8	2.2	1.8	2.1	1.9
20:1n-9[3]	8.9	4.1	16.6	9.8	5.8	18.4	5.4	0.9	3.9	3.1	1.2	2.0
22:1n-11[4]	12.6	5.9	14.6	13.5	8.0	15.8	7.3	0.3	3.9	3.7	0.7	1.8
24:1	1.2	0.9	0.9	0.9	1.1	0.8	0.8	0.2	0.4	0.4	0.3	0.3
Total monoenes	44.6	34.8	53.8	45.6	38.7	57.7	42.8	32.8	42.4	40.8	37.6	40.3
18:2n-6	5.4	10.4	6.2	3.2	2.9	3.1	11.3	22.9	17.8	13.4	19.1	17.0
20:4n-6	0.4	0.5	0.3	0.5	0.6	0.3	0.1	0.2	0.1	0.1	0.1	0.1
Total n-6PUFA[5]	7.0	11.7	7.2	5.0	4.3	4.0	12.0	23.2	18.1	14.0	19.3	17.1
18:3n-3	2.6	1.8	1.0	1.4	1.7	0.8	16.7	24.1	18.2	26.3	27.5	26.9
18:4n-3	2.7	2.3	2.8	3.2	2.7	3.0	1.3	0.4	0.5	0.7	0.2	0.4
20:4n-3	0.8	0.6	0.4	0.9	0.6	0.4	0.3	0.1	0.1	0.1	0.1	0.1
20:5n-3	6.2	8.6	6.7	6.7	9.6	6.5	3.0	2.1	3.0	1.6	1.4	1.6
22:5n-3	1.0	1.0	0.5	0.9	1.0	0.5	0.3	0.2	0.2	0.1	0.2	0.1
22:6n-3	11.3	11.9	6.0	11.3	13.1	5.4	6.0	3.5	3.7	3.3	2.3	2.1
Total n-3PUFA[6]	24.6	26.2	17.5	24.5	28.8	16.6	27.6	30.5	25.8	32.2	31.6	31.2
Total PUFA[7]	32.4	39.5	26.1	30.4	34.8	22.1	40.0	54.5	44.5	46.4	51.2	48.7
(n-3)/(n-6)	3.5	2.2	2.4	4.9	6.8	4.1	2.3	1.3	1.4	2.3	1.6	1.8

Results are means of two determinations. [1], totals include 15:0, 17:0, 20:0 and 22:0, present at up to 0.5%; [2]also contains n-9 isomer; [3]also contains n-11 and n-7 isomers; [4]also contains n-9 and n-7 isomers; [5]totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6 present at up to 0.5%; [6]totals include 20:3n-3 present at up to 0.2%; [7]totals include C16PUFA present at up to 1.5% in FO diets and up to 0.4% in VO diets; HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil; PUFA, polyunsaturated fatty acids.

4.2.2. Sampling and Analyses

At the end of the two year trial, seven fish per dietary treatment were sampled. Fish were killed by a blow to the head after anaesthesia in benzocaine. Livers and pyloric caeca were dissected from 4 fish per dietary treatment and immediately frozen

in liquid nitrogen. Total lipids of liver and pyloric caecal tissue were extracted and quantified as described in section 2.8. Samples of total lipid (2 mg) were applied as 2 cm streaks to thin-layer chromatography plates, the plates developed fully with isohexane/diethyl ether/acetic acid (90:10:1, by vol.) as developing solvent, and the origin area corresponding to total polar lipids were scraped into stoppered glass test tubes. Fatty acid methyl esters (FAME) of total polar lipids were prepared, extracted, purified and quantified by gas-liquid chromatography as described in section 2.10. Enterocytes and hepatocytes were prepared from three fish per dietary treatment essentially as described in section 2.2. The only major difference encountered in preparing the enterocytes from these large (2 Kg) salmon compared to the much smaller rainbow trout used in Chapter 3 was the large amount of mucous which could affect the pelleting of the cells at the centrifugation steps. This required slight modifications to the protocol including using a larger volume of HBSS/FAF-BSA at the initial isolation and first washing steps and centrifugation for longer at slightly higher speed (500 g for 10 min compared to 300 g for 3 min). Isolated enterocytes and hepatocytes were incubated with [1-^{14}C]18:3n-3 for 2 h at 20 °C as described in section 2.4, and HUFA synthesis and β-oxidation assays were performed as described in sections 2.5 and 2.6, respectively.

4.2.3. Statistical analysis

All the data are presented as means ± standard deviation (n = 3 or 4). The effects of dietary treatment on HUFA synthesis and β-oxidation in enterocytes and hepatocytes, and pyloric caecal and liver fatty acid compositions at the end of the two year trial were determined by one-way analysis of variance (ANOVA) followed, where

appropriate, by Tukey's comparison test. For selected data, significance of effects of dietary oil type and oil content on HUFA synthesis and oxidation, and fatty acid compositions were also determiined by two-way ANOVA. Differences in all analysis were regarded as significant when $P < 0.05$ (Zar, 1984).

4.3. Results

Dietary fatty acid compositions

The diets containing VO were characterized by having increased proportions of 18:1n-9, 18:2n-6 and 18:3n-3 and reduced proportions of saturated fatty acids, long chain monoenes (20:1 and 22:1) and n-3 HUFA (20:5n-3 and 22:6n-3) compared to diets containing FO (Table 4.2). These differences were slightly more prominent in the diets with higher oil contents due to the reduction in the relative contribution of fatty acids from the non-oil components of the diet, the fish and plant meals. The diets aimed to reflect current feeding practices and so the precise level of long-chain monoenes and n-3HUFA in the diets containing FO varied slightly depending on which fish oil (herring, capelin etc.) was utilised.

Effects of diet on fatty acid compositions of pyloric caeca and liver

The differences in the fatty acid compositions of the diets were reflected in the fatty acid compositions of the tissues of the fish at the end of the two year dietary trial. Thus, the fatty acid compositions of total polar lipids of livers from salmon fed the LVO and HVO diets were characterized by increased proportions of 18:1n-9, 18:2n-6 and 18:3n-3 and reduced proportions of 16:0 and total saturated fatty acids, long chain

monoenes (20:1 and 22:1), 20:4n-6, 20:5n-3 and 22:6n-3 compared to fish fed diets containing FO (Table 4.3). Again, the differences tended to be greatest between fish fed the high oil contents, HFO and HVO. Increased percentages of elongated and Δ6 desaturated products of 18:2n-6 and 18:3n-3, that is 20:2n-6 and 20:3n-6, and 18:4n-3, 20:3n-3 and 20:4n-3, respectively, were observed in fish fed the VO diets (Table 4.3). Essentially the same pattern was observed in the fatty acid composition of the pyloric caeca although the differences tended to be more pronounced both between FO and VO diets and also between low and high oil contents (Table 4.3). Two-way ANOVA showed that both oil level and oil type had significant effects on the amount of C_{18} PUFA in both pyloric caeca and hepatocytes. Similar results were obtained when the analysis was carried out with 20:5n-3 (EPA) and 22:6n-3 (DHA) in both types of cells, although the interaction between level and type of oil was not significant in the case of EPA in hepatocytes (Table 4.4).

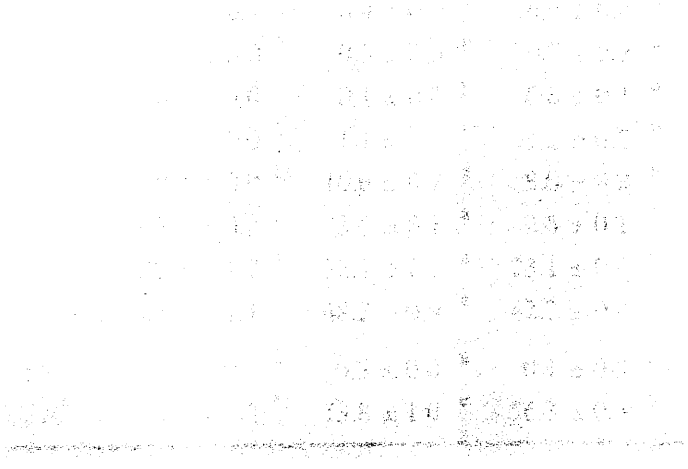


Table 4.3. Total lipid content (percentage of wet weight) and fatty acid composition (percentage of weight) of total polar lipid of liver at the end of the two-year dietary trial.

	LFO	HFO	LVO	HVO
Lipid content	3.0 ± 0.6 ^{c}	3.8 ± 0.6 ^{bc}	4.7 ± 0.7 ^{ab}	5.2 ± 0.8 ^{a}
Fatty acid composition				
14:0	1.6 ± 0.1 ^{b}	2.1 ± 0.2 ^{a}	0.6 ± 0.0 ^{c}	0.4 ± 0.0 ^{c}
16:0	18.8 ± 0.5 ^{a}	17.7 ± 0.8 ^{a}	13.8 ± 1.2 ^{b}	12.6 ± 0.7 ^{b}
18:0	5.0 ± 0.2 ^{b}	4.7 ± 0.2 ^{c}	5.9 ± 0.2 ^{a}	5.6 ± 0.4 ^{ab}
Total saturated[1]	26.0 ± 0.6 ^{a}	25.2 ± 0.8 ^{a}	20.8 ± 1.3 ^{b}	19.1 ± 0.8 ^{b}
16:1n-7[2]	1.9 ± 0.1 ^{b}	2.5 ± 0.2 ^{a}	0.9 ± 0.0 ^{c}	0.7 ± 0.0 ^{c}
18:1n-9	9.6 ± 0.2 ^{b}	8.7 ± 0.6 ^{b}	17.8 ± 1.5 ^{a}	18.1 ± 0.7 ^{a}
18:1n-7	2.0 ± 0.1 ^{b}	2.4 ± 0.1 ^{a}	1.4 ± 0.0 ^{c}	1.4 ± 0.1 ^{c}
20:1n-9[3]	3.5 ± 0.7 ^{a}	4.6 ± 0.5 ^{a}	1.2 ± 0.2 ^{b}	1.1 ± 0.2 ^{b}
22:1n-11[4]	0.6 ± 0.1 ^{b}	0.9 ± 0.1 ^{a}	0.3 ± 0 ^{c}	0.3 ± 0.1 ^{c}
24:1n-9	1.5 ± 0.1 ^{a}	1.4 ± 0.1 ^{a}	1.1 ± 0.1 ^{b}	1.1 ± 0.1 ^{b}
Total monoenes	19.1 ± 1.2 ^{b}	20.7 ± 1.2 ^{ab}	22.7 ± 1.7 ^{a}	22.7 ± 1.3 ^{a}
18:2n-6	2.9 ± 0.2 ^{c}	1.4 ± 0.1 ^{d}	8.8 ± 0.3 ^{b}	9.6 ± 0.3 ^{a}
20:2n-6	0.5 ± 0.1 ^{b}	0.3 ± 0.0 ^{b}	1.2 ± 0.1 ^{a}	1.1 ± 0.2 ^{a}
20:3n-6	0.4 ± 0.1 ^{b}	0.2 ± 0.0 ^{b}	1.0 ± 0.1 ^{a}	0.9 ± 0.1 ^{a}
20:4n-6	2.5 ± 0.1 ^{a}	2.5 ± 0.2 ^{a}	1.7 ± 0.2 ^{b}	1.2 ± 0.1 ^{c}
Total n-6 PUFA[5]	6.8 ± 0.2 ^{b}	4.8 ± 0.2 ^{c}	13.2 ± 0.3 ^{a}	13.1 ± 0.3 ^{a}
18:3n-3	0.4 ± 0.1 ^{c}	0.4 ± 0.0 ^{c}	6.4 ± 0.4 ^{b}	11.1 ± 0.9 ^{a}
18:4n-3	0.2 ± 0.0 ^{b}	0.3 ± 0.0 ^{b}	0.7 ± 0.2 ^{a}	0.9 ± 0.2 ^{a}
20:3n-3	0.1 ± 0.0 ^{c}	0.1 ± 0.0 ^{c}	0.8 ± 0.1 ^{b}	1.2 ± 0.1 ^{a}
20:4n-3	0.8 ± 0.0 ^{c}	1.1 ± 0.1 ^{bc}	1.2 ± 0.2 ^{b}	1.8 ± 0.1 ^{a}
20:5n-3	9.4 ± 0.9 ^{ab}	10.6 ± 0.7 ^{a}	8.0 ± 0.2 ^{b}	8.7 ± 0.8 ^{b}
22:5n-3	3.3 ± 0.3 ^{a}	3.6 ± 0.1 ^{a}	2.5 ± 0.1 ^{b}	2.1 ± 0.1 ^{b}
22:6n-3	32.8 ± 1.7 ^{a}	32.1 ± 1.3 ^{a}	23.1 ± 0.9 ^{b}	18.5 ± 0.6 ^{c}
Total n-3 PUFA	46.9 ± 1.1 ^{a}	48.2 ± 0.9 ^{a}	42.7 ± 0.8 ^{b}	44.2 ± 0.6 ^{b}
Total DMA	0.3 ± 0.1 ^{a}	0.3 ± 0.0 ^{a}	0.1 ± 0.0 ^{b}	0.1 ± 0.0 ^{b}
Total PUFA[6]	54.5 ± 1.2 ^{bc}	53.8 ± 1.0 ^{c}	56.3 ± 0.5 ^{b}	57.5 ± 0.9 ^{a}

Results are means ± SD (n = 3). [1], totals include 15:0, 17:0 and 20:0, present at up to 0.3%; [2]also contains n-9 isomer; [3]also contains n-11 and n-7 isomers; [4]also contains n-9 and n-7 isomers; [5]totals include 18:3n-6, 22:4n-6 and 22:5n-6 present at up to 0.3%; [6]totals include C16PUFA present at up to 0.6%; DMA, dimethylacetals; HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil; PUFA, polyunsaturated fatty acids. Different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post-test ($P<0.05$).

Table 4.4. Total lipid content (percentage of wet weight) and fatty acid composition (percentage of weight) of total polar lipid of pyloric caeca at the end of the dietary trial.

	LFO	HFO	LVO	HVO
Lipid content	2.9 ± 0.6 ^{b}	5.2 ± 1.5 ^{a}	2.5 ± 0.4 ^{b}	3.9 ± 0.8 ^{ab}
Fatty acid composition				
14:0	2.7 ± 0.2 ^{b}	5.5 ± 0.3 ^{a}	1.6 ± 0.1 ^{c}	1.0 ± 0.1 ^{d}
16:0	19.0 ± 0.6 ^{a}	18.1 ± 0.9 ^{a}	14.5 ± 1.1 ^{b}	9.8 ± 0.7 ^{c}
18:0	5.6 ± 0.2 ^{ab}	4.2 ± 0.4 ^{c}	6.2 ± 0.7 ^{a}	4.5 ± 0.2 ^{bc}
Total saturated[1]	27.6 ± 0.8 ^{a}	28.4 ± 1.2 ^{a}	22.6 ± 1.8 ^{b}	15.5 ± 1.0 ^{c}
16:1n-7[2]	2.6 ± 0.4 ^{b}	5.7 ± 0.7 ^{a}	1.2 ± 0.3 ^{c}	0.8 ± 0.0 ^{c}
18:1n-9	9.2 ± 0.3 ^{c}	11.0 ± 0.7 ^{c}	19.0 ± 3.1 ^{b}	31.0 ± 0.5 ^{a}
18:1n-7	2.6 ± 0.1 ^{b}	3.0 ± 0.1 ^{a}	2.1 ± 0.1 ^{c}	2.1 ± 0.1 ^{c}
20:1n-9[3]	4.4 ± 0.5 ^{b}	7.2 ± 0.9 ^{a}	2.4 ± 0.2 ^{c}	2.4 ± 0.2 ^{c}
22:1n-11[4]	3.5 ± 0.6 ^{b}	7.5 ± 1.4 ^{a}	1.7 ± 0.1 ^{c}	1.6 ± 0.1 ^{c}
24:1n-9	1.0 ± 0.8	1.0 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Total monoenes	23.3 ± 0.4 ^{b}	35.5 ± 0.9 ^{a}	27.0 ± 2.9 ^{b}	38.5 ± 0.3 ^{a}
18:2n-6	2.1 ± 0.1 ^{c}	2.5 ± 0.5 ^{c}	7.5 ± 1.3 ^{b}	13.4 ± 0.2 ^{a}
20:2n-6	0.4 ± 0.0 ^{b}	0.3 ± 0.0 ^{b}	0.8 ± 0.0 ^{a}	0.8 ± 0.0 ^{a}
20:3n-6	0.2 ± 0.0 ^{b}	0.2 ± 0.0 ^{b}	0.4 ± 0.1 ^{a}	0.5 ± 0.1 ^{a}
20:4n-6	2.1 ± 0.1 ^{a}	1.2 ± 0.2 ^{b}	1.6 ± 0.1 ^{b}	0.5 ± 0.0 ^{c}
Total n-6 PUFA[5]	5.4 ± 0.2 ^{c}	4.7 ± 0.4 ^{c}	10.6 ± 1.4 ^{b}	15.5 ± 0.1 ^{a}
18:3n-3	0.6 ± 0.0 ^{c}	1.0 ± 0.2 ^{c}	8.1 ± 1.1 ^{b}	15.8 ± 0.4 ^{a}
18:4n-3	0.8 ± 0.1 ^{bc}	1.8 ± 0.4 ^{a}	0.5 ± 0.1 ^{c}	1.3 ± 0.1 ^{ab}
20:3n-3	0.1 ± 0.0 ^{b}	0.1 ± 0.0 ^{b}	0.8 ± 0.0 ^{a}	1.0 ± 0.1 ^{a}
20:4n-3	0.7 ± 0.1	1.0 ± 0.3	0.6 ± 0.0	1.0 ± 0.2
20:5n-3	8.8 ± 0.6 ^{a}	8.2 ± 0.9 ^{a}	5.9 ± 1.2 ^{b}	2.2 ± 0.3 ^{c}
22:5n-3	2.2 ± 0.2 ^{a}	2.1 ± 0.6 ^{a}	1.4 ± 0.2 ^{ab}	0.7 ± 0.1 ^{b}
22:6n-3	30.1 ± 0.7 ^{a}	16.5 ± 1.3 ^{b}	18.9 ± 2.3 ^{b}	8.4 ± 0.2 ^{c}
Total n-3 PUFA	43.4 ± 0.7 ^{a}	30.8 ± 1.1 ^{bc}	36.3 ± 3.9 ^{b}	30.4 ± 1.1 ^{c}
Total DMA	0.4 ± 0.2	0.7 ± 0.1	0.6 ± 0.4	0.2 ± 0.1
Total PUFA	48.7 ± 0.9 ^{a}	35.5 ± 1.4 ^{b}	46.9 ± 3.9 ^{a}	45.9 ± 1.2 ^{a}

Results are means ± SD (n = 3). [1]totals include 15:0 present at up to 0.5%; [2]also contains n-9 isomer; [3]also contains n-11 and n-7 isomers; [4]also contains n-9 and n-7 isomers; [5]totals include 18:3n-6 and 22:5n-6 present at up to 0.4%; DMA, dimethylacetals; HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil; PUFA, polyunsaturated fatty acid. Different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post-test ($P<0.05$).

Effects of diet on HUFA synthesis and fatty acid oxidation in isolated hepatocytes and caecal enterocytes at the end of the two-year trial

HUFA synthesis, as measured by the conversion of [1-^{14}C]18:3n-3 to desaturated and elongated products was greater in both isolated hepatocytes and caecal enterocytes in fish fed VO (Figure 4.1). Both enterocytes and hepatocytes of fish fed with the HVO diet achieved the highest amount of converted substrate (1.3 and 1.9 pmol/h/mg protein respectively). Rates of conversion were generally higher in hepatocytes compared to enterocytes except for fish fed diet HFO, which showed the lowest HUFA synthesis activity in hepatocytes (0.4 pmol/h/mg protein), whereas the lowest activity in enterocytes was obtained with fish fed the LFO diet (0.3 pmol/h/mg protein) (Figure 4.1A). Thus HUFA synthesis in both enterocytes and hepatocytes was significantly affected by oil type and also oil level as determined by two-way ANOVA (Table 4.4).

Oxidation of [1-^{14}C]18:3n-3, as measured by the recovery of acid-soluble radioactivity, was 3- to 4-fold higher than conversion by the desaturation/elongation pathway in both hepatocytes and enterocytes but, in contrast to that pathway, fatty acid oxidation was not affected by diet in either cell type. Although there were no statistically significant differences between treatments, both enterocytes and hepatocytes of fish fed with LFO diet presented the highest values of fatty acyl β-oxidation (7.5 and 8.4 pmol/h/mg protein) (Figure 4.1B). In both cell types, two-way ANOVA showed that although there is not an effect of either oil type or oil level in the β-oxidation activity there is significant interaction between them, meaning that with FO the β-oxidation activity decreased with increasing oil content whereas, in contrast, with

VO, β-oxidation activity increased with increasing oil content (Figure 4.1B and Table 4.4).

Table 4.5. Significance of effects of dietary oil type and oil content on HUFA synthesis and oxidation in caecal enterocytes and hepatocytes and fatty acid compositions of pyloric caeca and liver of *Salmo salar* L. at the end of the two-year trial.

	Dietary oil type		Dietary oil level		Interaction	
Fatty acid	F value	significance	F value	significance	F value	significance
Pyloric caeca						
C_{18} PUFA	754.50	<0.0001	140.20	<0.0001	84.37	<0.0001
20:5n-3 (EPA)	109.90	<0.0001	25.85	0.0003	13.38	0.0033
22:6n-3 (DHA)	204.50	<0.0001	318.60	<0.0001	5.08	0.0437
HUFA synthesis	32.01	<0.0001	34.80	<0.0001	1.45	0.2519
β-oxidation	0.02	0.8881	0.87	0.3701	8.23	0.0141
Liver						
C_{18} PUFA	2772.03	<0.0001	49.44	0.0001	140.03	<0.0001
20:5n-3 (EPA)	16.50	0.0036	5.47	0.0475	0.38	0.5554
22:6n-3 (DHA)	285.68	<0.0001	14.11	0.0056	8.35	0.0202
HUFA synthesis	138.62	<0.0001	10.06	0.0132	16.26	0.0038
β-oxidation	0.32	0.5797	0.12	0.7345	6.18	0.0287

Data subjected to two-way ANOVA as described in the Methods section. EPA, eicosapentaenoic acid; DHA, docosahexanoic acid; HUFA, highly unsaturated fatty acids; PUFA, polyunsaturated fatty acids.

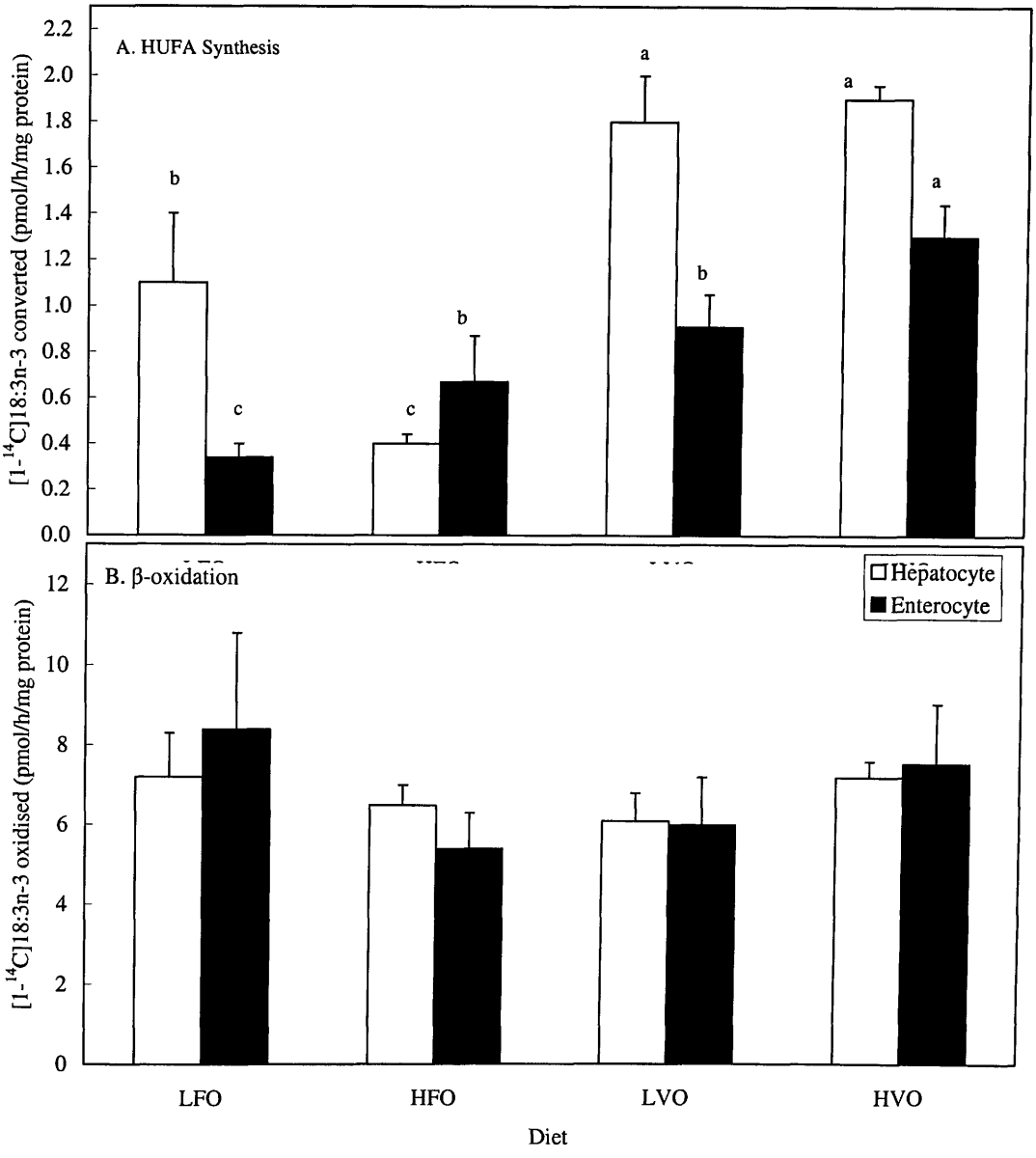


Figure 4.1. Metabolism of [1-^{14}C]18:3n-3 in isolated hepatocytes and caecal enterocytes at the end of the two-year dietary trial. HUFA synthesis (A) was determined by measuring total fatty acid desaturation/elongation activity and represents the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Fatty acid β-oxidation activity (B) was determined by the recovery (pmol/h/mg protein) of radioactivity from [1-^{14}C]18:3n-3 as acid soluble products. All results are means ± SD (n = 3). Columns for a specific activity and tissue with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$). HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil.

4.4. Discussion

Several studies have shown that both dietary oil type and oil content can have significant effects on fatty acid metabolism in Atlantic salmon (Bell *et al.* 1997; Tocher *et al.* 2000, 2002; Torstensen *et al.* 2000; Rosenlund *et al.* 2001; Jobling *et al.* 2002a, b, c). Some of the major consequences of these effects can be the alteration of lipid content and fatty acid composition of the flesh, both of which can compromise the nutritional quality of the fish as food for the human consumer (Sheehan *et al.,* 1996; Johansen and Jobling, 1998; Bell *et al.*, 2001a, 2002; Torstensen *et al.*, 2000; Rosenlund *et al.*, 2001). In order to determine how alterations in dietary lipid specifically affect the intestine, the present chapter aimed to investigate the effects of dietary oil content and fatty acid composition on key pathways of fatty acid biochemistry evaluated after feeding the experimental diets for an entire two-year growth cycle.

Two important pathways of lipid and fatty acid homeostasis whose relative activities contribute to final tissue fatty acid compositions are HUFA synthesis, through desaturation and elongation, and fatty acid oxidation through the β-oxidation pathway. It has been reported that a major fate of dietary 18:3n-3 in salmonids was oxidation to provide energy for growth (Bell *et al.*, 2001b). Thus, 18:3n-3 not only serves as a substrate for the synthesis of the important long-chain n-3 HUFA, but also plays a main role as an energy source. For this reason, a primary aim of the study reported in the present chapter was to determine the effects of the diets on the balance between these two different pathways, using [1-^{14}C]18:3n-3 as fatty acid substrate. The activities of the pathways in liver (hepatocytes), the major lipid metabolising organ, were also measured in order to act as a reference and for comparison with that of the intestine

(enterocytes), the first organ to encounter dietary fatty acids and also a site of significant HUFA synthesis (Bell *et al.*, 2003c).

Differences in the fatty acid compositions of the tissues at the end of the two-year dietary trial very largely reflected the fatty acid compositions of the diets as expected. The results were comparable with several other published studies on salmon fed diets containing vegetable oils (Bell *et al.*, 2001a, 2002; Tocher *et al.*, 1997, 2000, 2003b; Torstensen *et al.*, 2000). Furthermore, the fatty acid compositions of both liver and pyloric caeca indirectly indicated the existence of active HUFA synthesis in these tissues, as an increased percentage of elongated and Δ6 desaturated products were observed in fish fed the VO diets. There are several reports in the literature in which the effects of dietary VO on the metabolism of fatty acids via HUFA synthesis in hepatocytes from salmonids was determined (Buzzi *et al.*, 1996; Rodriguez *et al.*, 1997; Bell *et al.*, 2001a, 2002; Tocher *et al.*, 1997, 2000, 2001, 2003a, 2003b). The results in this chapter indicated that the HUFA synthetic activity in both isolated caecal enterocytes and hepatocytes of salmon fed diets containing VO was greater than in fish fed diets containing FO. This trend of increasing HUFA synthetic activity when using dietary VO as a substitute for FO has been reported previously for salmonid hepatocytes. Bell *et al.*, (2002) reported similar increased HUFA synthesis in salmon hepatocytes when diets containing palm oil were utilized as a substitute for FO, and Tocher *et al.*, (2001), also reported that HUFA synthesis was significantly increased in several different species and populations of salmonids when fed diets containing linseed and rapeseed oils compared to fish fed diets containing FO.

In the present study, and irrespective of the diet, the primary fate of the substrate [1-^{14}C]18:3n-3 was β-oxidation rather than HUFA synthesis in both enterocytes and hepatocytes. A similar pattern was reported previously. In an *in vivo* study in juvenile

rainbow trout using stable isotope (deuterium-labelled 18:3n-3), Bell *et al.*, (2001b) observed that large amounts of essential fatty acids were unexpectedly oxidised. It was suggested that any control over the selection of fatty acids for oxidation was mainly dependant on chain length rather than their potential as key precursors for functionally essential fatty acids. Subsequently, in a similar *in vivo* experiment with deuterium-labelled 18:3n-3, Bell *et al.*, (2003c), consistently found that the recovery of the substrate 18:3n-3 was significantly lower than the recovery of 21:4n-6 (added as a metabolically inert marker of dietary intake), confirming a very active catabolism of 18:3n-3 in these experiments. The *in vivo* studies had provided strong evidence that the intestine was an important site of HUFA synthesis in the rainbow trout, and, in addition, it suggested that this tissue may also plays an important role in β-oxidation of linolenate. The results of the present study using enterocytes *in vitro* has shown conclusively that intestine is potentially an important site of both HUFA synthesis and significant oxidation of dietary fatty acids including PUFA such as 18:3n-3.

Although there is a significant interaction between oil type and level, the type of dietary oil had no significant effect on β-oxidation either in enterocytes or hepatocytes, as there was no increased oxidation of 18:3n-3 in cells from fish fed VO compared to fish fed FO. Perhaps surprisingly though, dietary oil level also had no significant effect on β-oxidation activity when measured using [1-^{14}C]18:3n-3 as substrate in either enterocytes or hepatocytes. Thus, it is reasonable to deduce that the overall effect of the dietary treatments on fatty acid metabolism was that the balance between the two pathways competing for dietary 18:3n-3, was moved in the favour of HUFA synthesis in both intestine and liver in fish fed VO compared to fish fed FO. However, it is important to note that, even with the balance in fatty acid metabolism shifted towards HUFA synthesis in both hepatocytes and enterocytes thus increasing the amounts of

HUFA synthesized in intestine and liver, it is not sufficient to maintain n-3HUFA levels in the flesh or to prevent the deposition of excess dietary C_{18} PUFA in the tissues as observed in the present study and as reported in several previous studies (Bell *et al.*, 2001a, 2002, 2003b: Tocher *et al.*, 2002, 2003a, 2003b).

In the present chapter, apart from researching the effects of dietary oil content and VO substitution of FO, a major aim of the study was to determine the relative importance of the intestine specifically as an organ with HUFA synthetic activity, particularly in comparison with liver. This study was the first time that HUFA synthesis capacity had been investigated *in vitro* in pyloric caecal enterocytes, and it confirmed the previous *in vivo* trials that had suggested that pyloric caeca may be a site of significant HUFA synthesis, at least in juvenile rainbow trout (Bell *et al.*, 2003c). However, the earlier *in vivo* work was unable to draw any direct comparison between the levels of activity in liver and intestine although the suggestion was that intestine may be more active. The present study, directly measuring the HUFA synthesis activities of caecal enterocytes and hepatocytes, prepared identically, and assayed under identical *in vitro* conditions, indicated that caecal enterocytes were not more active than hepatocytes, as rates of conversion were generally higher in hepatocytes compared to those in enterocytes. However, there is a high variability in the number and size of caeca in salmonids (Buddington and Diamond, 1986). As observed in the present study, the variability is also dependent in the size of the fish. Therefore, it is important to consider that the comparison between liver and caeca is valid only when considering rates expressed as pmol/h/mg protein and by no means it expresses the total amount of synthesis that each organ can produce *in vivo*. With the *in vivo* studies, it is not possible to be certain of the relative activities in liver and intestine, but it is possible that the situation observed in the present trial was not the situation in the earlier study with

trout. The precise reason for any discrepancies between the *in vivo* and *in vitro* studies are not known but they can be speculated. The activities in the present trial were measured in cells from mature Atlantic salmon reared for over a year in seawater, whereas the fish in the *in vivo* experiment were juvenile rainbow trout reared in freshwater. Therefore, the size/age of the fish were different, as Bell's work was performed with small fish (2 g) whereas the present work was performed with commercial size salmon (2 - 3 kg). The *in vivo* trials with trout had shown that size/age of fish is an important factor affecting HUFA synthesis activity at least when comparing very small fish with much larger fish (Bell *et al* 2003c).

The FOSIS trial was the first in which VO replacement of FO had been used throughout the growth cycle of salmon from first feeding to harvest. The overall performance of the fish during the trial (see Tocher *et al.*, 2003a for full details of growth performance, etc.) showed that the combination of feeding high energy (oil) diets, and diets in which vegetable oils replace fish oil throughout the growth cycle of Atlantic salmon, had several effects on pathways of fatty acid metabolism in the fish, but that the combination of high dietary oil content and 100 % replacement of FO with VO did not significantly exacerbate the effects of feeding salmon VO. However, the major specific aim in this chapter was to determine the importance of the intestine in the metabolism of dietary fatty acids and this was achieved.

In summary, this was the first dietary trial in which HUFA synthesis, as measured by the conversion of [1-^{14}C]18:3n-3 to desaturated and elongated products, was evaluated and compared in caecal enterocytes and hepatocytes from Atlantic salmon. The HUFA synthetic activity of enterocytes showed a similar order of magnitude to that in hepatocytes albeit was generally slightly lower. HUFA synthesis in enterocytes, like that in hepatocytes, was increased in fish fed VO compared to fish

fed FO. In contrast, β-oxidation activity was very similar in enterocytes and hepatocytes and neither type of dietary oil nor dietary oil levels affected the activity in either cell type. Thus, the combined effects of VO feeding on fatty acid metabolism were that the balance was shifted towards HUFA synthesis in both enterocytes and hepatocytes although this was not sufficient to prevent the fatty acid compositions of the tissues being altered, to show lower levels of n-3HUFA and higher levels of C_{18} PUFA.

CHAPTER FIVE

5. EFFECT OF CRUDE PALM OIL IN HUFA SYNTHESIS AND β-OXIDATION ACTIVITIES OF ENTEROCYTES AND HEPATOCYTES OF RAINBOW TROUT

5.1. Introduction

As described in previous chapters, there is an important and considerable effort being directed towards developing alternative and better ways of managing nutrition in the commercial culture of fish. It is known that salmonid and marine fish culture at the present time relies profoundly on the available supplies of fish meal and fish oil. In addition, as mentioned previously, due to the increased exploitation pressure on global industrial fisheries, which are strictly limited, and also due to the increasing ecological and ethical objections to overexploitation of a finite resource, the fisheries industry is now placed in a vulnerable position (Sargent and Tacon, 1999; Barlow, 2000; Tidwell and Allan, 2002).

Plant/vegetable oils are sustainable alternatives to fish oil, but are rich in C_{18} PUFA such as 18:2n-6 and 18:3n-3, and devoid of the n-3HUFA abundant in fish oil (Sargent *et al.*, 2002). HUFA synthesis, that is the conversion of C_{18} PUFA to HUFA, is effected by a series of desaturase and elongase enzymes that are known to be under nutritional regulation in mammals (Brenner, 1981) and fish (Sargent *et al.*, 2002). Studies, both *in vivo* and in cell cultures, have shown that salmonid fish have the capability to produce EPA and DHA from 18:3n-3 (Tocher, 2003). In the previous chapter it was demonstrated that caecal enterocytes play an important role in fatty acid

metabolism in Atlantic salmon, and that HUFA synthesis was increased in fish fed vegetable oils compared to fish fed fish oil. However, the previous chapter also clearly demonstrated that, despite increased HUFA synthesis capacity, culture of salmonids on diets containing high levels of vegetable oils considerably alters the fatty acid composition of the tissues (including flesh) resulting in high levels of the C_{18} PUFA and much reduced levels of n-3HUFA as it has been shown before (Bell *et al.*, 2001a, 2002, 2003a, 2003b). Recently, it has been demonstrated that, although rainbow trout increased its 22:6n-3 production from 18:3n-3 with the use of diets totally lacking n-3HUFA, it was unable to synthesise sufficient 22:6n-3 to retain their body concentration of this fatty acid (Bell and Dick, 2004). Therefore, dietary trials, evaluated by *in vivo* or *in vitro* experiments, have clearly shown that the activity of the desaturation/elongation pathway cannot convert 18:3n-3 to EPA and DHA at an adequate rate to maintain tissue n-3HUFA levels (Tocher, 2003; Bell and Dick, 2004, present study). As a consequence, there is considerable interest in the regulation of the HUFA biosynthetic pathways in fish in order to determine how the conversion of C_{18} PUFA to HUFA can be improved in commercially important cultured fish species (Sargent *et al.*, 2002; Tocher, 2003).

Studies *in vivo* also showed a ten fold difference in rates between induced and repressed fish rather than the 2-3 fold difference found *in vitro*. Synthesis of 22:6n-3 was 5-10 times higher in fish reared on the VO diets than in fish those fed on the FO diet where 22:6n-3 synthesis was repressed.

As well as providing essential PUFA (Sargent *et al.*, 1995), dietary lipids are also a major source of energy in salmonid diets (Sargent *et al.*, 1989; Froyland *et al.*, 1998). Studies investigating mitochondrial β-oxidation suggested that saturated and monounsaturated fatty acids are preferred over PUFA for energy production in fish

(Henderson, 1996). Thus, in rainbow trout liver, 22:1n-11 and 16:0 were the best substrates for mitochondrial β-oxidation while in red muscle 16:0, 16:1, 18:1n-9 and 18:2n-6 were the preferred substrates (Henderson and Sargent, 1985b; Kiessling and Kiessling, 1993). Therefore, when selecting potential vegetable oils for substituting fish oil in diets of fish, energy availability as well as PUFA content must be considered. To minimise any reduction in quality, both in terms of the health of the farmed fish and its health benefits to human consumers, potential substitutes for fish oil should avoid excessive deposition of 18:2n-6, retain high levels of n-3HUFA and provide sufficient energy in the form of saturated and monounsaturated fatty acids to maintain growth rates (Bell *et al.*, 2002).

In this chapter, the suitability of crude palm oil (CPO) was investigated as a vegetable oil to replace fish oil (FO). There were a number of very good reasons for this choice. Firstly, palm (*Elaeis guineensis*) oil production is currently second in rank order to soybean oil production, but it is expected to exceed that within the next 10 years making it the most abundant vegetable oil in the world (Gunstone, 2001). Secondly, palm oil has some advantages compared to other vegetable oils that may enable it to have a potential use in the diets of a wide range of fish species because it has relatively low levels of 18:2n-6 and an abundance of 16:0 and 18:1n-9 (Ng 2002a,b). Thirdly, palm oil contains high levels of tocopherols (vitamin E) and, especially, tocotrienols and also β-carotenes that are potential antioxidants. Finally, in a previous study with CPO in Atlantic salmon, it was observed that the increase in HUFA synthesis induced by feeding CPO (Bell *et al.*, 2002) was greater than that observed with various other vegetable oils (Bell *et al.*, 1997, 2001; Tocher *et al.*, 2000). Furthermore, the use of CPO in diets for Atlantic salmon and rainbow trout has given growth and feed utilization efficiency comparable to fish fed equivalent levels of marine

fish oils (Torstensen *et al.*, 2000; Rosenlund *et al.*, 2001; Bell *et al.*, 2002; Caballero *et al.*, 2002). Investigating the impact of replacing at least 50% of the fish oil in high-energy salmon diets with different vegetable oils including CPO, Rosenlund *et al.* (2001), established that no significant effects of dietary lipid source were found on growth, survival, body traits or fillet quality comparable to fish fed equivalent levels of marine fish oils. Similar results were observed by Bell *et al.* (2002), however, they also reported that at levels of CPO inclusion above 50% of dietary lipid, significant reductions in muscle EPA, DHA and the (n-3)/(n-6) PUFA ratio occur, resulting in reduced availability of the essential (n-3) HUFA to the consumer.

Torstensen *et al.* (2000), reported that the apparent digestibility of fatty acids, measured by adding ytrium oxide as inert marker, was significantly lower in Atlantic salmon fed a diet containing CPO compared to other diets containing no CPO as a source of lipid when grown at 8 °C. There is some conflicting evidence on the influence of water temperature on lipid and fatty acid digestibility in salmonids. Windell *et al.* (1978), reported a significant decrease in lipid digestibility in small (~19 g) rainbow trout kept in water temperatures of 7 °C compared to fish in water temperatures of 11 or 15 °C, but this reduction in lipid digestibility was not observed in medium (~207 g) or large (~586 g) sized fish at the same temperature. Austreng *et al.* (1980), reported no significant differences in lipid and fatty acid digestibility in rainbow trout reared at 3 or 11 °C. Fatty acid digestibility varied by less than 5 % in Atlantic salmon fed increasing dietary levels of soybean oil and maintained at water temperatures of 5 or 12 °C (Johnsen *et al.*, 2000). In contrast, Olsen and Ringo (1998), reported that the digestibility of saturated fatty acids was significantly reduced in Arctic charr (*Salvelinus alpinus*) maintained at 0.6 °C compared to fish maintained at 10 °C, but the digestibility of monoenes and PUFA were hardly affected by the change in temperature regime. The

influence of water temperature on fatty acid metabolism has not been determined in enterocytes of salmonids.

In this chapter, the results of two dietary trials utilising CPO in the diet of rainbow trout are reported. In the first trial, the primary objective was to investigate the interaction between dietary CPO and water temperature in the modulation of fatty acid metabolism in caecal enterocytes from rainbow trout. This aimed to provide information that could be important in optimising the use of dietary vegetable oils, especially CPO, according to environmental temperature in the salmonid aquaculture industry. For this, rainbow trout, acclimatized to 7, 11 and 15 °C, were fed for 4 weeks on diets in which the FO was replaced in a graded manner by 25, 50 and 100 % CPO. The second trial in this study aimed to further investigate the effects of dietary CPO on intestinal fatty acid metabolism in rainbow trout in a fully replicated trial over a longer duration. Triplicate groups of rainbow trout were fed for 10 weeks on diets in which CPO replaced the fish oil in a similar graded manner as above. At the end of both trials, fatty acyl desaturation/elongation (HUFA synthesis) and oxidation activities were determined in isolated caecal enterocytes and hepatocytes using [1-^{14}C]18:3n-3 as substrate, and samples of flesh, liver and intestinal tissue were collected for analysis of fatty acid composition.

5.2. Material and methods

5.2.1. Experimental diets

Practical extruded diets (3 mm diameter) containing 47 % crude protein and 22.5 % crude lipid were formulated (BioMar Ltd, Scotland, UK) using the same basal ingredients and varying only in the content of CPO that was added at the expense of

marine FO (Table 5.1). CPO was fully melted and thoroughly mixed with the FO before the oils or oil mixtures were used to coat the extruded pellets to give the four diets with CPO replacing 0 % (P0), 25 % (P25), 50% (P50) and 100 % (P100) of the FO. The fatty acid compositions of the resultant diets are shown in Table 5.2. The diets were designed to meet all the known nutritional requirements of salmonid fish including n-3 EFA (US National Research Council, 1993).

Table 5.1. Ingredients and formulation (g / 100 g diet) of experimental diets

	Diet			
Component	P0	P25	P50	P100
Fishmeal[1]	34.3	34.3	34.3	34.3
Hi Pro Soya[2]	12.7	12.7	12.7	12.7
Wheat gluten[2]	10.0	10.0	10.0	10.0
Corn gluten[3]	10.0	10.0	10.0	10.0
Wheat[4]	10.0	10.0	10.0	10.0
Marine oil[1]	20.0	15.0	10.0	0.0
Palm oil[5]	0.0	5.0	10.0	20.0
Methionine	0.21	0.21	0.21	0.21
Lysine	0.79	0.79	0.79	0.79
Micronutrients[6]	2.41	2.41	2.41	2.41
Vitamin E[7]	0.015	0.011	0.0076	0.0
Ytrium oxide	0.02	0.02	0.02	0.02

[1]Norsemeal Ltd., London, UK; [2]Cargill, Swinderbury, UK. [3]Cerestar UK Ltd., Manchester, UK; [4]J.D. Martin, Tranent, UK. [5]United Plantations Bhd, Jenderata Estate, Teluk Intan, Malaysia. [6]Vitamins, minerals and astaxanthin (Carophyl pink®), BioMar A/S, Brande, Denmark; [7]Roche, Basel, Switzerland.

Table 5.2. Fatty acid composition (% of total fatty acids) of experimental diets
[1]Total includes 15:0 and 20:0; [2]Total includes 16:1n-9, 20:1n-7, 22:1n-9 and 24:1;

	Diet			
Fatty acid	P0	P25	P50	P100
14:0	6.4	4.6	3.7	1.6
16:0	18.6	26.7	30.6	37.9
18:0	3.4	3.8	4.2	4.2
Total saturated[1]	29.3	35.8	39.1	44.1
16:1n-7	6.7	4.5	3.5	1.0
18:1n-9	11.1	20.2	24.2	35.9
18:1n-7	2.5	1.9	1.8	0.6
20:1n-9	5.4	3.5	2.8	0.4
22:1n-11	7.2	4.5	2.5	0.4
Total monounsaturated[2]	34.7	35.6	35.5	38.5
18:2n-6	5.9	8.1	9.1	11.8
20:4n-6	0.6	0.4	0.3	0.2
Total n-6PUFA[3]	7.2	9.0	9.9	12.1
18:3n-3	1.3	1.0	0.9	0.6
18:4n-3	2.9	1.9	1.4	0.2
20:5n-3	9.2	6.0	4.7	1.2
22:5n-3	1.2	0.8	0.7	0.3
22:6n-3	11.3	7.9	6.4	2.7
Total n-3PUFA[4]	26.9	18.4	14.6	5.1
Total PUFA[5]	36.0	28.5	25.4	17.4
n-3/n-6	3.7	2.0	1.5	0.4

[3]Total includes 18:3n-6, 20:2n-6 and 22:5n-6; [4]Total includes 20:3n-3 and 20:4n-3;
[5]Total includes C16PUFA; PUFA, polyunsaturated fatty acids.

5.2.2. Experimental fish

Experiment 1

Rainbow trout with a mean initial body mass of approximately 45 g, were obtained from a local fish hatchery (Almondbank, Perthshire, Scotland) and stocked

into three 1000 L tanks on arrival at the Institute of Aquaculture (University of Stirling, Scotland). Over a 2-week period, the water temperature of two of the tanks was gradually increased to 11 ± 1 and 15 ± 1 °C, respectively, while the third tank was maintained at the ambient water temperature of 7 ± 1 °C. The indoor tanks were subjected to a photoperiod regime of 12 h light : 12 h dark. All fish were fed a commercial trout pellet during this acclimation period. After 2 weeks, each group of temperature-acclimatized fish was randomly distributed into four circular tanks of 100 L capacity (at 40 fish/tank) supplied with flow-through water of the same acclimation temperature at 1 L/min. Fish maintained at each temperature grouping were fed one of the four experimental diets for 4 weeks before sampling. Fish were fed to satiety three times daily.

Experiment 2

For the second trial using palm oil as a substitution of fish oil in the diet, rainbow trout with a mean initial body mass of approximately 27 g were obtained from a local fish hatchery (Almondbank, Perthshire, Scotland) and were stocked randomly (at 40 fish/tank) into twelve circular tanks of 100 L capacity on arrival at the Institute of Aquaculture. The tanks were supplied with flow-through water at a constant temperature of 13 °C at 1 L/min and were subjected to the same photoperiod regime (12 h light : 12 h dark). All fish were fed a commercial trout pellet during an initial one-week acclimation period before the experimental diets were randomly assigned to triplicate tanks. Fish were fed to satiety three times daily with one of the four experimental diets used in the previous experiment (Table 5.1 and 5.2) for ten weeks before sampling.

5.2.3. Sampling and Analyses

Fish were sampled at the end of the trials and killed by a blow to the head after anaesthesia in benzocaine. Pyloric caeca and livers were dissected from 3 (experiment I) and 4 (experiment II) fish per dietary treatment and used for preparation of enterocytes and hepatocytes as described in section 2.2. Isolated enterocytes and hepatocytes were incubated with [1-^{14}C]18:3n-3 for 2 h at 20 °C as described in section 2.4, and HUFA synthesis and β-oxidation assays were performed as described in sections 2.5 and 2.6, respectively. In experiment II, pyloric caeca, livers and flesh were also dissected from 4 fish per dietary treatment for lipid analyses. Total lipid was extracted as described in section 2.8 and FAME prepared, extracted, purified and quantified by gas-liquid chromatography as described in section 2.10.

5.2.4. Statistical analysis

All data are presented as means ± standard deviation (n = 3 or 4) and all statistical analyses were performed using PRISM 3 (Graphpad Software Inc., San Diego, USA). The effects of dietary CPO on HUFA synthesis and β-oxidation at each specific temperature in experiment I, and HUFA synthesis and β-oxidation in experiment II were analysed by one-way ANOVA followed, where appropriate, by Tukey's post-test to determine significant differences between individual treatments. In experiment I, the combined effects of both water temperature and dietary CPO and their interactions on HUFA synthesis and β-oxidation were also determined by two-way ANOVA. Percentage data and data which were identified as non-homogeneous (Bartlett's test) were subjected to arcsine transformation before analysis. Differences were regarded as significant when $P < 0.05$ (Zar, 1984).

5.3. Results

5.3.1. Dietary fatty acid composition

The control diet (P0), formulated with 100% FO, contained approximately 29% total saturates, mainly 16:0, almost 35% total monounsaturated fatty acids with approximately one-third as 18:1n-9 and one-third as the long chain monoenes 20:1 and 22:1, 7% n-6 fatty acids predominantly 18:2n-6 and 27% n-3 fatty acids predominantly n-3HUFA, 20:5n-3 and 22:6n-3 in approximately equal amounts (Table 5.2). Inclusion of graded amounts of CPO resulted in graded increased percentages of 16:0, 18:1n-9 and 18:2n-6 with concomitant decreased proportions of n-3HUFA, total PUFA and long chain monoenes. Thus, in the diet formulated with 100% CPO (P100), the levels of 16:0, 18:1n-9 and 18:2n-6 had risen to 38, 36 and 12% of total fatty acids, respectively, whereas 20:5n-3 and 22:6n-3 totalled only 4% (Table 5.2).

5.3.2. Experiment 1

Effects of CPO on enterocyte and hepatocyte HUFA synthesis activities

Dietary CPO had significant effects on HUFA synthesis as indicated by the desaturation of [1-^{14}C]18:3n-3, measured as the summed radioactivity recovered as desaturated fatty acid products (18:4, 20:4, 20:5, 22:5 and 22:6), in both caecal enterocytes and hepatocytes, but the effects were dependent on water temperature as indicated by the significant interaction (Table 5.3). Increasing dietary CPO gave a graded increase in fatty acid desaturation in caecal enterocytes from fish held at 11 °C, and generally increased activity in fish maintained at 15 °C but dietary CPO decreased fatty acid desaturation in fish maintained at the lowest water temperature (Fig. 5.1A). Thus, dietary CPO had no significant effect on fatty acid desaturation in caecal

enterocytes when analysed by two-way ANOVA because of the significant interaction with temperature (Table 5.3). The effect of temperature on fatty acid desaturation in caecal enterocytes was highly significant (Table 5.3) and a general pattern of decreasing activity with increasing water temperature was observed (Figure 5.1). Fatty acid desaturation in hepatocytes increased in a graded manner with dietary CPO inclusion at 11 °C, and similarly, CPO also increased desaturation at 7 °C but had little effect at 15 °C (Figure 5.1B). Temperature had a significant effect (Table 5.3) with generally lower activities in hepatocytes from fish held at the highest water temperature (Figure 5.1B). HUFA synthesis, that is total desaturation of [1-^{14}C]18:3n-3, in hepatocytes generally exceeded that in enterocytes other than that in fish fed diet P0 at 7 °C (Figures 5.1A and B).

Table 5.3. Significance of effects of dietary palm oil and water temperature on fatty acid desaturation/elongation and β-oxidation in enterocytes and hepatocytes.

Cells/Assay	Treatment	F-value	Significance
Enterocytes			
Desaturation	Palm oil	2.67	0.0645
	Temperature	89.83	<0.0001
	Interaction	18.00	<0.0001
β-oxidation	Palm oil	37.49	<0.0001
	Temperature	108.93	<0.0001
	Interaction	46.97	<0.0001
Hepatocytes			
Desaturation	Palm oil	58.70	<0.0001
	Temperature	31.18	<0.0001
	Interaction	16.90	<0.0001
β-oxidation	Palm oil	5.23	0.0064
	Temperature	40.21	<0.0001
	Interaction	0.57	0.7517

Data from Figures 5.1 and 5.2 were analysed by two-way ANOVA as described in the Methods section.

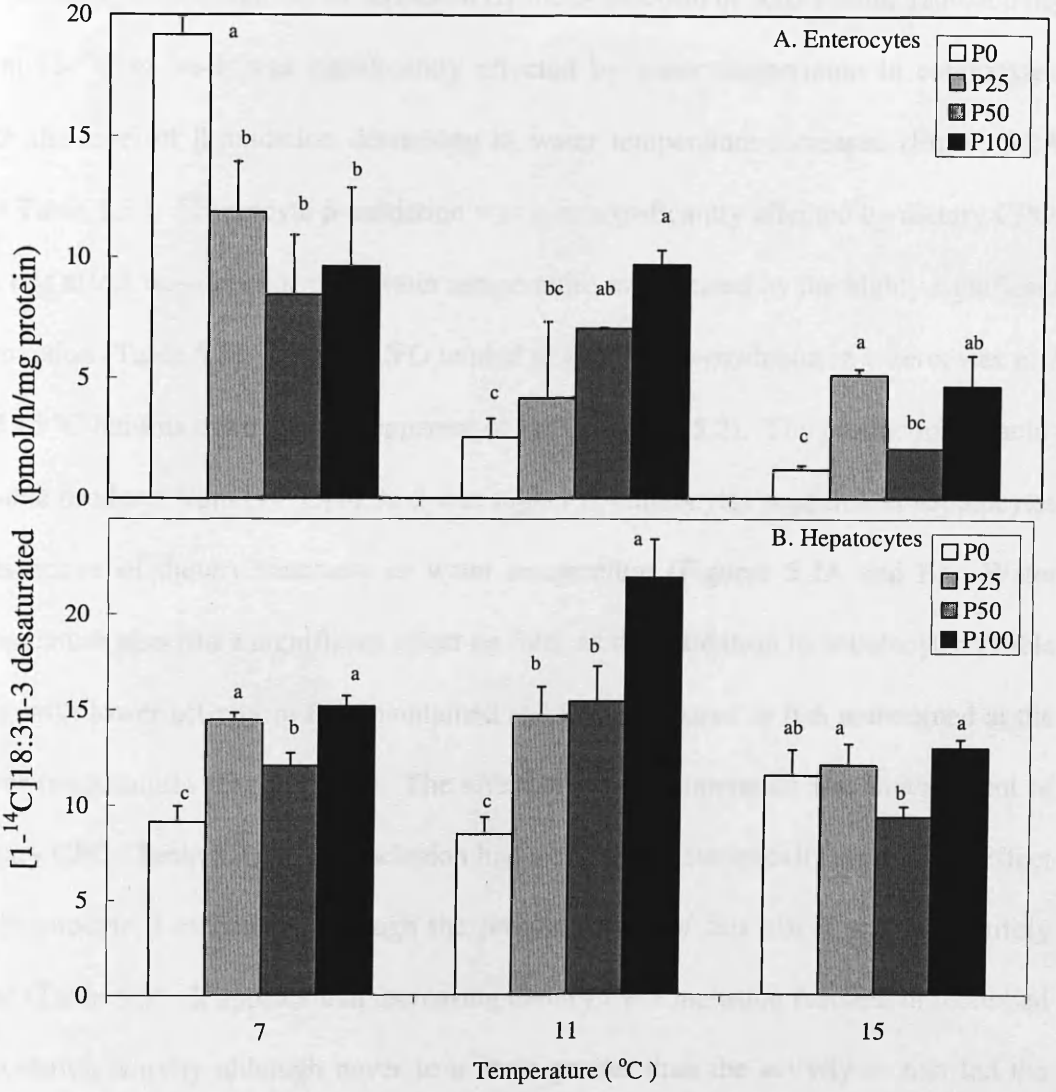


Figure 5.1. Total fatty acid desaturation/elongation activity in (A) caecal enterocytes and in (B) hepatocytes after feeding the experimental diets containing CPO for 4 weeks. Results are means ± standard deviation (n = 3) and represent the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Columns for a specific temperature with different superscript letters are significantly different ($P < 0.05$) as determined by one-way ANOVA.

Effects of CPO on enterocytes and hepatocyte fatty acid oxidation activities

Fatty acid oxidation, as measured by the production of acid-soluble radioactivity from [1-^{14}C]18:3n-3, was significantly affected by water temperature in enterocytes, with the level of β-oxidation decreasing as water temperature increased (Figure 5.2A and Table 5.3,). Enterocyte β-oxidation was also significantly affected by dietary CPO, but this effect was dependent on water temperature as indicated by the highly significant interaction (Table 5.3). Dietary CPO tended to decrease β-oxidation in enterocytes at 7 and 15 °C but this trend was not apparent at 11 °C (Figure 5.2). The production of acid-soluble products from [1-^{14}C]18:3n-3 was higher in enterocytes than that in hepatocytes irrespective of dietary treatment or water temperature (Figures 5.2A and B). Water temperature also had a significant effect on fatty acid β-oxidation in hepatocytes (Table 5.3), with lower activity in fish maintained at 15 °C compared to fish maintained at the lower temperatures (Figure 5.2B). The effect of water temperature was independent of dietary CPO (Table 5.3). CPO inclusion had a slight, but statistically significant, effect on hepatocyte β-oxidation, although the precise nature of this effect was not entirely clear (Table 5.3). It appears that increasing dietary CPO inclusion resulted in increased β-oxidation activity although never to a level greater than the activity in fish fed the 100% FO diet (PO) and, as a result, no statistically significant differences were obtained upon one-way ANOVA (Figure 5.2).

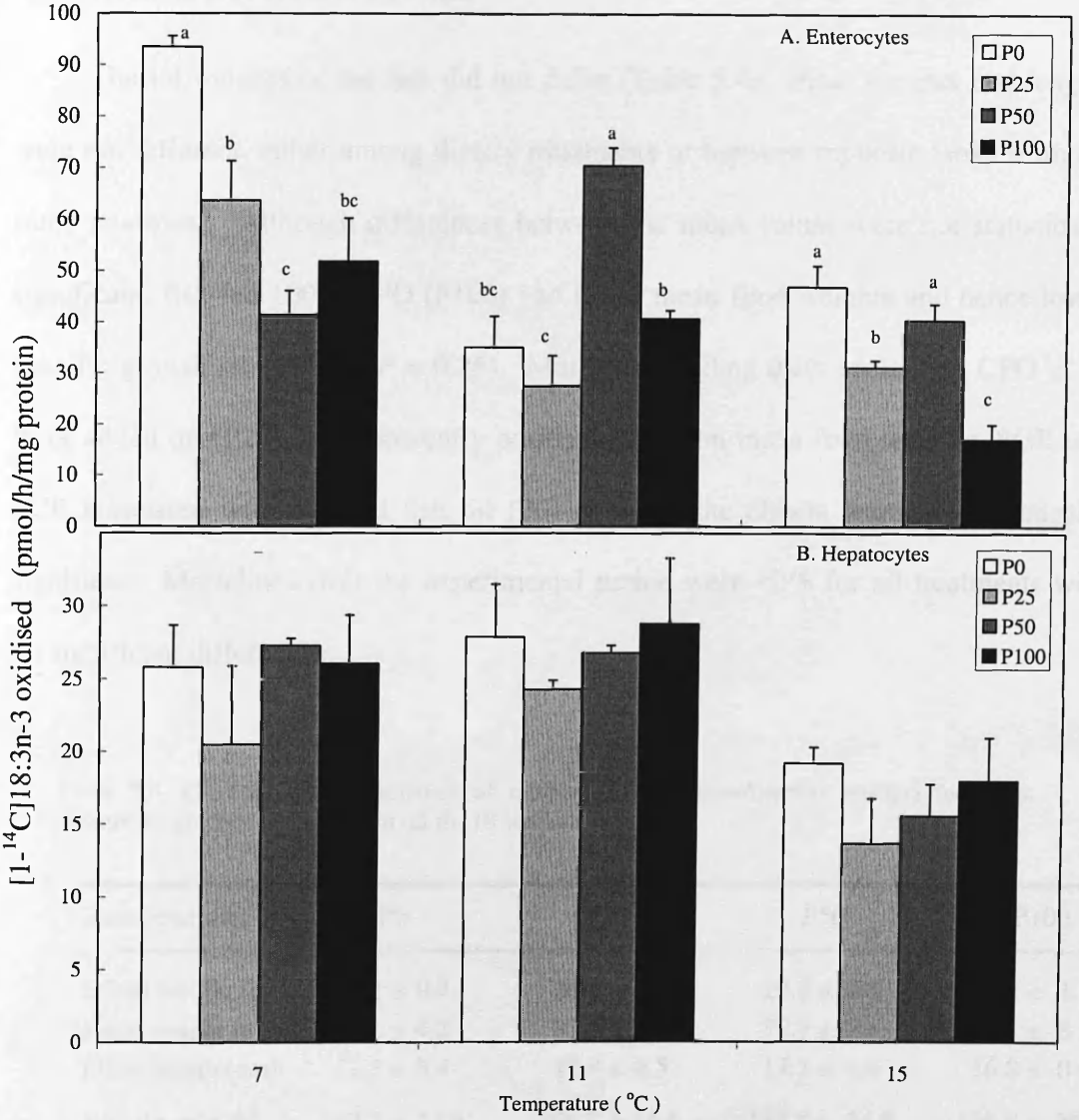


Figure 5.2. Fatty acid β-oxidation activity in (A) ceacal enterocytes and in (B) hepatocytes after feeding the experimental diets containing CPO for 4 weeks. Results are means ± standard deviation (n = 3) and represent the rate of oxidation (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to acid-soluble products. Columns for a specific temperature with different superscript letters are significantly different ($P < 0.05$) as determined by one-way ANOVA. There were no significant differences among dietary treatments at a specific temperature in hepatocytes.

5.3.3. Experiment 2

Effect of dietary CPO on fish growth

Initial weights of the fish did not differ (Table 5.4). Final weights and lengths were not different, either among dietary treatments or between replicate tanks from the same treatment. Although differences between the mean values were not statistically significant, fish fed 100% CPO (P100) had lower mean final weights and hence lower specific growth rate (SGR) ($P = 0.26$). Moreover, feeding diets containing CPO at 25 % of added oil (P25), had apparently positive effects on mean final weights, SGR and FCR compared with fish fed fish oil (P0) although the effects were not statistically significant. Mortalities over the experimental period were <2% for all treatments with no significant differences.

Table 5.4. Growth and performance of rainbow trout (*Oncorhynchus mykiss*) fed diets containing graded levels of palm oil for 10 weeks.

Parameter/diet	P0	P25	P50	P100
Initial weight (g)	27.2 ± 0.7	26.6 ± 0.5	27.8 ± 0.4	27.3 ± 1.1
Final weight (g)	76.1 ± 4.5	81.0 ± 2.9	76.7 ± 7.1	70.1 ± 8.5
Final length (cm)	17.5 ± 0.4	17.9 ± 0.5	17.3 ± 0.6	16.8 ± 0.4
Weight gain %[1]	180.2 ± 21.9	204.7 ± 16.6	175.8 ± 24.8	156.8 ± 26.8
SGR[2]	1.47 ± 0.11	1.59 ± 0.08	1.45 ± 0.13	1.34 ± 0.15
FCR[3]	1.50 ± 0.21	1.44 ± 0.12	1.79 ± 0.62	1.68 ± 0.36
Mortality (%)	1.0 ± 1.7	0.0 ± 0.0	1.7 ± 0.6	1.0 ± 0.0

Values are mean ± standard deviation (n = 3); [1]Weight gain = (final weight – initial weight) / (initial weight) x 100; [2]Specific growth rate (SGR) = [Ln (final weight) – Ln (initial weight)] / (days) x 100; [3]Feed conversion ratio (FCR) = feed offered / weight gain. Data were subjected to one-way ANOVA as described in the Methods section. There were no significant differences ($P > 0.05$) in initial weight, final weight or length of fish either among dietary treatments or between replicate tanks within the same treatment.

Effect of CPO on fatty acid composition of pyloric caeca, liver and flesh.

The rank order of the major fatty acids from total lipid of pyloric caeca from trout fed diet P0 (100% FO) was 16:0 > 18:1n-9 > 22:6n-3 > 16:1n-7 > 20:1n-9 > 20:5n-3 (Table 5.5). The changes in fatty acid composition of the diets in response to increasing replacement of FO with CPO, described above, were reflected in the caecal fatty acid compositions with graded increased proportions of 18:1n-9, 18:2n-6 and 16:0, and decreased proportions of n-3HUFA (Table 5.5). The rank order of the major fatty acids from total lipid of liver from fish fed diet P0 was 22:6n-3 > 16:0 > 18:1n-9 > 18:0 > 20:5n-3 > 16:1n-7 (Table 5.6). Increasing replacement of FO with CPO, was largely reflected in the liver fatty acid compositions with graded increased proportions of 18:1n-9 and 18:2n-6, but not 16:0, and decreased proportions of n-3HUFA (Table 5.6). The principal fatty acids of total lipid from flesh from fish fed diet P0 were, in rank order, 22:6n-3 > 16:0 > 18:1n-9 > 20:5n-3 > 16:1n-7 > 18:2n-6 (Table 5.7). Similar changes in fatty acid compositions of flesh in response to increasing dietary CPO content, as described above for caeca, were observed with increased percentages of 18:1n-9, 18:2n-6 and 16:0, and decreased proportions of virtually all other fatty acids, including n-3HUFA, other than 18:0 (Table 5.7).

Table 5.5. Fatty acid compositions (percentage of total fatty acids by weight) of total lipid of pyloric caeca from rainbow trout fed diets containing palm oil.

	Pyloric caeca			
Fatty acid	P0	P25	P50	P100
14:0	5.0 ± 0.5 [a]	3.8 ± 0.3 [b]	3.2 ± 0.2 [b]	1.6 ± 0.1 [c]
16:0	20.7 ± 3.7 [b]	25.9 ± 1.7 [ab]	27.2 ± 3.0 [a]	27.5 ± 1.3 [a]
18:0	4.2 ± 0.8	4.7 ± 0.6	5.0 ± 0.6	4.3 ± 0.2
Total saturated[1]	30.5 ± 5.1	34.9 ± 2.3	35.6 ± 3.8	33.6 ± 1.5
16:1n-7	7.4 ± 0.7 [a]	5.2 ± 0.1 [b]	4.4 ± 0.1 [b]	2.6 ± 0.5 [c]
18:1n-9	16.2 ± 1.4 [d]	24.8 ± 0.7 [c]	27.9 ± 0.6 [b]	38.1 ± 1.5 [a]
18:1n-7	3.3 ± 0.3 [a]	2.9 ± 0.1 [a]	2.3 ± 0.1 [b]	1.7 ± 0.1 [c]
20:1n-9	7.0 ± 0.7 [a]	4.8 ± 0.6 [b]	3.9 ± 0.0 [b,c]	2.8 ± 0.1 [c]
22:1n-11	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1n-9	1.0 ± 0.0 [a]	0.8 ± 0.1 [a,b]	0.7 ± 0.1 [b]	0.5 ± 0.0 [c]
Total monoenes[2]	35.6 ± 2.8 [a]	39.3 ± 0.6 [a]	39.8 ± 0.4 [a]	46.2 ± 2.1 [b]
18:2n-6	7.1 ± 0.6 [b]	8.3 ± 1.3 [a,b]	8.1 ± 1.3 [a,b]	10.4 ± 1.1 [a]
20:2n-6	0.4 ± 0.0 [b]	0.4 ± 0.0 [b]	0.4 ± 0.0 [b]	0.6 ± 0.1 [a]
20:3n-6	0.2 ± 0.0 [b]	0.2 ± 0.0 [b]	0.3 ± 0.0 [b]	0.4 ± 0.1 [a]
20:4n-6	0.7 ± 0.0 [a]	0.6 ± 0.1 [a,b]	0.5 ± 0.0 [a,b]	0.5 ± 0.1 [b]
Total n-6PUFA[3]	8.6 ± 0.6 [b]	9.7 ± 1.2 [a,b]	9.6 ± 1.4 [a,b]	12.2 ± 1.3 [a]
18:3n-3	1.3 ± 0.1 [a]	0.8 ± 0.1 [b]	0.7 ± 0.1 [b]	0.4 ± 0.1 [c]
18:4n-3	2.0 ± 0.3 [a]	1.0 ± 0.2 [b]	0.7 ± 0.1 [b,c]	0.3 ± 0.1 [c]
20:4n-3	1.1 ± 0.1 [a]	0.6 ± 0.1 [b]	0.6 ± 0.1 [b]	0.2 ± 0.1 [c]
20:5n-3	5.4 ± 1.0 [a]	2.8 ± 0.6 [b]	2.3 ± 0.5 [b,c]	0.8 ± 0.2 [c]
22:5n-3	1.3 ± 0.2 [a]	0.6 ± 0.1 [b]	0.6 ± 0.1 [b,c]	0.2 ± 0.0 [c]
22:6n-3	14.2 ± 2.5 [a]	9.7 ± 0.9 [b]	9.6 ± 1.3 [b]	5.8 ± 1.6 [b]
Total n-3PUFA[4]	25.3 ± 3.8 [a]	15.6 ± 1.4 [b]	14.4 ± 2.3 [b]	7.7 ± 2.0 [c]
Total PUFA	33.9 ± 1.1 [a]	25.9 ± 2.2 [b]	24.6 ± 3.5 [b]	20.2 ± 3.3 [b]
n3/n6	2.9 ± 0.5 [a]	1.6 ± 0.2 [b]	1.5 ± 0.0 [b]	0.6 ± 0.1 [c]

Results are means ± SD (n = 4). Values within a row for a particular tissue with a different superscript letter are significantly different ($P < 0.05$). PUFA, polyunsaturated fatty acids. [1]Totals include 15:0 present at up to 0.5%; [2]Totals include 16:1n-9 and 20:1n-7 present at up to 0.5%; [3]Totals include 18:3n-6, 22:4n-6 and 22:5n-6, present at up to 0.5%; [4]Totals include 20:3n-3 present at up to 0.2%.

Table 5.6. Fatty acid compositions (percentage of total fatty acids by weight) of total lipid of liver from rainbow trout fed diets containing palm oil.

	Liver			
Fatty acid	P0	P25	P50	P100
14:0	2.0 ± 0.3	2.1 ± 0.9	1.6 ± 0.0	1.0 ± 0.1
16:0	23.3 ± 1.6	20.7 ± 1.3	21.2 ± 2.1	21.6 ± 1.6
18:0	6.6 ± 0.5	5.6 ± 0.2	6.3 ± 0.6	6.6 ± 1.2
Total saturated[1]	32.5 ± 1.5	28.8 ± 2.5	29.5 ± 2.8	29.4 ± 1.5
16:1n-7	3.8 ± 1.4	4.6 ± 1.5	3.9 ± 0.1	2.6 ± 0.7
18:1n-9	12.1 ± 3.2 [b]	21.1 ± 2.0 [a,b]	22.0 ± 3.4 [a]	24.6 ± 5.6 [a]
18:1n-7	2.1 ± 0.3 [a,b]	2.4 ± 0.1 [a]	2.0 ± 0.2 [a,b]	1.7 ± 0.2 [b]
20:1n-9	2.6 ± 0.8	3.8 ± 0.8	3.4 ± 0.6	3.9 ± 1.1
22:1n-11	1.0 ± 0.5	1.7 ± 1.2	0.9 ± 0.5	0.4 ± 0.0
24:1n-9	1.0 ± 0.1 [a]	0.9 ± 0.1 [a,b]	0.9 ± 0.1 [a,b]	0.8 ± 0.1 [b]
Total monoenes[2]	22.6 ± 6.2	34.8 ± 3.4	33.2 ± 4.8	34.2 ± 7.4
18:2n-6	2.6 ± 0.3 [b]	4.1 ± 1.6 [a,b]	3.8 ± 0.2 [a,b]	5.5 ± 0.4 [a]
20:2n-6	0.6 ± 0.1 [b]	0.8 ± 0.1 [b]	0.8 ± 0.1 [b]	1.6 ± 0.1 [a]
20:3n-6	0.3 ± 0.0 [b]	0.5 ± 0.2 [b]	0.7 ± 0.3 [b]	1.5 ± 0.2 [a]
20:4n-6	2.1 ± 0.6	1.5 ± 0.5	1.6 ± 0.1	2.3 ± 0.3
Total n-6PUFA[3]	6.0 ± 0.1 [b]	7.5 ± 1.1 [b]	7.5 ± 0.4 [b]	12.3 ± 0.7 [a]
18:3n-3	0.4 ± 0.0	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.0
18:4n-3	0.3 ± 0.1	0.4 ± 0.3	0.2 ± 0.1	0.0 ± 0.0
20:4n-3	0.5 ± 0.0 [a]	0.3 ± 0.2 [a]	0.2 ± 0.0 [a]	0.0 ± 0.0 [b]
20:5n-3	5.0 ± 1.4 [a]	2.9 ± 0.4 [a,b]	2.3 ± 0.3 [b]	1.0 ± 0.7 [b]
22:5n-3	1.4 ± 0.1 [a]	0.9 ± 0.1 [b]	0.8 ± 0.1 [b,c]	0.4 ± 0.3 [c]
22:6n-3	31.2 ± 3.8	24.0 ± 6.4	26.0 ± 2.2	22.3 ± 5.3
Total n-3PUFA[4]	38.9 ± 4.6 [a]	28.9 ± 5.7 [a,b]	29.8 ± 1.9 [a,b]	24.0 ± 6.3 [b]
Total PUFA	44.9 ± 4.7	36.4 ± 5.3	37.3 ± 2.2	36.3 ± 6.9
n3/n6	6.4 0.7 [a]	3.9 1.1 [b]	3.9 0.1 [b]	1.9 0.4 [c]

Results are means ± SD (n = 4). Values within a row for a particular tissue with a different superscript letter are significantly different ($P < 0.05$). PUFA, polyunsaturated fatty acids. [1]Totals include 15:0 present at up to 0.5%; [2]Totals include 16:1n-9 and 20:1n-7 present at up to 0.5%; [3]Totals include 18:3n-6, 22:4n-6 and 22:5n-6, present at up to 0.5%; [4]Totals include 20:3n-3 present at up to 0.2%.

Table 5.7. Fatty acid compositions (percentage of total fatty acids by weight) of total lipid of flesh from rainbow trout fed diets containing palm oil

	Flesh			
Fatty Acid	P0	P25	P50	P100
14:0	4.2 ± 0.2 [a]	3.0 ± 0.3 [b]	2.7 ± 0.2 [b]	1.4 ± 0.1 [c]
16:0	22.4 ± 0.3 [b]	24.5 ± 1.9 [a,b]	24.2 ± 1.3 [a,b]	26.5 ± 1.3 [a]
18:0	4.3 ± 0.1 [a]	4.1 ± 0.2 [a,b]	3.8 ± 0.3 [b]	3.9 ± 0.2 [a,b]
Total saturated[1]	31.8 ± 0.5	32.3 ± 2.6	31.2 ± 1.9	32.3 ± 1.7
16:1n-7	5.2 ± 0.1 [a]	4.2 ± 0.3 [b]	4.0 ± 0.3 [b]	2.3 ± 0.3 [c]
18:1n-9	11.6 ± 0.7 [d]	16.4 ± 0.9 [c]	20.2 ± 1.9 [b]	26.9 ± 1.6 [a]
18:1n-7	2.4 ± 0.1 [a]	2.1 ± 0.1 [b]	2.0 ± 0.0 [b]	1.5 ± 0.2 [c]
20:1n-9	3.6 ± 0.4 [a]	2.8 ± 0.3 [b]	2.6 ± 0.1 [b]	2.0 ± 0.1 [c]
22:1	3.5 ± 0.3 [a]	2.5 ± 0.2 [b]	2.3 ± 0.2 [b]	1.0 ± 0.2 [c]
24:1n-9	0.7 ± 0.0 [a]	0.7 ± 0.1 [a]	0.6 ± 0.0 [a]	0.5 ± 0.0 [b]
Total monoenes[2]	27.2 ± 1.7 [c]	28.9 ± 1.6 [b,c]	32.0 ± 2.4 [a,b]	34.6 ± 1.2 [a]
18:2n-6	4.5 ± 0.2 [d]	5.7 ± 0.2 [c]	6.8 ± 0.4 [b]	9.0 ± 0.4 [a]
20:2n-6	0.4 ± 0.0 [b]	0.4 ± 0.0 [b]	0.4 ± 0.0 [b]	0.7 ± 0.1 [a]
20:3n-6	0.2 ± 0.0 [b]	0.2 ± 0.0 [b]	0.3 ± 0.0 [b]	0.6 ± 0.1 [a]
20:4n-6	0.7 ± 0.0 [b]	0.7 ± 0.0 [b]	0.6 ± 0.0 [b]	0.9 ± 0.1 [a]
Total n-6 PUFA[3]	6.1 ± 0.2 [c]	7.5 ± 0.3 [b]	8.5 ± 0.5 [b]	11.9 ± 0.7 [a]
18:3n-3	0.8 ± 0.0 [a]	0.7 ± 0.0 [b]	0.7 ± 0.0 [b]	0.4 ± 0.0 [c]
18:4n-3	1.1 ± 0.0 [a]	0.8 ± 0.0 [b]	0.7 ± 0.0 [b,c]	0.4 ± 0.2 [c]
20:4n-3	0.9 ± 0.1 [a]	0.7 ± 0.0 [b]	0.7 ± 0.0 [b]	0.3 ± 0.1 [c]
20:5n-3	5.9 ± 0.2 [a]	4.7 ± 0.3 [a,b]	4.2 ± 0.8 [b]	2.1 ± 0.5 [c]
22:5n-3	1.5 ± 0.0 [a]	1.2 ± 0.1 [b]	1.0 ± 0.0 [c]	0.5 ± 0.1 [d]
22:6n-3	24.5 ± 1.5 [a]	23.3 ± 1.7 [a]	21.0 ± 2.8 [a,b]	17.5 ± 2.5 [b]
Total n-3 PUFA[4]	34.9 ± 1.3 [a]	31.3 ± 1.5 [a]	28.2 ± 3.7 [a,b]	21.2 ± 3.3 [b]
Total PUFA	41.0 ± 1.1 [a]	38.8 ± 1.8 [a,b]	36.8 ± 3.2 [a,b]	33.1 ± 2.6 [b]
(n-3) / (n-6)	5.8 ± 0.5 [a]	4.2 ± 0.1 [b]	3.4 ± 0.7 [b]	1.8 ± 0.4 [c]

Results are means ± standard deviations (n = 4). Values within a row for a particular tissue with a different superscript letter are significantly different ($P < 0.05$). PUFA, polyunsaturated fatty acids. [1]Totals include 15:0 present at up to 0.5%; [2]Totals include 16:1n-9 and 20:1n-7 present at up to 0.5%; [3]Totals include 18:3n-6, 22:4n-6 and 22:5n-6, present at up to 0.5%; [4]Totals include 20:3n-3 present at up to 0.2%.

Effect of CPO on enterocyte and hepatocyte HUFA synthesis activity

Increasing levels of dietary CPO resulted in significantly increased HUFA synthesis in both enterocytes and hepatocytes as measured by the recovery of radioactivity in the summed desaturated products (18:4, 20:4, 20:5, 22:5 and 22:6) of [1-^{14}C]18:3n-3 (Figure 5.3). In both cell types, the rate of HUFA synthesis was highest in fish fed the P100 diet, being 2.8- and 2.9-fold higher in enterocytes and hepatocytes, respectively, than the lowest activity observed in fish fed 100% FO (P0). There was no significant difference in HUFA synthesis between fish fed the two intermediate diets, P25 and P50, in either cell type. The individual products of desaturation/elongation activities in enterocytes showed the highest rates of HUFA synthesis when CPO replaced 100 % of the FO in the diets (P100). The level of 18:4 in enterocytes correlated in a graded manner to CPO inclusion and the rest of the desaturation products were relatively low in comparison to 18:4 (Figure 5.4) and also in comparison with that of hepatocytes (Figure 5.5). Similarly to 18:4 products in enterocytes, the graded response in HUFA synthesis to graded increases in dietary CPO was observed in all the products of [1-^{14}C]18:3n-3 desaturation and elongation in hepatocytes, with most being increased in a step-wise manner (Figure 5.5). The largest increase in recovery of radioactivity in desaturated products between fish fed 100% CPO (P100) and 100% FO (P0) were in 22:6 (5.0-fold) and 20:5 (3.6-fold), followed by 18:4, 22:5 and 20:4 at 3.2-, 2.4- and 2.2-fold, respectively (Figure 5.3).

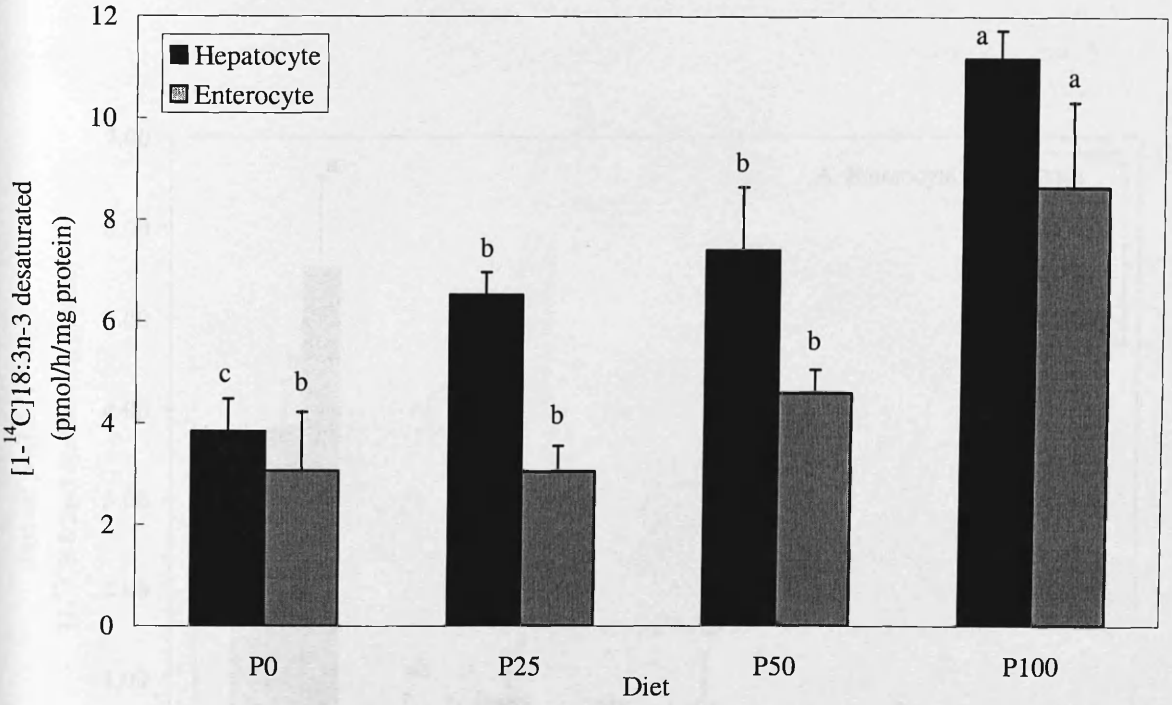


Figure 5.3. Total fatty acid desaturation/elongation activity in hepatocytes and caecal enterocytes after feeding the experimental diets for 10 weeks. Results are means ± S.D. (n = 4) and represent the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Columns assigned to a specific cell type with different superscript letters are significantly different.

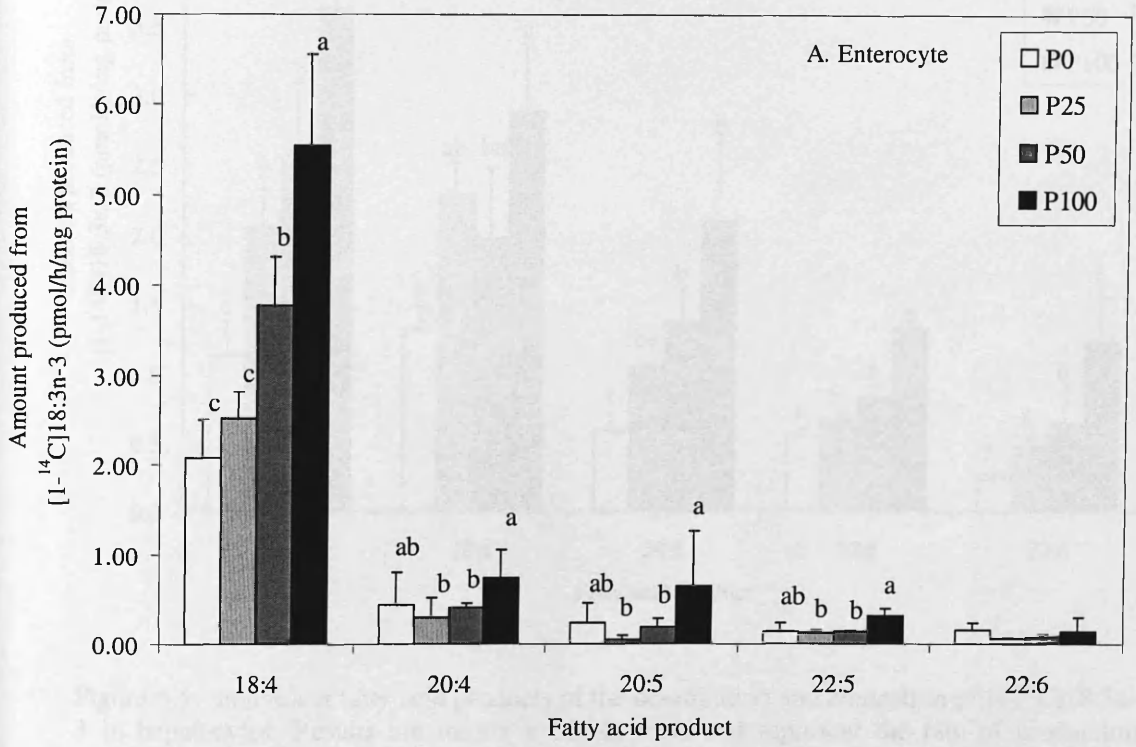


Figure 5.4. Individual fatty acid products of the desaturation and elongation of [1-^{14}C]18:3n-3 in enterocytes. Results are means ± SD (n = 4) and represent the rate of production (pmol/h/mg protein) of individual fatty acids as determined by the recovery of radioactivity in each fatty acid fraction. Columns referring to a specific fatty acid having different superscript letters are significantly different.

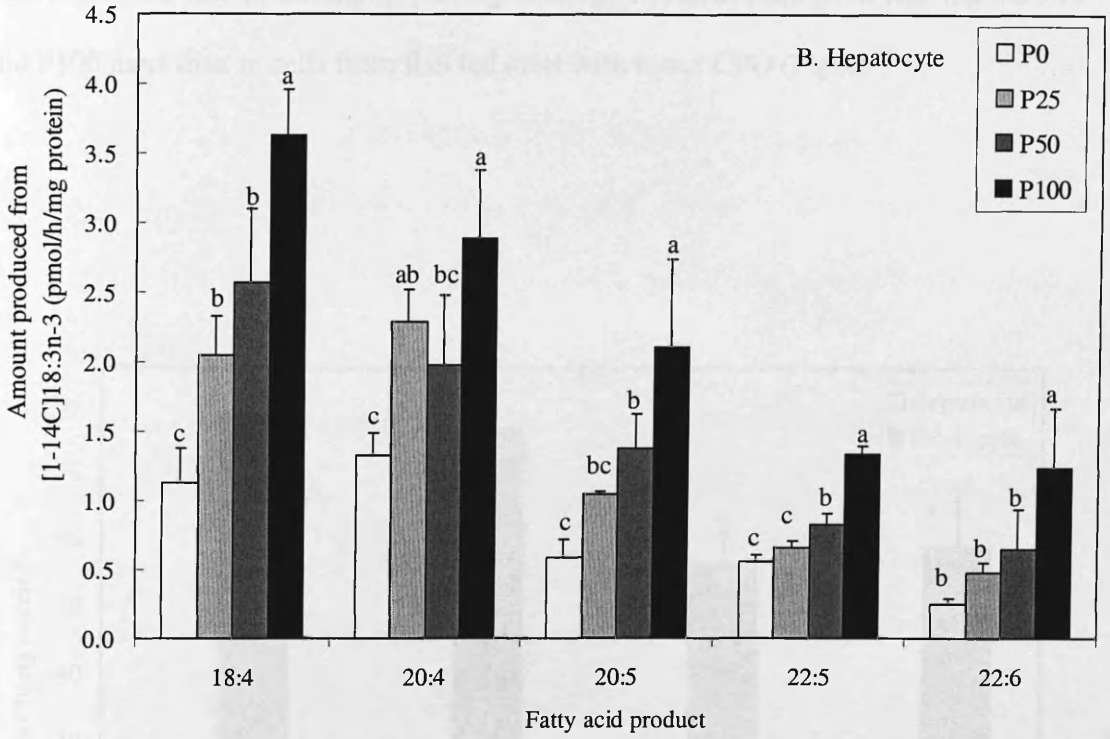


Figure 5.5. Individual fatty acid products of the desaturation and elongation of [1-^{14}C]18:3n-3 in hepatocytes. Results are means ± SD (n = 4) and represent the rate of production (pmol/h/mg protein) of individual fatty acids as determined by the recovery of radioactivity in each fatty acid fraction. Columns referring to a specific fatty acid having different superscript letters are significantly different.

Effects of CPO on enterocyte and hepatocyte fatty acid oxidation activities

The rate at which [1-^{14}C]18:3n-3 was oxidised was 2- to 3-fold higher in enterocytes than in hepatocytes (Figure 5.6). However, whereas the rate of oxidation of [1-^{14}C]18:3n-3 in hepatocytes was not significantly affected by dietary CPO, in caecal enterocytes the rate of oxidation was significantly lower in cells from fish fed the P75 and P100 diets than in cells from fish fed diets with lower CPO (Figure 5.6).

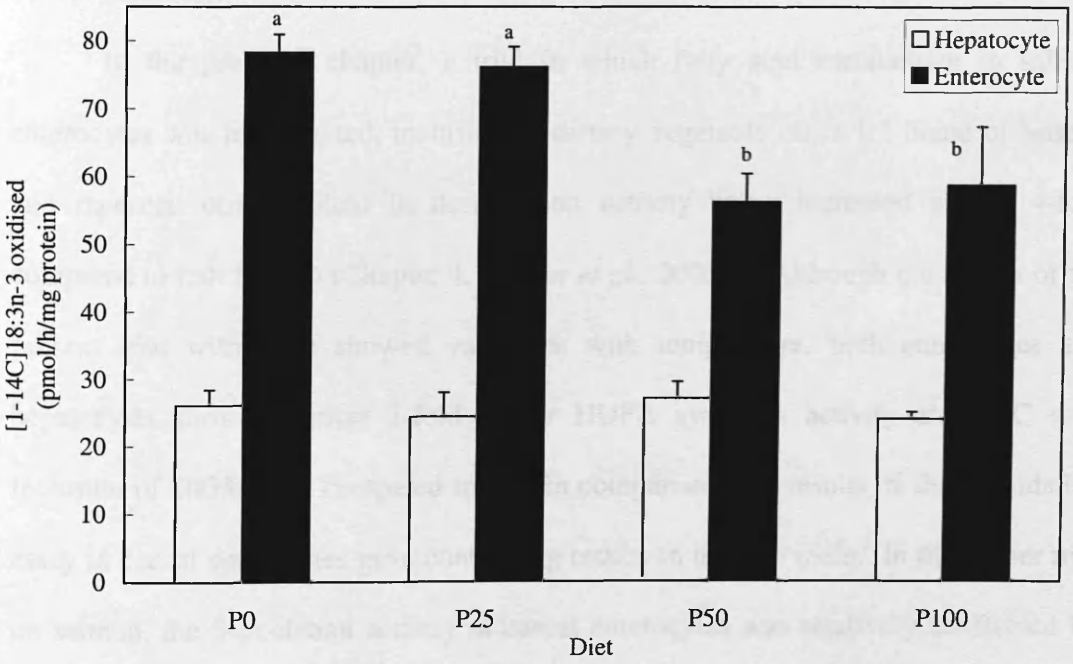


Figure 5.6. Fatty acid oxidation activity in caecal enterocytes and hepatocytes after feeding the experimental diets for 10 weeks. Results are means ± SD (n = 4) and represent the rate of oxidation (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to acid soluble products. Columns assigned to a specific cell type with different superscript letters are significantly different.

5.4. Discussion

Experiment 1

In the first experiment, the primary aim was to determine if there was an interaction between water temperature and nutritional factors in the regulation of fatty acid metabolism in caecal enterocytes of trout and this was achieved. The results clearly showed that both water temperature and diet had significant effects on fatty acid desaturation/elongation and β-oxidation. In addition, interaction or competition between the pathways was observed, as the effects on the anabolic pathway, HUFA synthesis, were at least partly dependent upon the effects on the catabolic pathway, fatty acid β-oxidation.

In the previous chapter, a trial in which fatty acid metabolism in salmon enterocytes was investigated, inclusion of dietary vegetable oil, a 1:1 blend of linseed and rapeseed oils, resulted in desaturation activity being increased almost 4-fold compared to fish fed FO (Chapter 4, Tocher *et al.*, 2003a). Although the results of the present trial with trout showed variations with temperature, both enterocytes and hepatocytes showed almost 3-fold higher HUFA synthesis activity at 11 °C with inclusion of 100% CPO compared to FO. In comparison, the results of the β-oxidation assay in caecal enterocytes gave contrasting results in the two trials. In the earlier trial on salmon, the β-oxidation activity in caecal enterocytes was relatively unaffected by the inclusion of the dietary VO and the amount of radioactivity recovered was never more than 10 pmol/h/mg protein. In the present study with trout, dietary CPO tended to reduce β-oxidation of the [1-^{14}C]18:3n-3 substrate in enterocytes. Although the radioactivity recovered was higher in trout enterocytes than that in hepatocytes, both cell types showed greater catabolic activity compared to the activity obtained with the

salmon cells in the previous chapter, ranking between 10 and 100 pmol/h/mg protein. The differences between the results from these two trials were interesting and suggested that further work was indeed necessary to investigate fatty acid metabolism in intestinal cells, and the interaction of fatty acid desaturation and β-oxidation pathways in hepatocytes and other cell types.

Irrespective, it was clear that the most obvious effect of temperature was that activities of the desaturation/elongation pathway and β-oxidation were lower at the highest water temperature of 15 °C in both caecal enterocytes and hepatocytes. The highest desaturation/elongation activity was observed in fish maintained at 7 °C in enterocytes and 11 °C in hepatocytes. In a much earlier study, it had been reported that Δ6 and Δ5 desaturase activities of liver microsomes were 2- and 4-fold higher at 16 °C compared to 30 °C in *Pimelodus maculates* (Ninno *et al.*, 1974). In a similar way, the activity of Δ6 desaturase was increased in liver microsomes from common carp (*Cyprinus carpio*) (Schuenke and Wodke, 1983) and rainbow trout (Hagar and Hazel, 1985) acclimated to 10 °C and 5° C, respectively, compared to fish acclimated to 30 °C and 20 °C, respectively. DeTorrengo and Brenner (1976) showed that when fish acclimated to 28 °C were shifted to 18 °C, the activity of Δ6 desaturase activity was initially decreased one day after transfer, probably due to a kinetic effect, but subsequently increased by one week after transfer, presumed to be due to induction of gene transcription, although this was unable to be confirmed as this was long before any animal desaturase genes had been cloned. The activity of stearoyl Δ9 fatty acid desaturase, responsible for the production of 18:1n-9 from 18:0, is also increased in fish in response to lower environmental temperature (Schuenke and Wodtke, 1983; Hagar and Hazel, 1985; Wodke and Cossins, 1991). The Δ9 SCD gene was cloned from carp (Tiku *et al.*, 1996) and subsequent work showed that the cold-induced increase in Δ9

SCD activity in carp was indeed due to increased gene expression, the first demonstration of transcriptional control of desaturase activity in fish (Trueman *et al.*, 2000). It also indicated an important role for this enzyme in cold acclimation in fish. Interestingly though, the specific activity of carp Δ9 activity was also increased isothermally by addition of 5 % foetal calf serum to the culture medium (Macartney *et al.*, 1996). The increased activity was also associated with increased transcript levels although it was not determined whether this was due to increased transcript stability or increased transcription (Macartney *et al.*, 1996). However, it showed that the fish Δ9 SCD activity could be regulated by both temperature and nutrition, almost certainly through transcriptional regulation.

The results of the combined fatty acid desaturation/elongation and β-oxidation assay showed that, in both hepatocytes and enterocytes, more radioactivity was recovered in acid soluble products than was recovered in desaturated products, indicating that a greater proportion of the exogenously added [1-^{14}C]18:3n-3 was being oxidised. The fatty acid oxidation activity was generally higher in trout enterocytes compared to hepatocytes as observed in the salmon study reported in the previous chapter (Tocher *et al.*, 2003a). As with desaturation/elongation activities, less 18:3n-3 was β-oxidised at 15 °C compared to 11 °C or 7 °C, but the physiological explanation for this is not obvious in a poikilothermic animal like fish. There are surprisingly few data in the literature on β-oxidation of fatty acids in fish, particularly in hepatocytes or intestinal tissue (Small and Connock, 1981; Sidell *et al.*, 1995; Frøyland *et al.*, 1998; Nanton *et al.*, 2003; Torstensen and Stubhaug, 2004; Stubhaug *et al.*, 2005). The effect of temperature has not been studied, but it is perhaps noteworthy that liver is a site of significant β-oxidation in Antarctic fish with peroxisomal β-oxidation possibly accounting for up to 30 % of total hepatic activity (Crockett and Sidell, 1993), whereas,

in contrast, liver is not regarded as a major site of β-oxidation activity in salmon (Frøyland *et al.*, 2000).

A previous study with Atlantic salmon in seawater showed that graded increases in dietary palm oil in post-smolts gave similarly graded increases in fatty acid desaturation and elongation in isolated hepatocytes (Bell *et al.*, 2002). In that study, replacement of 100 % of FO with CPO resulted in a 10-fold increase in hepatocyte desaturation activity, which was greater than reported in several other studies in salmonids both in freshwater and seawater, in which increases of 2.4- to 2.8-fold were observed in fish fed diets in which 100 % of the fish oil was replaced by single vegetable oils or blends (Bell *et al.*, 1997, 2001a; Tocher *et al.*, 1997, 2000, 2001, 2003a). The present trial with trout in freshwater compares well with these latter studies, with hepatocyte desaturation activity at 11 °C in fish fed P100 being 2.6-fold greater than in fish fed P0. Thus, the very great stimulation of activity (10-fold) in the earlier trial with palm oil in salmon was not repeated in the present trial with trout. However, the total desaturation activity recorded in hepatocytes from salmon fed 100 % CPO in the earlier trial was around 21 pmol/h/mg protein which compares with the 22 pmol/h/mg protein recorded in hepatocytes from trout fed 100% CPO in this chapter (Bell *et al.*, 2002). Therefore, it appears the very large stimulation in the salmon trial was more the result of a rather low activity in fish fed 100 % FO rather than an unusually high level of activity in hepatocytes from fish fed 100 % CPO. This may be related to the mechanism of the increased HUFA synthesis activity in fish fed vegetable oils. However, it is still unclear whether increased desaturation activity in fish fed vegetable oils is the result of decreased levels of n-3HUFA in membrane lipids, and, thus, mediated by decreased product inhibition, or whether it is a result of increased

levels of membrane 18:2n-6 and/or 18:3n-3 and, thus, mediated by increased substrate provision.

In summary, this dietary trial using CPO as a replacement for FO in trout showed that, first, temperature had significant effects on fatty acid metabolism in rainbow trout, some of which were, at least partly, dependent upon dietary fatty acid composition. The most obvious effect was that fatty acid desaturation/elongation and β-oxidation activities were reduced in both caecal enterocytes and hepatocytes from fish maintained at the highest water temperature (15 °C). There were differences between the two cell types with the highest desaturation/elongation and β-oxidation activities tending to be in fish held at 7 °C in enterocytes, but 11 °C in the case of hepatocytes. Graded effects on fatty acid metabolism in response to graded dietary palm oil were most clearly observed in desaturation/elongation activities in both enterocytes and hepatocytes at 11 °C which was the ambient water temperature at the time of the experiment. The highest β-oxidation activities were generally observed in fish fed fish oil alone in both enterocytes and hepatocytes with palm oil inclusion having differential effects in the two cell types.

Experiment 2

The second trial aimed to further investigate the effects of dietary CPO on intestinal fatty acid metabolism in rainbow trout in a fully replicated trial over a longer duration allowing effects on growth performance and feed utilization efficiency to be determined. Therefore, triplicate groups of rainbow trout were fed for 10 weeks on diets in which CPO replaced the fish oil in a similar graded manner as the first trial above. A number of earlier studies had utilized vegetable oils in feed formulations for salmonids at replacement levels of up to 100 %, and no changes in growth rates and

feed conversion had been observed (Bell *et al.*, 2001a, 2002; Caballero *et al.*, 2002; Torstensen *et al.*, 2004). The results of this second trial with CPO were consistent with these findings. Feeding diets containing CPO, in the range of 25 – 100 % of added oil, had no significant effect on growth rate and feed conversion ratio, compared with fish fed 100 % fish oil (P0). However, fish fed 100 % CPO (P100) had lower mean final weights and hence lower SGR than those fed the other three diets, although the differences were not statistically significant. Interestingly, a similar non-significant reduction in final weight was observed in other trials in which 100 % of dietary fish oil was replaced by vegetable oil, specifically rapeseed oil in salmon (Bell *et al.*, 2001a; Torstensen *et al.*, 2004). In the present study, the lower final weight of fish fed 100 % CPO could be explained by the lower digestibility of the saturated fatty acids, especially 16:0, in diets containing 100% CPO compared to those containing fish oil (Ng *et al.*, 2003).

The fatty acid compositions of tissue lipids of salmonids are readily influenced by the fatty acid composition of dietary lipid and linear correlations exist between individual fatty acids in tissue total lipid and their concentrations in dietary lipid (Torstensen *et al.*, 2000; Bell *et al.*, 2001a, 2002, 2003a, b; Tocher *et al.*, 2003a). Fatty acid compositions of pyloric caeca and liver in the present study responded in a similar manner to the inclusion of CPO in the diet. Dietary palm oil inclusion was reflected in the fatty acid compositions of total lipid of all the tissues by graded increased percentages of 18:1n-9 and 18:2n-6, and decreased proportions of n-3 HUFA. The changes in liver fatty acid composition were very similar to those reported in the earlier trial in which trout were fed similar diets, but over a period of only four weeks at three different temperatures (Ng *et al.*, 2003). The magnitudes of the changes in the present trial, run at 13 °C, were most similar to the fish grown at 11 °C in the earlier trial. In the

present trial the initial size of the fish was half that used in the earlier trial (27 g v. 45 g), and the duration of the trial was 2.5 x longer (10 weeks v. 4 weeks). Therefore, it is noteworthy that the data are so similar between the trials, suggesting that the dietary influence on the tissue lipids had reached an equilibrium after only 4 weeks in rainbow trout weighing less than 50 g. Similar effects of dietary CPO were observed in both pyloric caeca and flesh, with the important exception that 16:0 levels in these tissues were also increased by increased dietary inclusion of CPO, whereas this was not observed in liver lipid in trout, either in this trial or in the previous trial (see Tocher *et al.*, 2004). Previously, the level of 16:0 was increased in a graded manner, although only moderately compared to 18:1n-9 and 18:2n-6, in the flesh of salmon fed incremental dietary CPO (Bell *et al.*, 2002). These data suggest that liver metabolism may be predisposed to treat 16:0 differently to most other fatty acids and that its level in liver is more tightly controlled than in other tissues, although all tissues appear to heavily oxidise 16:0 for energy. The difference in metabolism between liver and the other tissues may be related to lipid class compositions, with liver having less triacylglycerol and more phospholipid than muscle and caeca (Henderson and Tocher, 1987; Sargent *et al.*, 1989; Tocher, 1995). Palmitic acid is an important component of phospholipid, especially in the sn-1 position of phosphatidylcholine and, to a lesser extent, phosphatidylethanolamine (Henderson and Tocher, 1987; Tocher, 1995). Thus, the levels of 16:0 incorporated in tissues may be related to the level of phospholipid, or the phospholipid/triacylglycerol ratio, in specific tissues.

The present trial, showing that HUFA synthesis was increased in a generally incremental manner in isolated enterocytes and hepatocytes in trout fed increasing levels of dietary CPO was entirely consistent with previous data. Several earlier studies have shown that HUFA synthesis, as measured by the desaturation and elongation of

18:3n-3, is increased in salmonids, including trout, fed diets in which dietary fish oil is replaced with vegetable oils (Tocher *et al.*, 1997, 2000, 2001). In some trials, graded increments of dietary vegetable oil have been reflected in graded increases in the activity of the HUFA biosynthetic pathway. Specifically, it has been shown that graded increases in dietary palm oil, rapeseed oil and linseed oil all resulted in graded increases in HUFA synthesis in salmon (Bell *et al.*, 2001a, 2002; Tocher *et al.*, 2002). A similar incremental increase in HUFA synthetic activity in isolated caecal enterocytes was found in trout fed palm oil in the first experiment described in this chapter. In that experiment, which also focused on investigating the effects of growth temperature on fatty acid digestibility (see Ng, 2003), the effects of temperature significantly impacted on the effects of diet, and HUFA synthetic activity only correlated with dietary CPO at a growth temperature of 11 °C and not at 15 °C. The growth temperature in the present trial, at 13 °C, was chosen to be between these two values so it was noteworthy that the response was similar to that at 11 °C rather than 15 °C.

The previous experiment with palm oil had also indicated that both water temperature and diet affected the balance between desaturation/ elongation to HUFA and oxidation as possible fates of 18:3n-3, but also that the effects on HUFA synthesis and oxidation were, at least partly, inter-dependent. This second trial, without the complication of differential effects of temperature, gave some clear effects. In fish fed 100 % FO (P0), the rate of 18:3n-3 oxidation was around 7- and 25-fold greater than the rate of HUFA synthesis from 18:3n-3 in hepatocytes and enterocytes, respectively. However, increasing dietary CPO shifted this balance by increasing the rate of HUFA synthesis while either not affecting oxidation, as in hepatocytes, or decreasing it, as in enterocytes. As a consequence, in fish fed 100 % CPO (P100), the amount by which oxidation of 18:3n-3 exceeded HUFA synthesis was reduced to only 7- and 2-fold in

enterocytes and hepatocytes, respectively. Thus, although oxidation was still the primary fate of 18:3n-3 in both cell types, there was a 3- to 4-fold shift towards utilization of 18:3n-3 for HUFA synthesis rather than for energy via β-oxidation in response to dietary crude palm oil.

These data are generally consistent with those in the previous chapters and those from previously reported trials which have shown that more radioactivity from [1-^{14}C]18:3n-3 was recovered in acid soluble (oxidation) products than was recovered in desaturated/elongated products, in both enterocytes and hepatocytes, indicating that a greater proportion of the exogenously added [1-^{14}C]18:3n-3 was being oxidised (Tocher *et al.*, 2003a). The data are consistent with *in vivo* experiments with stable isotopes that have shown that the majority of dietary 18:3n-3 was catabolised in trout, as is the case in many other species, including humans (Bell *et al.*, 2001b). There are relatively few data in the literature on the effects of diet on β-oxidation of fatty acids in fish, particularly in intestinal tissue or liver (Small and Connock, 1981; Crockett and Sidell, 1993; Sidell *et al.*, 1995; Frøyland *et al.*, 2000; Norgarden *et al.*, 2003). However, different dietary oils including palm oil did not significantly affect β-oxidation capacity in red muscle of Atlantic salmon (Torstensen *et al.*, 2000). Also in the previous experiment as in other earlier trials, we have noted that oxidation of [1-^{14}C]18:3n-3 was not increased in hepatocytes by dietary vegetable oil in salmonids (Tocher *et al.*, 2003a; previous experiment). In the present experiment, dietary CPO tended to reduce oxidation of [1-^{14}C]18:3n-3 in isolated enterocytes.

The overarching objective of both trials described in this chapter was to determine the effects of dietary CPO on lipid and fatty acid metabolism in rainbow trout enterocytes in comparison with hepatocytes. In summary, the results showed that the inclusion of CPO in the diet of rainbow trout had significant effects on tissue fatty acid

compositions and also on fatty acid desaturation/elongation and oxidation, in both caecal enterocytes and hepatocytes, but without major detrimental effects on growth. One possible conclusion of this chapter is that CPO can be used as an effective substitute for fish oil in rainbow trout in terms of permitting similar growth and feed conversion, and having no apparent detrimental effects on fish health. The data have further confirmed that intestine of fish is an important site of HUFA synthesis and fatty acid β-oxidation, and that these processes are affected by changing the dietary fatty acid composition as in vegetable oil replacement of fish oil. Nevertheless, the complete understanding of these pathways in this organ/tissue is not yet achieved and will require further research.

CHAPTER SIX

6. RESEARCHING ALTERNATIVES TO FISH OIL IN AQUACULTURE. (RAFOA 1)

6.1. Introduction

Qualitative and quantitative EFA requirements vary between fish species and, although salmonids require both linoleate (18:2n-6) and α-linolenate (18:3n-3) at a combined level of around 1 % of the diet, their essentiality actually derives from their desaturation and elongation to the functionally active HUFA eicosapentaenoate (20:5n-3), docosahexaenoate (22:6n-3) and arachidonate (20:4n-6) (Sargent *et al.*, 1995, 1999). The activity of the fatty acyl desaturase enzymes involved in the conversion of C_{18} EFA to $C_{20/22}$ HUFA are known to be under nutritional regulation in mammals (Brenner, 1981) and this has also been demonstrated in fish (Tocher *et al.*, 1996; Sargent *et al.*, 2002). The regulation of the HUFA biosynthetic pathways in fish has become of great interest recently due to the urgent need to develop new aquaculture diets. Briefly, over-exploitation of wild fisheries has meant that an increasing proportion of fish for human consumption is provided by farmed fish (Tidwell and Allan, 2002). Paradoxically though, diets for aquaculture, including salmonid (salmon, trout and char) culture, have been based traditionally on fish meals and oils, derived from feed grade fisheries, as the predominant protein and lipid sources (Sargent and Tacon, 1999) and as a consequence, demand for fish oils is rapidly increasing and will soon exceed supply. For aquaculture to continue to expand and supply more of the global demand for fish, alternatives to fish oil must be found (Barlow, 2000), with the plant (vegetable) oils, which are rich in C_{18}

PUFA but devoid of the n-3HUFA abundant in fish oils, the only practical and sustainable alternative (Sargent *et al.*, 2002). Knowledge of endogenous synthesis of HUFA from C_{18} PUFA in fish is required to determine the effectiveness with which the fatty acids in vegetable oils can be utilized by commercially important cultured fish species like salmon (Sargent *et al.*, 2002).

The previous chapters have clearly demonstrated that the intestine, the first tissue to come into contact with the feed and thus the first to experience changed dietary formulations, is not simply a passive uptake and transport organ but can also participate in initial metabolic processing of dietary fatty acids. Thus, it was demonstrated that isolated caecal enterocytes are a site of significant HUFA synthesis that may be of physiological significance, not only to the intestine itself, but perhaps also to the whole fish. It was also demonstrated in the previous chapters that replacing fish oil with vegetable oils, specifically a 1:1 blend of rapeseed oil and linseed oil or palm oil in the diets of Atlantic salmon and rainbow trout, respectively, resulted in increased activity of the fatty acyl desaturation/elongation pathway in both isolated enterocytes and hepatocytes.

In replacing dietary fish oil with vegetable oils, an important consideration is that the levels of n-3HUFA in the flesh are not significantly reduced, as this would compromise the nutritional quality of the product to the human consumer. Endogenous production of n-3HUFA may alleviate this problem, but it requires the supply of sufficient dietary n-3HUFA precursor fatty acids, specifically 18:3n-3. The two previous trials described above, have shown that replacing dietary fish oil with vegetable oils resulted in a stimulation of the activity of the HUFA synthesis pathway in enterocytes as measured by the desaturation and elongation of [1-^{14}C18:3n-3. However, the extent to which this stimulation was the result of reduced dietary n-3HUFA,

reducing suppression of desaturase activities, or due to increased levels of C_{18} PUFA, increasing activity through increased substrate availability, was unclear. The possibility existed that the extent of stimulation of HUFA synthesis through desaturation/elongation may have been limited in the previous trials by the amount of dietary 18:3n-3 available. Palm oil is rich in 16:0 and 18:1n-9, but contains virtually no 18:3n-3 (Ng, 2002a). In the other trial, half the oil was provided by rapeseed oil which is rich in 18:2n-6 and 18:1n-9, but contains only low levels of 18:3n-3 (Padley *et al.*, 1986). Therefore, the results of previous chapters regarding the extent to which vegetable dietary oils can activate HUFA production in caecal enterocytes may be partially obscured.

The aim of this chapter was to determine the extent to which the HUFA synthesis capacity of caecal enterocytes can be stimulated when supplied with abundant substrate 18:3n-3. Few plant oils contain high levels of 18:3n-3, a notable exception being linseed oil which can contain up to 56 % 18:3n-3 with an 18:3n-3:18:2n-6 ratio of over 3 in selected strains (Padley *et al.*, 1986). Therefore, in this trial, due to its specific characteristics, linseed oil alone was chosen to replace dietary fish oil in order to raise the available substrate for conversion to EPA and DHA in enterocytes. Therefore, the primary hypothesis tested in the present chapter was that replacing fish oil with 18:3n-3-rich linseed oil in salmon diets, and therefore, increasing substrate fatty acid available would enable the fish to maximally synthesize n-3HUFA through maximally increased desaturation/elongation activity. However, *in vivo* studies using stable isotopes (deuterium-labelled 18:3n3), that had initially suggested that intestine may be an organ/tissue with hitherto unappreciated fatty acyl desaturation activity (Bell *et al.*, 2003c), had also shown that a large portion of dietary 18:3n-3 was actually oxidized (Bell *et al.*, 2001b). Therefore, in addition, a secondary aim was to determine the extent

to which the 18:3n-3 tracer was oxidized and compare that with the amount desaturated/elongated by measuring these pathways simultaneously in both enterocytes and hepatocytes.

Salmon smolts were stocked randomly into five seawater pens and, after acclimatization for three weeks, were fed for 40 weeks on diets in which the fish oil (FO) was replaced in a graded manner (0, 25, 50, 75 and 100 % replacement) by linseed oil (LO). At the end of the trial, fatty acyl desaturation/elongation and oxidation activities were determined in isolated caecal enterocytes and isolated hepatocytes using [1-^{14}C]18:3n-3 as substrate, and samples of intestinal tissue and liver were collected for analysis of lipid and fatty acid composition.

6.2.Materials and methods

6.2.1. Experimental fish and diets

In June 2001, Atlantic salmon post-smolts (*Salmo salar* L.) were randomly assigned to 5 cages (5 m x 5 m; 600 fish per cage), with the mean initial weight across the five cages being 127 ± 3 g (range 123 to 130 g). The smolts were fed one of five diets, consisting of a control diet containing fish oil alone (FO) and four diets in which the FO was replaced in a graded manner by linseed oil (LO). Specifically, the five diets were 100 % FO (FO), 100 % LO (LO100) and FO/LO in ratios of 3:1 (LO25), 1:1 (LO50) and 1:3 (LO75). The experimental diets were prepared by the Nutreco Aquaculture Research Centre, Stavanger, Norway and were fed by hand. The formulation, proximate analyses and fatty acid compositions of the diets (6 mm pellet) are shown in Tables 6.1 and 6.2. All diets were formulated to satisfy the nutritional requirements of salmonid fish (US National Research Council, 1993). The fish were

fed the experimental diets for 40 weeks with the experiment terminating in March 2002. The final weights of the fish were 1.78 ± 0.40 kg (FO), 1.89 ± 0.34 kg (LO25), 1.90 ± 0.33 kg (LO50), 1.87 ± 0.35 kg (LO75) and 1.87 ± 0.33 kg (LO100) and there were no significant differences between different dietary treatments (Tocher *et al.*, 2002). At the end of the feeding trial, HUFA synthesis and β-oxidation was determined in isolated hepatocytes and intestinal caecal enterocytes from 3 fish of each treatment, and samples of pyloric caeca and liver collected for lipid and fatty acid analyses.

Table 6.1. Formulation (g / kg) of feed.

Component	FO	LO25	LO50	LO75	LO100
Fishmeal[1]	338	338	338	338	338
Maize gluten	200	200	200	200	200
Soya (Hi Pro	100	100	100	100	100
Fish oil[4]	258	193.5	129	64.5	0
Linseed oil[5]	0	64.5	129	193.5	258
Micronutrien	25	25	25	25	25

[1]Scandinavian LT-fish meal (Nordsildmel, Norway). [2]Cargill/ADM, Decatur, Illinois. [3]Soybean meal (Denofa, Fredrikstad, Norway). [4]Capelin oil (Nordsildmel, Norway) supplemented with 200 ppm BHT. [5]Crude E.C.C. linseed oil (N.V. Oliefabriek Lictervelde, Belgium) supplemented with 500ppm Ronoxan A (Roche, Switzerland). [6]Vitamin, mineral and carotenoid pigment premix formulated to Nutreco specification (Farmix, Trouw Nutrition, The Netherlands).

Table 6.2. Proximate (% of weight of feed) and fatty acid compositions (% of total fatty acids by weight) of the experimental diets.

	FO	LO25	LO50	LO75	LO100
Protein	44.1	44.1	44.2	44.4	43.6
Lipid	28.9	28.9	30.2	29.5	31.1
Ash	6.5	6.9	7.2	7.5	7.6
Moisture	5.9	6.3	5.7	6.1	5.6
Fatty acid					
14:0	6.3	4.7	3.4	2.0	0.4
16:0	12.1	10.6	9.3	8.1	6.9
18:0	1.1	1.7	2.1	2.7	3.1
Total saturates	19.9	17.2	15.1	13.0	10.5
16:1n-7	8.1	6.1	4.2	2.3	0.5
18:1n-9	11.9	13.6	15.1	16.0	17.0
18:1n-7	3.3	2.6	2.2	1.6	1.0
20:1n-9	17.9	13.1	9.0	5.0	1.1
22:1n-11	13.3	10.1	7.1	4.3	1.1
22:1n-9	2.1	1.5	1.0	0.5	0.1
Total monoenes	58.4	48.4	39.6	30.5	21.1
18:2n-6	4.2	7.4	9.8	12.3	15.1
20:4n-6	0.2	0.2	0.1	0.1	0.1
Total n-6PUFA	5.0	8.0	10.2	12.6	15.2
18:3n-3	0.9	14.0	25.6	37.8	50.4
18:4n-3	2.9	2.1	1.6	0.9	0.2
20:5n-3	5.9	4.6	3.5	2.2	1.0
22:6n-3	5.0	4.0	3.4	2.4	1.5
Total n-3PUFA	15.7	25.6	34.6	43.7	53.3
Total PUFA	21.7	34.4	45.3	56.5	68.5

[1]Totals include 15:0, present at up to 0.3%;[2]Totals include 16:1n-7, 20:1n-11, 20:1n-7 and 24:1, present at up to 0.5%; [3]Totals include 18:3n-6, 20:2n-6 and 20:3n-6, present at up to 0.2%; [4]Totals include 20:3n-3, 20:4n-3 and 22:5n-3, present at up to 0.4%. PUFA, polyunsaturated fatty acids.

6.2.2. Sampling and Analyses

Fish were sampled at the end of the dietary trial, and were killed by a blow to the head after anaesthesia in benzocaine. Pyloric caeca and livers were dissected from 3 fish per dietary treatment and used for preparation of enterocytes and hepatocytes as described in section 2.2. Preparation of the enterocytes required the modifications described in section 4.2.2 to counteract the presence of mucous. Isolated enterocytes and hepatocytes were incubated with [1-^{14}C]18:3n-3 for 2 h at 20 °C as described in section 2.4., and HUFA synthesis and β-oxidation assays were performed as described in sections 2.5 and 2.6, respectively. Further fish were sampled for lipid and fatty acid analyses. Intact livers were dissected from 18 fish (pooled into 6 samples of 3 livers each) per dietary treatment and immediately frozen in liquid nitrogen. For pyloric caecal samples, four fish were sampled per dietary treatment and caeca cleaned of adhering adipose tissue and luminal contents washed away before being frozen in liquid nitrogen. Total lipids of liver and pyloric caecal tissue were extracted and quantified as described in section 2.8, and FAME of total lipids were prepared, extracted, purified and quantified by gas-liquid chromatography as described in section 2.10.

6.2.3. Statistical analysis

All the data are presented as means ± standard deviation (n = 3, 4 or 6 as stated). The relationships between dietary fatty acid contents and growth, pyloric caeca and liver fatty acid compositions, and between enterocyte and hepatocyte HUFA synthesis activity and both dietary and pyloric caeca and liver fatty acid compositions were determined by regression analyses (Prism 3, Graphpad Software, Inc., San Diego,

USA). Some data such as growth (final weight), and the effects of diet on HUFA synthesis and oxidation were also analysed by one-way ANOVA followed where appropriate by Tukey's post test or the student's t-test to determine significant differences between individual treatments. Percentage data and data which were identified as non-homogeneous (Bartlett's test) were subjected to either arcsine or log transformation before analysis. Differences were regarded as significant when $P < 0.05$ (Zar, 1984).

6.3. Results

Dietary fatty acid compositions

The control diet (FO), formulated with 100 % FO, contained approximately 20 % total saturates, mainly 16:0, almost 60 % total monounsaturated fatty acids over half of which were the long chain monoenes, 20:1 and 22:1, 5 % n-6 fatty acids predominantly 18:2n-6, and 16 % n-3 fatty acids, predominantly the n-3HUFA, 20:5n-3 and 22:6n-3 in approximately equal amounts, and less than 1 % 18:3n-3 (Table 6.2). Inclusion of graded amounts of LO in the diets resulted in graded increased percentages of 18:3n-3, 18:2n-6 and 18:1n-9 with concomitant decreased proportions of n-3HUFA, 16:0, total saturates, 20:1, 22:1 and total monoenes. Thus, in the diet formulated with 100 % LO (LO100), the levels of 18:3n-3 and 18:2n-6 had risen to 50 % and 15 % of total fatty acids, respectively, whereas 20:5n-3 and 22:6n-3 totalled only 2.5 %, and total saturates and monoenes were reduced to 10 % and 21 %, respectively (Table 6.2).

Effects of diet on pyloric caecal and liver fatty acid compositions

The principal fatty acids of pyloric caecal total lipid from fish fed diet FO were, in rank order, 16:0 (16 %), 18:1n-9 (14 %), 20:1n-9 (14 %), 22:6n-3 (13 %), 22:1 (8 %), 16:1n-7 (6 %) and 20:5n-3 (5 %) (Table 6.3). The graded change in fatty acid compositions of the diets in response to increasing LO content, described above, was reflected in the pyloric caeca fatty acid compositions with graded increased percentages of 18:3n-3, 18:2n-6 and 18:1n-9 and decreased proportions of n-3HUFA, 16:0, total saturates, 20:1, 22:1 and total monoenes. In addition, however, there were increased proportions of 18:4n-3, 20:3n-3 and 20:4n-3 and decreased proportions of 20:4n-6 and 22:5n-3 (Table 6.3). These effects were highly significant as shown by regression analyses with positive correlations (slopes) between dietary 18:3n-3 and pyloric caecal 18:3n-3 and negative correlations between dietary 18:3n-3 and caecal 20:5n-3 and 22:6n-3 levels (Table 6.5).

The fatty acid composition of total lipid from liver of fish fed the FO diet showed lower levels of monoenes than pyloric caeca, particularly 20:1 and 22:1 and higher levels of n-3HUFA, with levels of 20:5n-3 and 22:6n-3 about twice the levels observed in caeca (Table 6.4). However, the effects of increased inclusion of dietary LO in liver were identical to those of pyloric caeca with the levels of 18:3n-3 and 18:2n-6 increased to almost 33 % and 12 % of total fatty acids, respectively, and 20:5n-3 and 22:6n-3 reduced to around 3 % and 6 %, respectively, in fish fed the LO100 diet (Table 6.4). As with pyloric caeca, these effects were highly significant as shown by regression analyses with positive correlations (slopes) between dietary 18:3n-3 and liver 18:3n-3, and negative correlations between dietary 18:3n-3 and liver 20:5n-3 and 22:6n-

3 levels (Table 6.5). Similar highly significant correlations between dietary level and pyloric caeca and liver level can be derived for any of the major fatty acids.

Table 6.3. Fatty acid compositions (percentage of total fatty acids by weight) of total lipid of pyloric caeca from Atlantic salmon (*Salmo salar* L.) fed the experimental diets for 40 weeks.

	FO	LO25	LO50	LO75	LO100
14:0	4.0 ± 0.5	3.4 ± 0.2	2.5 ± 0.2	1.5 ± 0.1	0.5 ± 0.0
16:0	16.1 ± 0.6	13.6 ± 0.8	13.0 ± 1.0	10.7 ± 0.6	9.7 ± 0.5
18:0	3.5 ± 0.3	3.5 ± 0.3	4.4 ± 0.4	4.4 ± 0.2	5.0 ± 0.3
Total saturated[1]	24.0 ± 0.5	20.8 ± 1.0	20.1 ± 1.4	16.7 ± 0.7	15.3 ± 0.8
16:1n-7	5.5 ± 0.5	4.5 ± 0.2	3.0 ± 0.2	1.9 ± 0.1	0.6 ± 0.0
18:1n-9	14.4 ± 1.0	15.2 ± 0.4	15.8 ± 0.6	16.2 ± 0.6	17.3 ± 0.2
18:1n-7	3.9 ± 0.2	3.3 ± 0.1	2.6 ± 0.1	1.8 ± 0.0	1.2 ± 0.0
20:1n-9	13.8 ± 1.5	11.3 ± 0.9	7.9 ± 0.8	4.6 ± 0.5	1.4 ± 0.1
22:1	7.9 ± 1.3	7.4 ± 0.6	4.9 ± 0.7	3.2 ± 0.5	1.1 ± 0.1
24:1n-9	0.9 ± 0.0	0.8 ± 0.0	0.7 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
Total monoenes[2]	47.1 ± 4.0	43.0 ± 1.8	35.4 ± 2.2	28.4 ± 1.8	22.0 ± 0.4
18:2n-6	3.3 ± 0.2	5.9 ± 0.2	7.3 ± 0.6	10.5 ± 0.4	12.7 ± 0.4
20:2n-6	0.5 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
20:4n-6	1.1 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
Total n-6 PUFA[3]	5.2 ± 0.2	7.8 ± 0.2	9.5 ± 0.5	12.1 ± 0.4	14.1 ± 0.3
18:3n-3	0.7 ± 0.2	8.9 ± 0.6	13.5 ± 1.5	27.1 ± 1.5	34.6 ± 1.9
18:4n-3	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	0.8 ± 0.1	1.3 ± 0.1
20:3n-3	0.1 ± 0.1	0.8 ± 0.1	1.7 ± 0.2	2.5 ± 0.5	2.4 ± 0.2
20:4n-3	1.0 ± 0.1	1.4 ± 0.2	1.9 ± 0.0	1.7 ± 0.3	1.9 ± 0.2
20:5n-3	5.3 ± 1.2	4.9 ± 0.3	5.1 ± 0.9	3.3 ± 0.3	2.7 ± 0.5
22:5n-3	2.1 ± 0.5	1.8 ± 0.2	1.9 ± 0.3	1.0 ± 0.1	0.7 ± 0.1
22:6n-3	13.4 ± 2.5	9.4 ± 1.1	9.6 ± 1.9	6.2 ± 1.2	4.9 ± 0.7
Total n-3 PUFA	23.5 ± 3.6	28.2 ± 1.8	34.7 ± 1.7	42.7 ± 1.4	48.4 ± 1.0
Total PUFA	28.8 ± 3.5	36.0 ± 1.7	44.3 ± 1.6	54.7 ± 1.2	62.5 ± 0.9

Results are means ± standard deviation (n = 6). [1] Includes 15:0 present at up to 0.3%; [2] Includes 16:1n-9, 20:1n-7, 20:1n-11 and 22:1n-9 each present at up to 0.4%; [3] Includes 18:3n-6, 20:3n-6 and 22:5n-6 present in at up to 0.4%. FO, fish oil; LO, linseed oil; PUFA, polyunsaturated fatty acids.

Table 6.4. Fatty acid compositions (percentage of total fatty acids by weight) of total lipid of liver from Atlantic salmon (*Salmo salar* L.) fed the experimental diets for 40 weeks.

	FO	LO25	LO50	LO75	LO100
14:0	2.8 ± 0.2	2.2 ± 0.2	1.7 ± 0.1	1.1 ± 0.0	0.5 ± 0.0
16:0	17.9 ± 1.1	15.0 ± 1.6	13.3 ± 1.0	11.3 ± 1.1	7.3 ± 0.8
18:0	3.1 ± 0.2	3.8 ± 0.2	4.1 ± 0.2	4.6 ± 0.1	4.9 ± 0.2
Total saturated[1]	24.1 ± 1.2	21.3 ± 1.8	19.3 ± 1.0	17.0 ± 1.1	12.7 ± 1.0
16:1n-7	3.5 ± 0.2	3.0 ± 0.2	2.4 ± 0.2	1.5 ± 0.1	0.7 ± 0.1
18:1n-9	11.9 ± 1.1	14.7 ± 0.8	14.1 ± 1.5	15.7 ± 1.3	19.1 ± 2.4
18:1n-7	3.5 ± 0.1	3.0 ± 0.1	2.3 ± 0.1	1.7 ± 0.1	1.2 ± 0.1
20:1n-9	6.8 ± 1.2	6.1 ± 0.5	4.2 ± 0.7	3.0 ± 0.2	1.4 ± 0.3
22:1	2.1 ± 0.4	1.9 ± 0.3	1.6 ± 0.5	1.0 ± 0.1	0.5 ± 0.1
24:1n-9	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.4 ± 0.1
Total monoenes[2]	30.2 ± 3.1	31.1 ± 1.9	26.4 ± 3.0	24.5 ± 1.9	23.9 ± 2.9
18:2n-6	2.6 ± 0.1	4.4 ± 0.1	6.0 ± 0.3	8.0 ± 0.2	11.6 ± 0.6
20:2n-6	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
20:4n-6	1.4 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.3 ± 0.1
Total n-6 PUFA[3]	4.9 ± 0.2	6.6 ± 0.2	8.0 ± 0.3	10.0 ± 0.2	13.3 ± 0.7
18:3n-3	0.5 ± 0.2	6.8 ± 0.5	14.4 ± 0.6	20.3 ± 1.1	32.6 ± 1.2
18:4n-3	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	1.1 ± 0.2
20:3n-3	0.1 ± 0.0	0.8 ± 0.1	1.6 ± 0.2	2.5 ± 0.2	3.8 ± 0.4
20:4n-3	1.6 ± 0.1	1.9 ± 0.1	2.4 ± 0.2	3.0 ± 0.1	3.1 ± 0.4
20:5n-3	10.0 ± 0.4	8.5 ± 0.5	7.6 ± 0.7	6.4 ± 0.8	3.1 ± 1.1
22:5n-3	3.7 ± 0.4	2.8 ± 0.2	2.3 ± 0.3	1.6 ± 0.1	0.8 ± 0.3
22:6n-3	24.4 ± 1.6	19.7 ± 1.5	17.2 ± 1.7	13.7 ± 1.4	5.6 ± 1.6
Total n-3 PUFA	40.6 ± 2.1	40.8 ± 2.0	46.0 ± 2.4	48.3 ± 1.1	50.0 ± 3.3
Total PUFA	45.7 ± 2.0	47.6 ± 1.9	54.3 ± 2.2	58.5 ± 0.9	63.4 ± 2.6

Results are means ± standard deviation (n = 6). [1] Includes 15:0 present at up to 0.3%; [2] Includes 16:1n-9, 20:1n-7, 20:1n-11 and 22:1n-9 each present at up to 0.4%; [3] Includes 18:3n-6, 20:3n-6 and 22:5n-6 present in at up to 0.4%. FO, fish oil; LO, linseed oil; PUFA, polyunsaturated fatty acids.

Table 6.5. Correlation (regression) analyses (r^2, slope values and significance) for dietary 18:3n-3 and percentages of caecal and liver 18:3n-3, 20:5n-3 and 22:6n-3, and fatty acyl desaturation and β-oxidation activities.

	Caeca			Liver		
Fatty acid	r^2	slope	significance	r^2	slope	significance
18:3n-3	0.98	0.700 ± 0.06	0.0013	0.98	0.633 ± 0.05	0.0011
20:5n-3	0.83	-0.055 ± 0.01	0.0306	0.94	-0.130 ± 0.08	0.0070
22:6n-3	0.93	-0.165 ± 0.03	0.0077	0.96	-0.356 ± 0.04	0.0036
Total desaturation	0.43	-0.030 ± 0.02	0.2265	0.98	0.051 ± 0.00	0.0010
β-Oxidation	0.41	0.058 ± 0.04	0.2433	0.06	-0.003 ± 0.01	0.6946

Effects of diet on enterocyte and hepatocyte HUFA synthesis activities

HUFA synthesis as indicated by the desaturation of [1-^{14}C]18:3n-3, measured as the summed radioactivity recovered as desaturated fatty acid products (18:4, 20:4, 20:5, 22:5 and 22:6), was 2-fold higher in enterocytes than hepatocytes in fish fed the diet containing FO (Figure 6.1). However, the desaturation of [1-^{14}C]18:3n-3 in hepatocytes was increased in a graded manner by increasing dietary LO, and regression analyses confirmed a significant positive correlation with a very high degree of confidence ($p = 0.001$ and $r^2 = 0.98$) between dietary 18:3n-3 levels and hepatocyte fatty acid desaturation (Figure 6.1 and Table 6.5). In contrast, total desaturation of [1-^{14}C]18:3n-3 in enterocytes was significantly decreased in fish fed dietary LO at levels of 50 % or more (Figure 6.1), but there was no direct correlation between dietary 18:3n-3 levels and caecal fatty acid desaturation activity (Table 6.5). In both, enterocytes and hepatocytes, the main products of 18:3n-3 desaturation were the Δ6 desaturated products, 18:4n-3 and 20:4n-3 (Figure 6.2). In enterocytes, the recovery of radioactivity in all products of 18:3n-3 desaturation, with the exception of 20:4n-3, was significantly reduced in fish fed the LO100 diet compared to fish fed the FO diet (Figure 6.2). Furthermore, the major effect of feeding diets containing LO on the desaturation of 18:3n-3 in hepatocytes was to increase the amount of Δ6 desaturated products whereas recovery of radioactivity in 20:5n-3 was only slightly, but significantly, increased, and recoveries in 22:5n-3 and 22:6n-3 were unaffected.

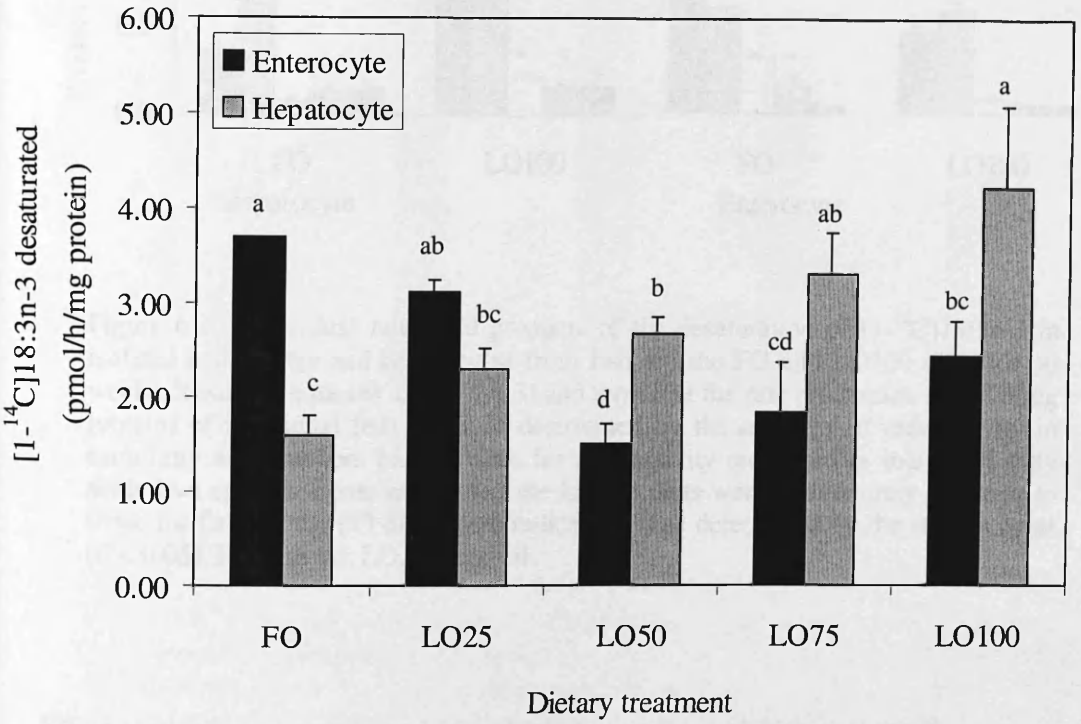


Figure 6.1. Total fatty acid desaturation/elongation activity in isolated enterocytes and hepatocytes after feeding the experimental diets for 40 weeks. Results are means ± SD (n = 3) and represent the rate of conversion (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Columns for a specific a tissue with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$). FO, fish oil; LO, linseed oil.

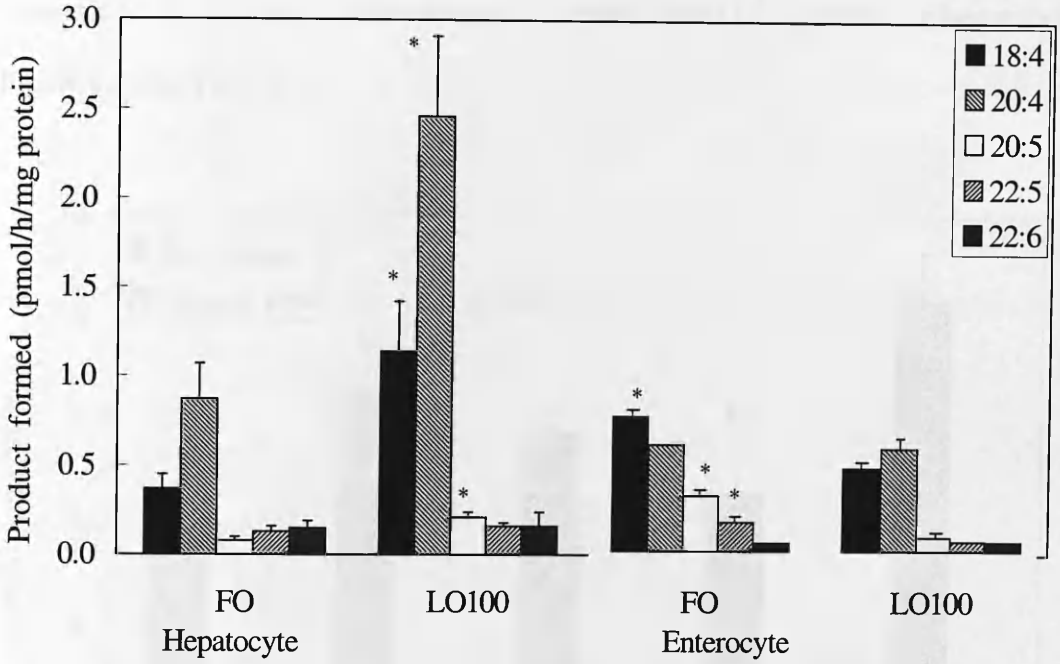


Figure 6.2. Individual fatty acid products of the desaturation of [1-^{14}C]18:3n-3 in isolated enterocytes and hepatocytes from fish fed the FO and LO100 diets for 40 weeks. Results are means ± SD (n = 3) and represent the rate production (pmol/h/mg protein) of individual fatty acids as determined by the recovery of radioactivity in each fatty acid fraction. Mean values for radioactivity recovered as individual fatty acids in a specific tissue in fish fed the LO100 diets were significantly different to those for fish fed the FO diet where indicated (*) as determined by the student t-test ($P < 0.05$). FO, fish oil; LO, linseed oil.

Effect of diet on enterocyte and hepatocyte fatty acid oxidation activities.

Fatty acid oxidation as measured by the production of acid soluble products from [1-^{14}C]18:3n-3 was higher in enterocytes than in hepatocytes irrespective of dietary treatment (Figure 6.3). In enterocytes, the production of acid-soluble products from [1-^{14}C]18:3n-3 was significantly higher in fish fed diets containing LO compared to fish fed the FO diet (Figure 6.3), but there was no significant correlation with dietary 18:3n-3 levels (Table 6.5). In contrast, there was no significant effect of dietary

treatment on the production of acid-soluble products from [1-^{14}C]18:3n-3 in hepatocytes (Figure 6.3 and Table 6.5).

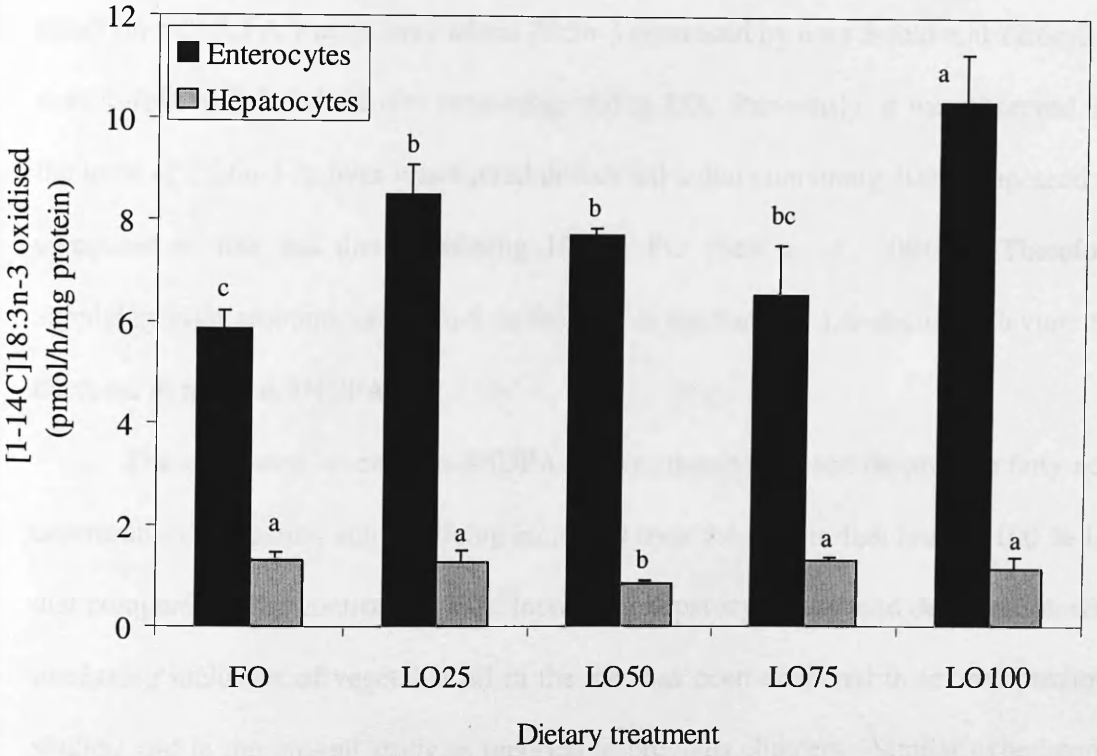


Figure 6.3. Fatty acid β-oxidation activity in isolated enterocytes and hepatocytes after feeding the experimental diets for 40 weeks. Results are means ± standard deviation (n = 3) and represent the rate of oxidation (pmol/h/mg protein) of [1-^{14}C]18:3n-3 to acid soluble products. Columns for a specific a tissue with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$). FO, fish oil; LO, linseed oil.

6.4. Discussion

The overall hypothesis tested in this chapter was that replacing FO with 18:3n-3-rich LO in salmon diets would enable the fish to maintain tissue n-3HUFA levels through a combination of increased desaturation/elongation activity and increased substrate fatty acid provision. The results of this dietary trial and the metabolic studies with enterocytes and hepatocytes showed that, unfortunately, this was not the case.

In the present study, both intestinal and liver tissue had very large increments in the proportions of 18:3n-3, and also 18:2n-6, with n-3HUFA decreasing significantly with increasing dietary LO, particularly at inclusion levels above 50 %. The greater effect on n-3HUFA was in liver where 20:5n-3 decreased by over 3-fold and 22:6n-3 by over 4–fold in fish fed the diet containing 100 % LO. Previously, it was observed that the level of 22:6n-3 in liver was halved in fish fed a diet containing 100 % rapeseed oil compared to fish fed diet containing 100 % FO (Bell *et al.*, 2001a). Therefore, supplying high amounts of 18:3n-3 in the diet in the form of LO did not alleviate the decrease in tissue n-3HUFA.

The decreased levels of n-3HUFA in liver tissue occurred despite the fatty acid desaturation/elongation activity being increased over 2.6-fold in fish fed the 100 % LO diet compared to the control diet FO. Increasing hepatocyte fatty acid desaturation with increasing inclusion of vegetable oil in the diet has been observed in several previous studies, and in the present study as reported in previous chapters. Similar experiments on salmon in seawater using graded levels of rapeseed oil and palm oil gave comparable graded increases in hepatocyte fatty acid desaturation activity, with 100 % replacement of FO giving increases of 2.7- (Bell *et al.*, 2001a) and 10-fold (Bell *et al.*, 2002), respectively. In freshwater, salmonid hepatocyte desaturation activities were up to 2.5-fold (Bell *et al.*, 1997), 2.4-fold (Tocher *et al.*, 2000) and 2.8-fold (Tocher *et al.*, 2001) greater in fish fed vegetable oils compared to fish fed FO. A *in vivo* study with rainbow trout fed VO with no n-3HUFA showed 10 times more DHA production than fish fed FO (Bell and Dick 2004). It is not entirely clear whether the increased desaturation activity in fish fed vegetable oils is the result of decreasing levels of 20:5n-3 and 22:6n-3 in liver membrane lipids, and, thus, mediated by decreased product inhibition, or whether it is a result of increased levels of membrane 18:2n-6 and/or 18:3n-3 and, thus,

mediated by increased substrate provision. In the previous chapter it was suggested that both decreased product inhibition and increased substrate provision were factors determining hepatocyte fatty acid desaturation activity. In this respect, it is important to note that the correlations reported in Table 6.5 were made between dietary 18:3n-3 levels and the various outcomes, including hepatocyte fatty acyl desaturation and elongation. However, similar correlations are obtained if comparisons of any of the other dietary fatty acids that vary in a graded manner across the diets are made, which includes the major fatty acids 18:2n-6, 20:5n-3, 22:6n-3, 18:1n-9, 16:0, 20:1n-9 and 22:1. Therefore, regression analyses of dietary 20:5n-3 or 22:6n-3 with hepatocyte desaturation also shows a significant correlation, albeit negative compared to the correlation with dietary 18:3n-3, but with a similar significance and r^2 value. Therefore, with the data obtained in the present study of linseed oil replacement, it is not possible to elucidate the precise mechanism, but it does demonstrate clearly that, irrespective of mechanism, the increased hepatic fatty acyl desaturation/elongation induced by feeding Atlantic salmon with vegetable oils was ineffective in maintaining tissue levels of either 20:5n-3 or 22:6n-3, even in the presence of high levels of dietary 18:3n-3.

Previous chapters had indicated that caecal enterocytes were a site of significant HUFA synthesis in salmonids, including salmon, and so the specific aim in this chapter was to determine the extent to which intestinal fatty acid desaturation/elongation could be increased by diets rich in 18:3n-3. In this trial, enterocytes were more active than hepatocytes in desaturating and elongating the exogenously added [1-^{14}C]18:3n-3 substrate in fish fed 100 % FO. However, in fish fed dietary LO, the intestinal enterocytes did not appear to have the capacity to increase their desaturation activity as occurred with hepatocytes. In fact, a trend of decreasing fatty acid desaturation/elongation activity in pyloric caecal enterocytes with increasing dietary LO

(up to 50 % LO inclusion) was observed. This result was unexpected and in contrast to the data obtained in this trial with hepatocytes. It was also completely different to the data obtained with enterocytes fed vegetable oils in the form of either a rapeseed/linseed oil blend or palm oil as reported in previous chapters of the present work, or as reported in all previous studies on hepatocytes from salmonids fed a variety of different vegetable oils and blends (Bell *et al.*, 1997, 2001a, 2002: Tocher *et al.*, 1997, 2000, 2001). One possible explanation is that fatty acyl desaturation and elongation in the two tissues serves different purposes with different regulatory mechanisms. Thus, the primary purpose of hepatic fatty acid desaturation may be to provide the body with sufficient long-chain HUFA for membrane biosynthesis and as such it is nutritionally induced when membrane HUFA declines as it does when feeding vegetable oil diets. In contrast, the fatty acyl desaturation activity in enterocytes may be simply to supply its own needs for HUFA for biomembrane synthesis. Being a tissue with a high turnover of cells/membranes, fatty acid desaturation/elongation may be relatively high and unaffected by diet. However, the activities in enterocytes from pyloric caeca were not maintained in the present study, and the effect was not observed in enterocytes in our previous trials when palm oil or the rapeseed/linseed oil blend was used, suggesting that this is not a likely explanation.

An alternative explanation is that the particular dietary fatty acid composition of a diet rich in linseed oil affected the actual assay. Thus, it is important to note that desaturation/elongation activity was measured by incubating cells with [1-^{14}C]18:3n-3 and determining the amount of radioactivity recovered as desaturated and elongated fatty acid products. If cells being assayed contained high intracellular levels of unesterified fatty acids derived from the diet, this may compete in the assay, particularly in fish fed diets very rich in 18:3n-3 as in the present trial with salmon fed high levels of

linseed oil alone. Certainly the fish in this trial were not specifically starved for any significant period before being sampled. As a result, it is highly likely that enterocytes, as the first organ/tissue in contact with dietary fatty acid intake, may have contained high levels of intracellular unesterified 18:3n-3 that competed with the labelled 18:3n-3 for enzyme binding sites and produced an artifactual inhibition of the enzyme assay. Intracellular unesterified fatty acid compositions were not determined in the present study but subsequent studies here in the Nutrition Laboratory in the Institute of Aquaculture have indicated that enterocytes can indeed have high levels of intracellular unesterified dietary fatty acids (Oxley and Tocher, personal communication), and much higher than ever found in hepatocytes (Stubhaug and Tocher, personal communication). Therefore, although there is no direct evidence in the present study, it is possible that the differences between fatty acid desaturation/elongation activity observed between enterocytes and hepatocytes may be explained as a result of competition during the HUFA synthesis assay between the exogenously added [1-^{14}C]18:3n-3 substrate and the dietary 18:3n-3 absorbed by enterocytes and retained intracellularly. Hepatocytes in contrast, would not contain high levels of intracellular unesterified dietary-derived 18:3n-3. Nevertheless, a full explanation of the apparent difference in fatty acyl desaturation and elongation activities in enterocytes and hepatocytes and their nutritional regulation in intestine and liver *in vivo* will require further research.

As noted above, HUFA synthesis activity was measured by determining the amount of radioactivity from [1-^{14}C]18:3n-3 recovered as desaturated and elongated fatty acid products. In both enterocytes and hepatocytes, desaturation of [1-^{14}C]18:3n-3 was primarily observed through the levels of radioactivity recovered as 18:4n-3 and 20:4n-3 whereas recovery of radioactivity as 20:5n-3 and 22:6n-3 was much lower. In the same way, the effects of feeding LO were mainly manifested in changes in the

recovery of radioactivity in these two main products, 18:4n-3 and 20:4n-3. These data suggest that production of 20:5n-3 and, especially 22:6n-3, was low within the 2 h time-scale of the assay. Nevertheless, the fact that desaturation of 18:3n-3 in fish fed increasing levels of LO was not sufficient to maintain tissue levels of 20:5n-3 and 22:6n-3 in pyloric caeca and liver suggests that these steps of HUFA synthesis were also low *in vivo*. Therefore, it can be deduced that the reduced level of dietary n-3HUFA with increased dietary inclusion of LO was the major factor in determining the tissue levels of 20:5n-3 and 22:6n-3. Consistent with this, only the intermediates 18:4n-3 and 20:4n-3 were increased in both intestinal and liver tissue lipids with LO feeding. Interestingly, there was no evidence for desaturation of dietary 18:2n-6 as, in addition to declining 20:4n-6, there was no increase in the levels of the intermediates 18:3n-6 or 20:3n-6 in either intestinal tissue or liver. This is probably due to the excess of dietary 18:3n-3 effectively blocking the Δ6 desaturation of 18:2n-6. A similar effect has been observed previously in salmon fed vegetable oil in seawater (Bell *et al.*, 1993). The only indication of metabolism of 18:2n-6 in fish fed LO was the increased levels of the so-called "dead end" products, 20:2n-6 (and 20:3n-3 from 18:3n-3) in both intestinal tissue and liver.

A further aim in this chapter was to determine the extent to which 18:3n-3 was oxidised in comparison with the amount desaturated/elongated. The results showed that in hepatocytes from fish fed the FO diet, there was a similar amount of radioactivity recovered in acid soluble products as there was in desaturated products indicating that approximately equal amounts of the exogenously added [1-^{14}C]18:3n-3 were being oxidised and desaturated. In contrast, fatty acid oxidation activity in enterocytes of fish fed the FO diet was about 1.5- fold higher than desaturation activity. In addition, fatty acid oxidation activity was around 5-fold greater in caecal enterocytes compared to

hepatocytes in fish fed the FO diet. There are few results in the literature with which to compare the present data. Relatively high levels of fatty acid oxidation have been reported for red muscle and heart in trout, whereas liver, kidney and white muscle have only limited capacity to oxidise fatty acids (Henderson and Tocher, 1987). More recently though, white muscle has shown to be a quantitatively important site of fatty acid catabolism in salmon, particularly in juveniles, when data is expressed on a *per tissue* basis (Frøyland *et al.*, 1998, 2000). However, in that study, liver was not a major site of mitochondrial or peroxisomal β-oxidation irrespective of how the data were presented (Frøyland *et al.*, 2000). Certainly, the level of fatty acid oxidation appeared relatively low in the hepatocytes, but there are few data on the fatty acid oxidative capacity of fish intestinal tissues (Small and Connock, 1981). In the previous chapter, trout caecal enterocytes displayed more β-oxidation activity than hepatocytes. Therefore, the results of the present study with salmon fed LO are generally similar in comparison to the results observed in the trial with trout fed CPO.

It terms of dietary effects, in the previous chapter, β-oxidation activity of trout enterocytes was decreased when fish were fed diets containing CPO compared to FO. In the present trial, there was also a difference between salmon enterocytes and hepatocytes in fatty acid oxidation in response to dietary LO. In hepatocytes, and in contrast to the desaturation pathway, there was no dietary effect on the oxidation of [1-^{14}C]18:3n-3. However in enterocytes, fatty acid oxidation activity was generally increased in fish fed diets containing LO, whereas desaturation activity generally decreased, although there was no correlation with the dietary level of 18:3n-3. This was interesting in relation to the discussion above on the effects of diet on desaturation/elongation activities, because the apparent reciprocal manner in which the oxidation and desaturation assays varied in enterocytes suggested that there was the

possibility of competition between the two pathways for available labelled fatty acid substrate. Whether this is the case and whether such competition between the two pathways for fatty acid could occur *in vivo* is unclear and requires further research.

In summary, in this chapter three objectives were accomplished. Firstly, it was found that providing high levels of fatty acid substrate for n-3HUFA synthesis by replacing FO with 18:3n-3 rich LO in salmon diets did not enable the fish to maintain tissue n-3HUFA levels. Secondly, enterocytes were more active than hepatocytes in desaturating and elongating the [1-^{14}C]18:3n-3 substrate in fish fed FO. However, in fish fed dietary LO, the intestinal enterocytes did not appear to have the capacity to increase their desaturation activity as occurred with hepatocytes. This, however may be due to a competition between the exogenously added [1-^{14}C]18:3n-3 substrate and the dietary 18:3n-3 absorbed by enterocytes. Thirdly, fatty acid oxidation activity in enterocytes was generally increased in fish fed diets containing LO in comparison to hepatocytes where there was no dietary effect on oxidation of 18:3n-3. The fact that oxidation in enterocytes increased whereas desaturation activity generally decreased may also be interpreted as competition between the two fatty acid metabolic pathways.

CHAPTER SEVEN

7. RESEARCHING ALTERNATIVES TO FISH OIL IN AQUACULTURE. (RAFOA 2)

7.1. Introduction

The previous chapters have highlighted the fact that the aquaculture industry is increasing year on year at a high rate (at least 10 %/year) and will soon outstrip fish oil supplies, and have stressed the importance of researching alternatives to dietary fish oil for aquaculture. The study has focused on the ability of salmonids, Atlantic salmon included, to convert dietary C_{18} PUFA, found in vegetable oil alternatives, into HUFA. Specifically, fatty acyl desaturation/elongation and β-oxidation in isolated caecal enterocytes have been investigated, and the effects of replacement of dietary fish oil with vegetable oils determined. In those trials, the dietary fish oil was replaced with different vegetable oils that were selected based on, and to take advantage of, their particular fatty acid composition. The results have shown that different vegetable oils in diets for salmon can modulate the ability of both enterocytes and hepatocytes to desaturated/elongate and even oxidize [1-^{14}C]18:3n-3.

In the previous chapter, the use of linseed oil as the only dietary substitute for fish oil provided interesting and unexpected data in that HUFA synthesis in caecal enterocytes, as measured by the desaturation/elongation of [1-^{14}C]18:3n-3, was apparently reduced or inhibited at high levels (75-100 %) of dietary linseed oil replacement. This was not observed with hepatocytes and it was suggested that this effect was perhaps an artifact of the assay system due to high levels of cellular 18:3n-3

derived from the diet interfering in the assay through competition with the exogenously added labelled 18:3n-3 tracer. However, irrespective of the extent to which linseed oil could increase HUFA synthesis (or not), the tissue fatty acid compositions suggested that very high levels of dietary linseed oil alone were not effective in maintaining tissue n-3HUFA levels. Nevertheless, a source of 18:3n-3 is certainly required in any fish oil replacement and Chapter 4 showed that linseed oil, as part of an oil blend together with rapeseed oil, was a useful dietary component that contributed to the increased extent to which HUFA were synthesized. Consequently, one aim of the present chapter was to investigate the effects of a dietary formulation containing blended vegetable oils based on the information accumulated both in the previous trials and other studies reported in the literature. One factor considered in the development of this oil blend was the relative amounts of specific fatty acids that would enable high conversion of 18:3n-3 to DHA and EPA in salmon.

As mentioned previously, rapeseed oil is rich in 18:2n-6 and 18:1n-9; linseed oil is rich in 18:3n-3 and palm oil is rich in 16:0 and 18:1n-9, and so, between them, these three oils are able to supply virtually the whole range of fatty acids commonly available in vegetable oils. It was therefore possible, through quantitative calculations of the amount of fatty acids contained in the individual oils, to design a blend that partially mimicked the fatty acid composition of fish oil, at least in relation the relative proportions of saturated, monounsaturated and polyunsaturated fatty acids, only with the n-3HUFA replaced by C_{18} PUFA. In theory, the oil blend would suit the nutritional requirements of salmonids, Atlantic salmon included, without compromising the health of the fish, and would enhance their endogenous ability to convert C_{18} PUFA to HUFA. Therefore the first aim in the present trial was to evaluate HUFA synthesis (PUFA desaturation/elongation) and β-oxidation activity in isolated caecal enterocytes of

salmon fed a diet containing rapeseed, linseed and palm oils blended to produce a dietary fatty acid composition as closely resembling fish oil as possible.

The Atlantic salmon is an anadromous fish, which means that it begins life in freshwater and migrates to the sea after 1-2 years, before returning to freshwater to breed after spending 1-3 years at sea. The migration towards the sea of salmonids juveniles, or parr, is accompanied by profound changes in morphology, behavior, and physiology, know collectively as parr-smolt transformation or smoltification (Folmar and Dickhoff, 1980; McCormick and Saunders, 1987). The smoltification process involves the activation of neuroendocrine and endocrine systems which results in biochemical reorganisations within the tissues which prepare the fish, while still in freshwater, for life in the marine environment (Hoar, 1976; Wedemeyer *et al.*, 1980). Previous studies had suggested that lipid metabolism could be modified in response to ambient salinity changes (Li and Yamada, 1992; Sheridan *et al.*, 1985), and effects of parr-smolt transformation on HUFA synthesis in hepatocytes had been reported previously (Bell *et al.*, 1997; Tocher *et al.*, 2000). However none of these previous studies have investigated relationships between smoltification and intestinal fatty acid metabolism of salmonids, let alone the effects of feeding vegetable oils.

There are a number of other reports in the literature describing trials in which different vegetable oils were used to replace dietary fish oil in salmon culture including rapeseed oil (Bell *et al.*, 2001a; Bell *et al.*, 2003a; Bell *et al.*, 2003b), linseed oil (Bell *et al.*, 1993) and palm oil (Bell *et al.*, 2002; Ng *et al.*, 2003). There are also other trials in which blends of different vegetable oils have been utilised (Torstensen and Frøyland, 2000; Caballero, 2002; Bell *et al.*, 2003b; Torstensen *et al.*, 2004). However, the above trials have been of relatively short duration and only one covered the whole production cycle of salmon (Bell *et al.*, 2003b). In that trial (FOSIS), a blend of vegetable oils was

used as the dietary oil throughout the whole growth cycle of salmon, but fatty acyl desaturation/elongation and β-oxidation activity in enterocytes was only measured at the end of the trial (see Chapter 4). Therefore, the effects of dietary vegetable oil on the capacity of enterocytes to synthesise HUFA at different time-points in the growth cycle of Atlantic salmon had not been investigated, and consequently possible interactions between dietary vegetable oil and developmental and/or seasonal/environmental effects on enterocyte fatty acid metabolism were unknown. Therefore, a further aim of the present study was to determine fatty acid metabolism, specifically HUFA synthesis and β-oxidation, in caecal enterocytes throughout the entire two-year growth/production cycle of salmon from first-feeding to harvest, to determine the effects of season and development including specific stages such as parr-smolt transformation.

Thus, a large-scale dietary trial was designed to investigate the replacement of fish oil with alternative oils in diets for Atlantic salmon culture over the entire growth cycle. Atlantic salmon were grown from first feeding to harvest size on diets in which fish oil was replaced by a blend of vegetable oils, rapeseed, linseed and palm oil. In this chapter, the effects of the experimental diets and season/developmental stage on specific aspects of fatty acid metabolism, namely HUFA synthesis (fatty acyl desaturation/elongation) and β-oxidation in isolated caecal enterocytes are reported.

7.2. Materials and methods

7.2.1. Experimental fish and diets

In March 2002, Atlantic salmon fry were distributed randomly into 6 tanks (3 m x 3 m, depth 0.5 m) at a stocking level of 3000/tank at the Marine Harvest Ltd. hatchery facility at Invergarry, Highland, Scotland. The fry were weaned onto extruded feeds

containing 20 % added oil which was either fish oil (FO; capelin oil) or a vegetable oil blend (VO), containing rapeseed, palm and linseed oils in a 3.7 : 2 : 1 ratio, replacing 75 % of the FO. Fish were fed the diets described above for around one year until sea water transfer in April 2003, at which point fish (average weight ~ 50 g) were transferred at 700 fish/pen into six 5 m x 5 m net pens at the Marine Harvest Ltd. Feed Trial Unit, Loch Duich, Lochalsh, Scotland. The fish were fed the same diet in seawater as in freshwater although the dietary oil levels were increased to 25 % (3mm pellet) rising to 32 % (9mm pellets) through the year long seawater phase. The diets aimed to be practical, and were formulated and manufactured by Nutreco ARC, Stavanger, Norway according to current practices in the salmon feed industry. All diets were formulated to satisfy the nutritional requirements of salmonid fish (US National Research Council, 1993). The dietary formulations are given in Table 7.1, and the measured proximate and fatty acid compositions of the diets are given in Table 7.2. There was no significant difference in final weights between fish fed the FO diet (2.54 ± 0.14 kg) or the 75% VO diet (2.37 ± 0.13 kg).

Table 7.1. Formulation of feed (according to pellet size mm; g/kg).

Component	0.3-2.0[3] FW[1]	2 FW	3 FW	3 SW[2]	4 SW	6 SW	9 SW
Fishmeal (LT Nordsildmel, Norway)	672	567	553	147			386
Fishmeal (Consortio, Peru).				407	506	473	
Corn gluten (Cargill, USA).		100	100	100	100	100	100
Soybean meal extracted (Denofa, Norway).				54	100	100	100
Wheat (Statkorn, Norway).	164	155	166	60	46	80	99
Oil. [4]	139	153	153	208	223	223	291
Vitamines and minerals.[5]	25	25	25	25	25	25	25

[1]Freshwater stage. [2]Cargill/ADM, Decatur, Illinois. [3]Includes five different pellet sizes. Composition of the largest two diet sizes (1.2 and 2.0 mm) is given. [4]Oil is capelin oil (Nordsildmel, Norway) for fish oil based diet, or a mixture of 55% rapeseed oil (Oelmuhle, Germany), 30% palm oil (Denofa, Norway) and 15% linseed oil (NV Oliefabriek, Belgium) for vegetable oil diets. [5]Vitamin and mineral supplementation is estimated to cover requirements according NRC, 199.

Table 7.2. Proximate (g / 100 g) and fatty acid compositions (% of total fatty acids by weight) of representative diets used in the freshwater and marine stages.

	Freshwater (3mm)		Marine (9 mm)	
	FO	VO	FO	VO
Protein	46.5	45.8	42.1	41.2
Lipid	19.6	18.2	30.2	32.8
Ash	8.5	6.8	7.0	6.4
Moisture	7.8	7.9	7.0	7.1
Fatty acid composition				
14:0	6.0	2.7	6.3	2.1
16:0	12.2	15.5	14.2	16.3
18:0	1.4	2.4	2.0	2.6
Total saturates	20.3	20.8	23.0	21.2
16:1n-7	7.5	3.0	4.6	1.5
18:1n-9	11.7	30.6	13.0	35.2
18:1n-7	3.2	2.5	2.3	2.6
20:1n-9	18.8	6.9	11.0	3.7
22:1n-11	13.8	5.7	16.6	4.7
22:1n-9	1.7	0.7	1.4	0.6
Total monoenes	59.4	50.6	50.5	49.1
18:2n-6	3.9	11.5	3.2	13.5
20:4n-6	0.2	0.2	0.4	0.1
Total n-6PUFA	4.4	12.2	4.1	13.8
18:3n-3	0.6	6.6	1.3	9.5
18:4n-3	1.9	0.9	2.6	0.8
20:5n-3	5.9	3.5	6.4	2.0
22:6n-3	6.0	4.1	9.5	2.9
Total n-3PUFA	14.9	15.9	21.3	15.7
Total PUFA	20.3	28.6	26.4	29.7

Totals include 15:0, 16:1n-9, 20:1n-11, 20:1n-7, 24:1, 18:3n-6, 20:2n-6, 20:3n-6, 20:3n-3, 20:4n-3 and 22:5n-3 present at up to 0.5%. FO, fish oil; VO, vegetable oil; PUFA, polyunsaturated fatty acids.

Sampling and Analyses

Fish were sampled at regular intervals during the trial, specifically in September and December 2002, February, March (just prior to seawater transfer), April (just after seawater transfer), July and November 2003, and March 2004 at the end of the dietary trial, corresponding to months 6, 9, 11, 12, 12.75, 16, 20 and 24, respectively. Fish were killed by a blow to the head after anaesthesia in benzocaine. At each time point, pyloric caeca and livers were dissected from six fish per dietary treatment and used for preparation of enterocytes and hepatocytes as described in section 2.2. Preparation of the enterocytes in fish from the seawater stage required the modifications described in section 2.2 to counteract the presence of mucous. Isolated enterocytes and hepatocytes were incubated with [1-^{14}C]18:3n-3 for 2 h at 20 °C as described in section 2.4., and HUFA synthesis and β-oxidation assays were performed as described in sections 2.5 and 2.6, respectively.

7.2.2. Statistical analysis

All the data are presented as means ± standard deviation (n = 6). The effects of dietary treatment on HUFA synthesis in enterocytes and hepatocytes was determined by analysis of variance (ANOVA) followed by the Tukey post test ($P < 0.05$) and in the case of β-oxidation in enterocytes they were determined by a Mann Whitney test. Differences were regarded as significant when $P < 0.05$ (Zar, 1984).

7.3. Results

Dietary fatty acid compositions

In freshwater, the control diet (FO), formulated with 100% FO, contained approximately 20 % total saturates, mainly 16:0 and 14:0, almost 60 % total monounsaturated fatty acids over half of which were the long chain monoenes, 20:1 and 22:1, 4.4 % n-6 fatty acids predominantly 18:2n-6, and 15 % n-3 fatty acids, predominantly the n-3HUFA, 20:5n-3 and 22:6n-3 in approximately equal amounts, and less than 1 % 18:3n-3 (Table 7.2). Replacement of FO with the vegetable oil blend resulted in increased percentages of 18:3n-3, 18:2n-6 and 18:1n-9 with concomitant decreased proportions of n-3HUFA, 20:1, 22:1 and total monoenes. The FO diet in seawater was characterized by having lower 20:1 and higher 22:6n-3 compared to the freshwater phase due to seasonal variation in batches of capelin oil. However, replacement of FO with the VO blend had similar effects in seawater diets as in the freshwater diets other than the fact that total monoene levels were unchanged by substitution (Table 7.2). The vegetable oil (VO) blend was formulated to mimic FO in total saturated, monounsaturated and polyunsaturated fatty acid content and this was largely achieved, particularly in the seawater diets.

Effects of life cycle and diet on enterocyte and hepatocyte HUFA synthesis activities

In both enterocytes and hepatocytes, HUFA synthesis as indicated by the desaturation of [1-^{14}C]18:3n-3, measured as the summed radioactivity recovered as desaturated fatty acid products (18:4, 20:4, 20:5, 22:5 and 22:6), increased during the

freshwater phase, particularly in the latter stages of smoltification, peaking around the point of seawater transfer (Figures 7.1 and 7.2). After the peak in activity at seawater transfer, HUFA synthesis activity in both enterocytes and hepatocytes decreased. However, in enterocytes a second peak in activity occurred around November 2004 (month 20) whereas in hepatocytes there was a small, but consistent, increase in HUFA synthesis activity throughout the remaining year in seawater. These seasonal changes in HUFA synthesis activity in both enterocytes and hepatocytes were unaffected by diet, with basically the same pattern being observed in both cell types from fish fed either FO or the VO blend (Figure 7.1 and 7.2). However, HUFA synthesis in both enterocytes and hepatocytes was significantly affected by diet throughout the life cycle. Specifically, HUFA synthesis activity in both cell types was higher in fish fed the diet containing VO compared to fish fed the FO diet at virtually every time point sampled in the life cycle (Figures 7.1 and 7.2).

Each one of the individual fatty acid products of desaturation of [1-^{14}C]18:3n-3 in enterocytes and hepatocytes was higher in fish fed with the diet containing VO compared to fish fed FO (Figure 7.3). In enterocytes and hepatocytes, the primary products of 18:3n-3 desaturation were the Δ6 desaturated products, 18:4n-3 and 20:4n-3 (Figure 7.3). Moreover, the most important effect of feeding diets containing VO on the desaturation of 18:3n-3 in both enterocytes and hepatocytes, was to increase the amount of Δ6 desaturated products, whereas recovery of radioactivity in 20:5n-3, 22:5n-3 and 22:6n-3 was only slightly, but significantly, increased, (Figure 7.3).

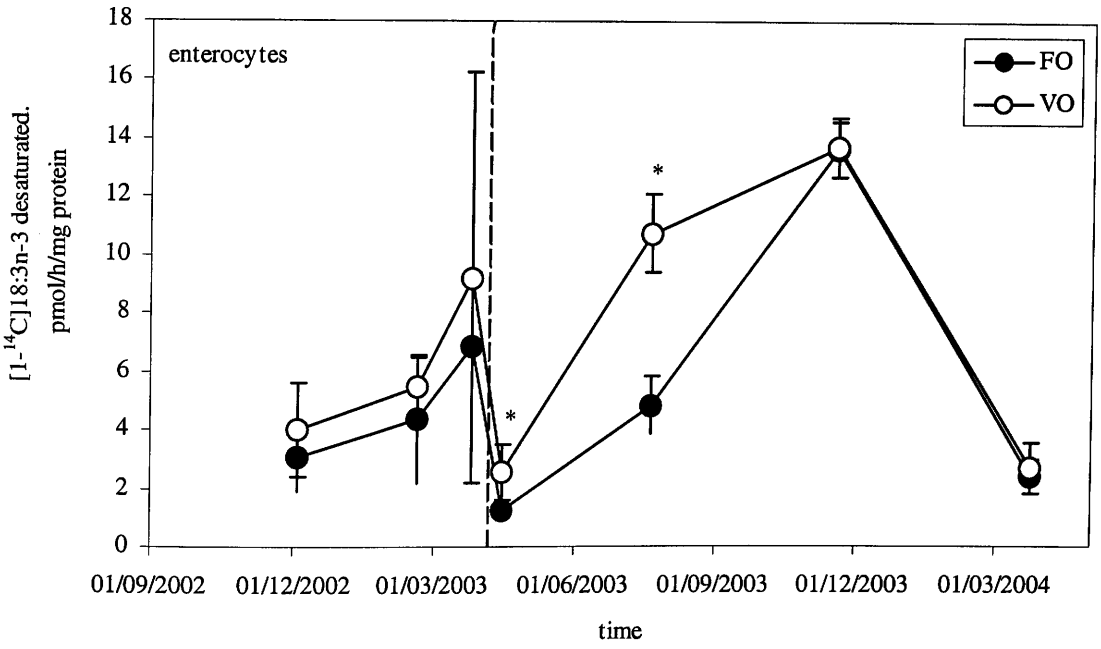


Figure 7.1. Desaturation/elongation of [1-^{14}C]18:3n-3 in isolated caecal enterocytes throughout the production cycle of *Salmo salar* L. fed with diets containing fish oil (FO) and vegetable oil (VO). Dashed line indicates the date of transfer of fish from freshwater to seawater. Results are means ± SD (n = 6). Asterisks indicate the mean values for FO and VO at an individual time point are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$).

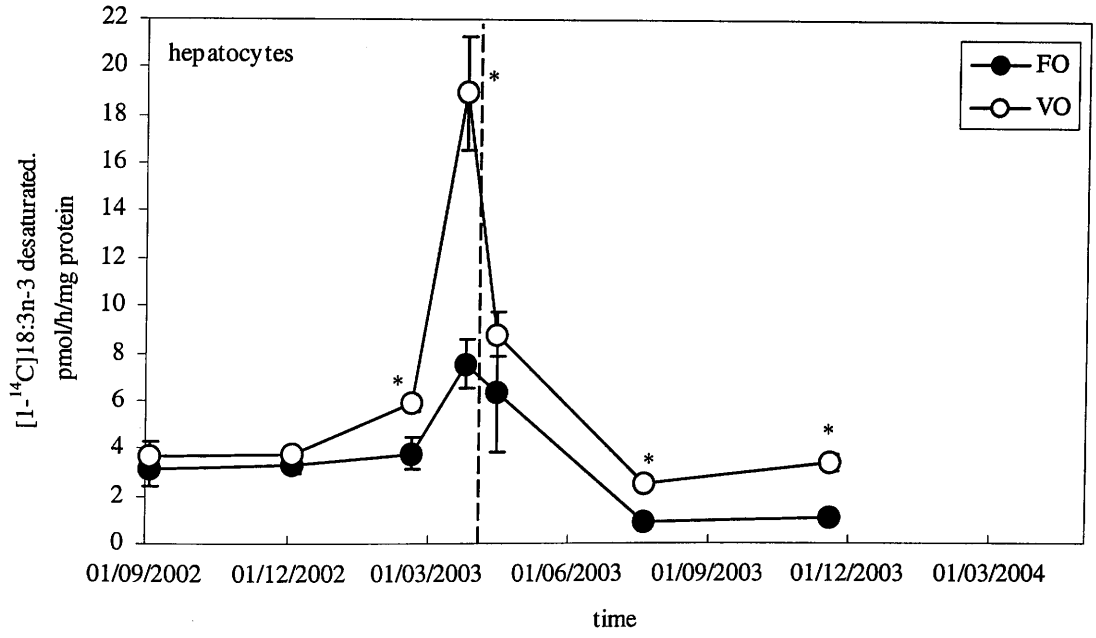


Figure 7.2. Desaturation/elongation of [1-^{14}C]18:3n-3 in isolated hepatocytes throughout the production cycle of *Salmo salar* L. fed with diets containing fish oil (FO) and vegetable oil (VO). Dashed line indicates the date of transfer of fish from freshwater to seawater. Results are means ± SD (n = 6). Asterisks indicate the mean values for FO and VO at an individual time point are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$).

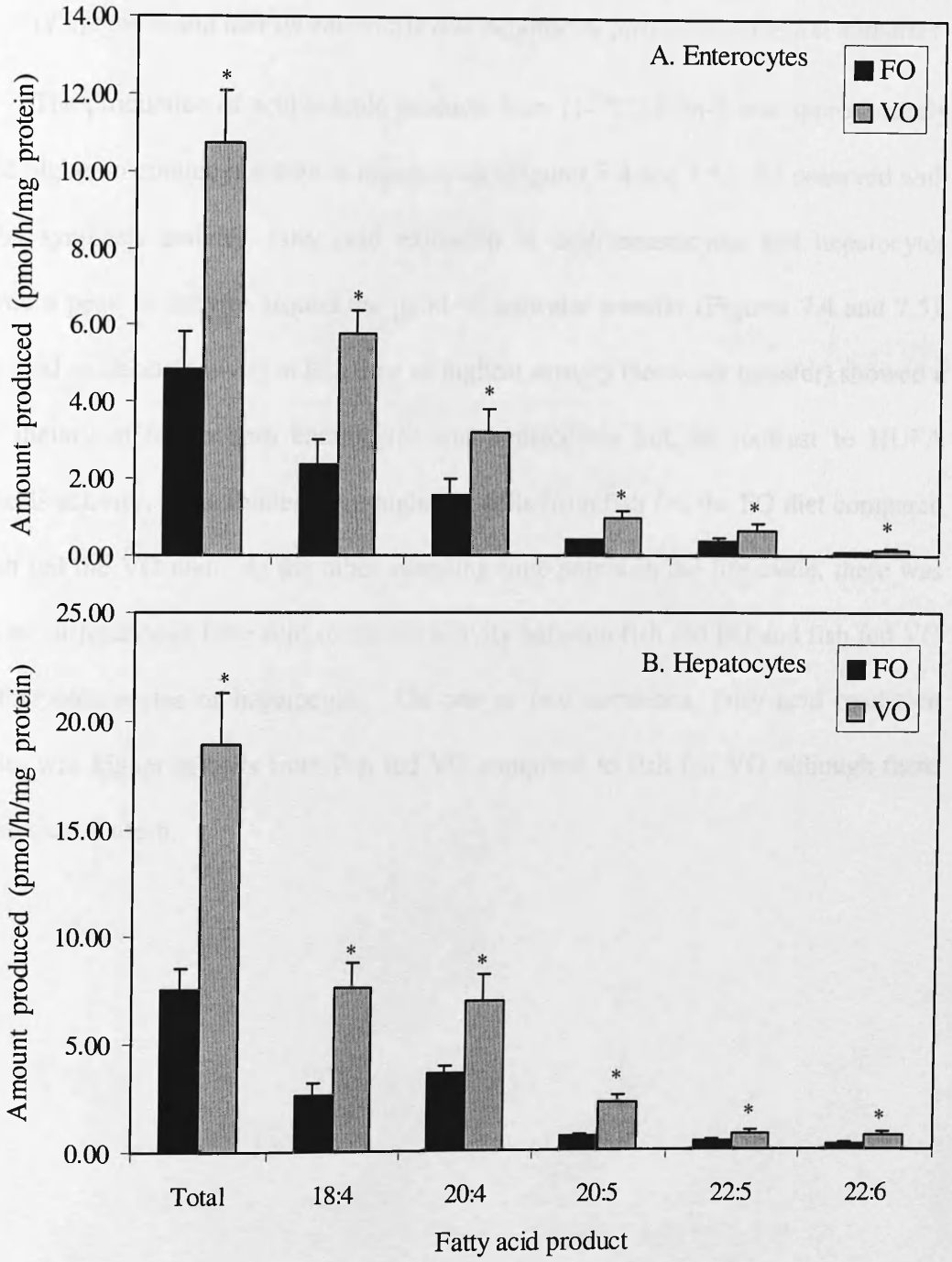


Figure 7.3. Individual fatty acid products of the desaturation and elongation of [1-^{14}C]18:3n-3 in enterocytes (A) and hepatocytes (B) of *Salmo salar* L. fed with diets containing vegetable oil (VO) and fish oil (FO) at the highest point of activity. Results are means ± SD. (n = 3) Asterisks indicate the mean values for FO and VO for an individual fatty acid product are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$).

Effects of life cycle and diet on enterocyte and hepatocyte fatty acid oxidation activities.

The production of acid soluble products from [1-^{14}C]18:3n-3 was approximately 2-fold higher in enterocytes than in hepatocytes (Figures 7.4 and 7.5). As observed with HUFA synthesis activity, fatty acid oxidation in both enterocytes and hepatocytes showed a peak in activity around the point of seawater transfer (Figures 7.4 and 7.5). Fatty acid oxidation activity at this time of highest activity (seawater transfer) showed a clear dietary effect in both enterocytes and hepatocytes but, in contrast to HUFA synthesis activity, the activities were higher in cells from fish fed the FO diet compared to fish fed the VO diet. At the other sampling time points in the life cycle, there was often no difference in fatty acid oxidation activity between fish fed FO and fish fed VO in either enterocytes or hepatocytes. On one or two occasions, fatty acid oxidation activity was higher in cells from fish fed VO compared to fish fed VO although there was no clear pattern.

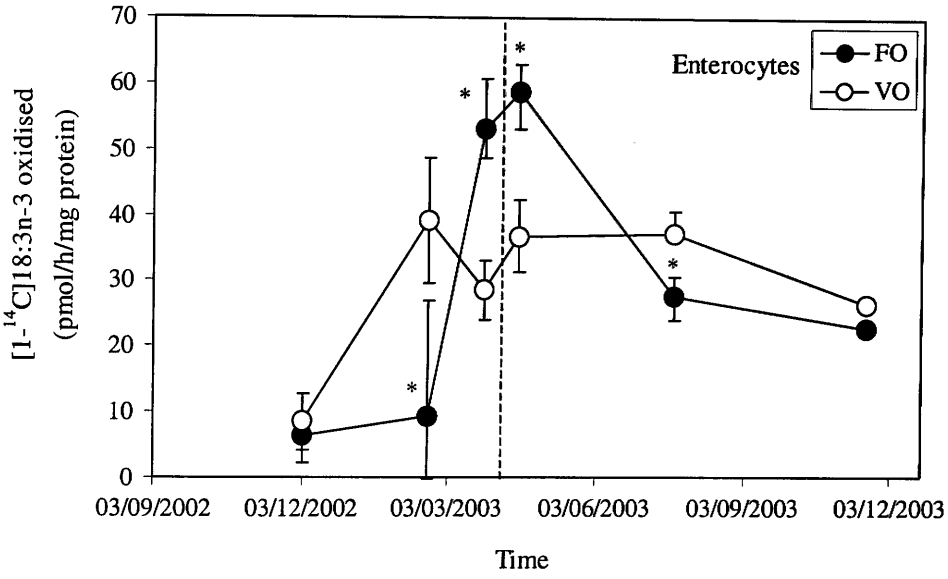


Figure 7.4. β-oxidation of [1-^{14}C]18:3n-3 in isolated enterocytes throughout the production cycle of *Salmo salar* L. fed with diets containing fish oil (FO) and vegetable oil (VO). Dashed line indicates the date of transfer of fish from freshwater to seawater. Results are means ± SD (n = 6). Asterisks indicate the mean values are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$).

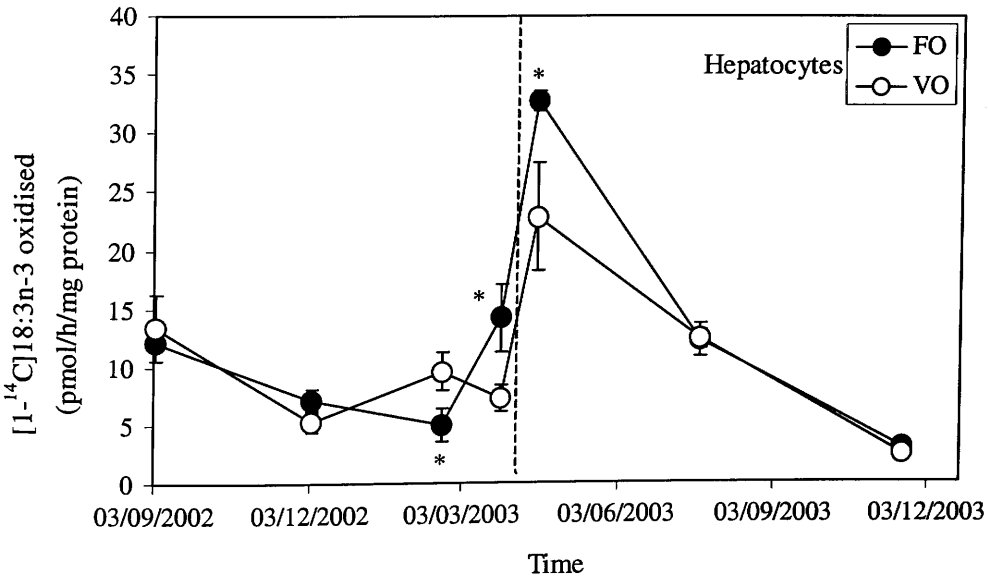


Figure 7.5. β-oxidation of [1-^{14}C]18:3n-3 in isolated hepatocytes throughout the production cycle of *Salmo salar* L. fed with diets containing fish oil (FO) and vegetable oil (VO). Dashed line indicates the date of transfer of fish from freshwater to seawater. Results are means ± SD (n = 6). Asterisks indicate the mean values are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$).

7.4. Discussion

One aim in the present trial was to evaluate HUFA synthesis activity of isolated caecal enterocytes in salmon fed a diet containing rapeseed, linseed and palm oils blended to give a fatty acid composition that mimicked FO with respect to the overall balance of saturates, monounsaturates and polyunsaturated fatty acids, albeit that the n-3HUFA were replaced in the VO blend by C_{18} PUFA. This goal was achieved and the results showed a positive influence of the dietary VO blend on PUFA desaturation/elongation activity. In the dietary trial reported in the previous chapter, it was observed that increasing vegetable oil, in the form of 18:3n-3 rich linseed oil, resulted in decreased desaturation/elongation activity in caecal enterocytes. The mechanisms for this apparent reduced activity were difficult to elucidate, but it was suggested that the assay may be compromised by high levels of 18:3n-3 in the enterocytes, as a result of feeding 18:3n-3 rich diets. The results of the present trial were consistent with that explanation as they show that feeding the blend of VO, that partially mimics the fatty acid composition of fish oil, resulted in increased desaturation and elongation of [1-^{14}C]18:3n-3 substrate used in the assay implying increased HUFA synthesis activity in the enterocytes. Therefore, the diet formulated for the present trial had a substantial influence on HUFA synthesis activity in both caecal enterocytes and hepatocytes, confirming that it is possible to upregulate desaturation activity in fish by nutritional intervention, specifically feeding vegetable oils as replacements for fish oil.

In the present study, both enterocytes and hepatocytes of fish fed the VO blend showed higher metabolic activity than fish fed FO. This trend of increasing HUFA synthetic activity when using dietary VO as a substitute for FO has been reported previously for salmon hepatocytes. Bell *et al.* (2002) reported similar increased HUFA

synthesis activity in salmon hepatocytes when diets containing palm oil were utilized as a substitute for FO, and Tocher *et al.* (2001), also reported that HUFA synthesis was significantly increased in several different species and populations of salmonids when fed diets containing linseed and rapeseed oils compared to fish fed diets containing FO. The results in this study with the VO blend are also consistent with the data presented in earlier trials chapters of this project (FOSIS and CPO).

There have been several other dietary trials investigating the use of vegetable oils as replacements for fish oil in salmonids, but in most of these fatty acid desaturation/elongation activities have not been measured (Bell *et al.*, 1993, 2001a, 2003b; Torstensen and Frøyland, 2000; Caballero, 2002; Ng *et al.*, 2003). Furthermore, none of the abovementioned trials studied HUFA synthesis in enterocytes, a major reason for the present project. In all the other trials reported in the previous chapters, the effect of VO on the capability of enterocytes to synthesize HUFA was evaluated at the end of the period of VO feeding. A further aim of the present trial was to investigate fatty acid metabolism, including HUFA synthesis and β-oxidation, at several important time-points throughout the entire growth cycle of salmon. The results indicated that season and/or developmental stage also affected fatty acid metabolism in enterocytes with parr-smolt transformation having a major effect. Therefore, in addition to the changes in the morphology, behaviour, and physiology of the fish that occur during the process of smoltification, there were profound changes in the rates of fatty acid metabolism in caecal enterocytes.

The high fatty acid desaturation/elongation activities shown in both enterocytes and hepatocytes around the time of transfer of fish from fresh water to seawater are evidence that fatty acyl desaturation/elongation in Atlantic salmon is influenced by environmental and seasonal factors in addition to dietary or nutritional factors as

reported previously (Eriksson and Lundqvist, 1982; Duston and Saunders, 1992; Solbakken *et al.*, 1994; Stead *et al.*, 1996; Bell *et al.*, 1997; Helland and Grisdale-Helland, 1998). Although relationships and interactions between seasonal and nutritional factors have been proposed with regards to effects on growth, the direct effects of nutrition and dietary factors on smoltification are poorly understood (Woo, *et al.*, 1978; Birt and Green, 1986; Helland and Grisdale-Helland, 1998). However, similar results to those presented here were obtained previously, where the HUFA synthesis activity of hepatocytes in Atlantic salmon increased during the freshwater phase, peaking around the time of seawater transfer after which point it decreased (Bell *et al.*, 1997; Tocher *et al.*, 2000). This effect was more pronounced with a higher peak of activity in fish fed a diet containing linseed and rapeseed oils either individually or in a blend (Bell *et al.*, 1997; Tocher *et al.*, 2000).

It is interesting to compare the patterns of changes in HUFA synthesis activity in enterocytes and hepatocytes. The highest value of HUFA synthesis activity in hepatocytes was observed just after seawater transfer, followed by a trend of decreased activity post-transfer. In contrast, the peak in activity in enterocytes occurred just prior to the seawater transfer point and, after an initial decrease in activity immediately post transfer, HUFA synthesis showed a second peak in activity later on in the seawater phase that was not observed in hepatocytes. The reason of this difference between the tissues is not clear but it is possible that water temperature may have played a role in enterocyte fatty acid metabolism as observed previously in Chapter 5. Although none of the earlier experiments with trout were specifically designed to investigate the effects of season, it was observed that rainbow trout enterocytes were generally much more active during the winter months than in the summer months indicating seasonal variation in HUFA synthesis. The pattern of HUFA synthesis activity in enterocytes

observed in the present whole growth cycle trial in salmon clearly showed that the activity rose from a minimum after seawater transfer in May to a peak in mid-winter (December) again indicating seasonal variation. It is not entirely clear whether photoperiod or water temperature or both contribute to this effect. The two most obviously environmental factors are temperature (colder in winter) and photoperiod (short days in winter). The light regime in the aquarium that housed the trout used in the laboratory studies was a constant 12 h light : 12 h dark regime throughout the year, suggesting that photoperiod was not the main factor behind the increased activity in winter and that decreasing temperature was more important. Certainly, HUFA synthesis activity was higher at 7 °C than at 11 and 15 °C in trout in the first trial involving CPO (see Chapter 5). However, the subject of environmental factors and the regulation of HUFA synthesis in salmonid enterocytes requires further research.

The oxidation of [1-^{14}C]18:3n-3 was higher in enterocytes compared to that observed in hepatocytes throughout the growth cycle of salmon. In general, a similar situation was observed previously (Tocher *et al.*, 2002; 2004 and previous chapters). In addition, the results of the β-oxidation activity assay showed that, in general, a greater proportion of [1-^{14}C]18:3n-3 was oxidised rather than being desaturated/elongated throughout the growth cycle of salmon. However, the β-oxidation activity assay in enterocytes gave contrasting results in the different trials performed during this research project. In the earlier trial on salmon fed linseed oil, the β-oxidation activity in caecal enterocytes tended to be increased in fish fed linseed oil compared to fish oil (Chapter 6), whereas, in Chapter 5, dietary CPO tended to reduce enterocyte β-oxidation activity in trout. In contrast, hepatocyte β-oxidation activity was generally unaffected by dietary oil in both these earlier trials, although the total β-oxidation activity was much higher in

the trout hepatocytes in the CPO trial, irrespective of water temperature and dietary oil, compared to the salmon hepatocytes previously (Tocher *et al.*, 2003a; Chapter 4).

In the present chapter, the differences between β-oxidation activity of fish fed with VO or FO are not consistent across all the time points measured throughout the two year trial. However, as with HUFA synthesis activity, it was interesting that in both enterocytes and hepatocytes, and in fish fed with both diets, important changes were observed around the point of seawater transfer. In both cell types, the highest levels of β-oxidation of [1-^{14}C]18:3n-3 were observed just after seawater transfer. Another very interesting point appeared to be consistent with competition between the two pathways, HUFA synthesis and fatty acid oxidation. Specifically, at the peak of highest HUFA synthesis activity in both enterocytes and hepatocytes the greater value was expressed by cells from fish fed with VO, whereas in contrast, at the same time point of sampling, β-oxidation activity showed the greater value in both types of cell from fish fed with FO. This metabolic dynamic may be interpreted as an apparent competition between the two fatty acid pathways for the labelled fatty acid substrate. It is noteworthy that this was most apparent at perhaps the most critical physiological phase of the salmon life cycle, the parr-smolt transformation.

There are studies suggesting that lipid metabolism could be modified in response to ambient salinity changes (Li and Yamada, 1992; Sheridan *et al.*, 1985). It has been reported that masu salmon smolts (*Oncorhynchus masou*) can modify their lipid metabolism to adapt to ambient salinity changes by altering the proportion of (n-3) PUFA. This particularly occurred in polar lipids and in osmoregulatory organs such as gut and gills, which were seen to be critical in defining lipid types of freshwater- or sea water-adapted fish (Li and Yamada, 1992). Nevertheless, in a study that aimed to investigate the effects of salinity acclimation on parr–smolt transformation and post-

smolt performance in off-season salmon (*Salmo salar* L.) smolts, defining lipid types of freshwater- or sea water-adapted fish, the authors suggested that there are different factors involved in the morpho-physiological changes of the fish (species, developmental stage, size, temperature, feeding, etc.) and it does not depend upon salinity alone or photoperiod (Handeland and Stefansson, 2002).

In relation to the abovementioned, the mechanism of changes in HUFA synthesis around smoltification, irrespective of diet, is one issue, but it is also interesting to speculate on the effect that the differences between the dietary treatments may have on the smoltification process. Increasing HUFA synthesis occurred in the salmon prior to seawater transfer and so was a pre-adaptive biochemical change in both hepatocytes and enterocytes. However, HUFA synthesis in both cell types began to increase earlier in fish fed with VO than in fish fed with FO. This could be an indication of the importance of dietary long chain HUFA in salmon at this critical point. The fatty acid composition of the FO in the present trial probably gave to the fish the HUFA required for an optimal pre-adaptation to the up-coming physiological changes at smoltification whereas fish fed VO required to reach a maximum point of desaturation/elongation activity. Conversely, of course, the mechanism of changes in HUFA synthesis are very likely to be driven by fatty acid composition so it can be said that the VO fatty acid composition conferred the requirements for greater increases in fatty acid desaturation/elongation. Further research is required in order to understand the exact metabolic changes that smoltification period triggers and the effects that specific dietary treatments may have on this process.

In summary, the primary objectives of this trial were to acquire a general understanding of the role of intestine in the metabolism of fatty acids throughout the growth cycle and to determine the extent to which enterocyte HUFA synthesis was

affected by a diet of carefully formulated blend of vegetable oils. Based on the data accumulated in the previous trials and other studies reported in the literature on salmon, a crucial factor considered in the development of this vegetable oil blend was the relative amounts of specific fatty acids that would enable high conversion of 18:3n-3 to EPA and DHA. The formulation of the diet appeared to provide the enterocytes with the appropriate requirements to induce increased desaturation/elongation of the exogenously added [1-^{14}C]18:3n-3 to a greater extent than acquired with the FO diet. In fish fed this VO blend diet, the peak HUFA synthesis activity in enterocytes around the point of seawater transfer resulted in the highest substrate conversion compared with results obtained in all the previous trials described in the present project. In addition, the proportion of substrate [1-^{14}C]18:3n-3 that was β-oxidised in enterocytes from salmon fed the blended VO diet was lower than that observed in the previous trials. Although there is still much to discover about the metabolism of fatty acids by intestinal cells of fish, the present trial has revealed some important points of interest for salmon aquaculture and FO substitution with VO. The complete life/growth cycle of Atlantic salmon was investigated and important effects on intestinal fatty acid metabolism were observed and evaluated, and can be attributed to either environmental/seasonal and/or nutritional factors. These results give a solid basis for more profound studies on the role(s) of this generally under-studied organ in HUFA production in salmonids.

CHAPTER EIGHT

8. GENERAL DISCUSSION

The overall aim of this research work was to determine the importance of intestine and caecal enterocytes in fatty acid metabolism in salmonids, focusing on their potential role in HUFA synthesis and the effects of replacement of fish oil with vegetable oils in the diets. The first step required to achieve these objectives was the establishment of a simple, rapid and reliable method for the isolation of viable enterocytes from salmonids. This resulted in the development and establishment of an isolation technique suitable for use in both laboratory and fish farm conditions. The resultant technique enabled the production of enterocyte preparations with good cell viability that enabled the development of the HUFA synthesis and metabolism assay. With these achievements as a starting point, it was possible to determine that pyloric caeca were the ideal intestinal tissue, primarily in terms of HUFA synthesis capacity, for the routine preparation of enterocyte suspensions. Additionally, it was possible to determine how the caecal enterocyte preparation performed in terms of HUFA synthesis under a range of experimental conditions, which enabled the adoption of a standardized assay protocol for use in all comparative studies, that gives the most appropriate conditions for the HUFA synthesis analysis. With relatively minor changes the assay was modified to enable the determination of fatty acid β-oxidation and the desaturation/elongation pathway in a single, combined assay. As mentioned previously, measurement of fatty acid oxidation in Atlantic salmon using isolated hepatocytes and [1-^{14}C]16:0, rather than labelled acyl CoA substrates as used in mitochondrial assays,

had been described previously (Ji *et al.*, 1996) and, in the present study, only slight modifications to existing methods (Frøyland *et al.*, 1995, 2000; Ji *et al.*, 1996) were required in order to combine the HUFA synthesis and fatty acid oxidation assays in isolated cells (and hepatocytes) for first time.

The success in the establishment of the methods for both enterocyte isolation and the assay of enterocyte HUFA synthesis and fatty acid β-oxidation were decisive steps that allowed the pursuit of a primary aim of the research work: the determination of the effects of replacing fish oil with vegetable oils in salmonid diets on fatty acid metabolism in enterocytes. In order to understand how enterocytes respond to fish oil replacement, a series of dietary trials, at an industrial scale in the case of Atlantic salmon, investigating the effects of different vegetable oils and blends, dietary oil content, and interaction with seasonal/environmental factors during an entire Atlantic salmon production cycle, were carried out. The overarching aims of these dietary trials were to determine if salmonids can be grown on diets containing vegetable oils at practical levels without deleterious effects on the fish itself or its value as an important nutritious food for human consumption.

The first trial was designed to investigate the twin problems of replacement of fish oil with alternative oils, specifically a blend of rapeseed and linseed oils, in combination with the high-energy (oil) diets presently used in Atlantic salmon culture. This study was the first time that HUFA synthesis capacity had been investigated *in vitro* in pyloric caecal enterocytes, and it produced data entirely consistent with previous *in vivo* trials that had suggested that pyloric caeca may be a site of significant HUFA synthesis, at least in juvenile rainbow trout (Bell *et al.*, 2003c). However, the HUFA synthesis activities determined in caecal enterocytes and hepatocytes, in identical preparations, and assayed under identical *in vitro* conditions, indicated that

caecal enterocytes were not more active than hepatocytes, as rates of conversion were generally higher in hepatocytes compared to those in enterocytes. In addition, irrespective of the diet in this trial, the primary fate of the substrate [1-^{14}C]18:3n-3 was β-oxidation rather than HUFA synthesis, again consistent with earlier in vivo stable isotope studies in trout (Bell *et al.*, 2003c). Regarding the type of dietary oil, HUFA synthesis in enterocytes was increased in fish fed VO compared to fish fed FO, as had previously been shown in hepatocytes. In contrast, β-oxidation activity was similar in enterocytes and hepatocytes and neither type of dietary oil nor dietary oil levels affected the activity in either cell type. Therefore, the combined effects of feeding VO on fatty acid metabolism were that the balance was shifted towards HUFA synthesis in both enterocytes and hepatocytes although this was not sufficient to prevent the fatty acid compositions of the tissues being altered, to show lower levels of n-3HUFA and higher levels of C_{18} PUFA.

The effects of vegetable oil substitution on salmonid fatty acid metabolism were further studied in two experiments using crude palm oil (CPO) as a substitute for fish oil in diets for rainbow trout. The first trial was primarily aimed at providing information important for optimising the use of dietary CPO in the salmonid aquaculture industry, specifically the effects of environmental temperature, through investigating the interaction between dietary CPO and water temperature, with the modulation of fatty acid metabolism in caecal enterocytes as one parameter investigated. The encouraging results of the first trial stimulated a second trial that aimed to further investigate the effects of dietary CPO on intestinal fatty acid metabolism in rainbow trout in a fully replicated trial over a longer duration. In summary, the inclusion of CPO in the diet had significant effects on tissue fatty acid compositions and also on fatty acid desaturation/elongation and oxidation, in both caecal enterocytes and hepatocytes, but

without major detrimental effects on growth. In particular, HUFA synthesis in both enterocytes and hepatocytes was increased in trout fed CPO compared to fish fed FO, confirming that the fatty acid desaturation and elongation pathway in enterocytes may be regulated similarly in both liver and intestinal tissue. In contrast, β-oxidation activity in enterocytes tended to be reduced by dietary CPO whereas it was generally unaffected in hepatocytes perhaps suggesting different methods of regulation in the two tissues. Overall, it was concluded that CPO can be used as an effective substitute for fish oil in rainbow trout in terms of permitting similar growth and feed conversion, and having no apparent detrimental effects on fish health. At this point of the research, data of three trials had confirmed that intestine of fish was an important site of HUFA synthesis and fatty acid β-oxidation, and that these processes are affected by changing the dietary fatty acid composition as in vegetable oil replacement of fish oil.

At this stage of the project, the mechanism underlying vegetable oil stimulation of HUFA synthesis in enterocytes and hepatocytes was still unclear. Thus it was not known whether dietary vegetable oil stimulated the activity of the HUFA synthesis pathway as a result of reduced dietary n-3HUFA, reducing suppression of desaturase activities, or due to increased levels of C_{18} PUFA, increasing activity through increased substrate availability. An experiment to definitively answer this question is difficult to design as diets that are high in one (eg n-3HUFA) will consequently be low in the other (C_{18} PUFA) and *vice versa*. However, the next trial aimed to determine if very high substrate levels could maximally activate the HUFA synthesis pathway. For this purpose, linseed oil (LO) alone was chosen to replace dietary fish oil in order to raise the available substrate for conversion to EPA and DHA in enterocytes to the highest possible level. Although this aim of the experiment was achieved, one of the most interesting results of this project was obtained in that dietary LO, as would have been

expected, increased HUFA synthesis in hepatocytes, but not in enterocytes. Indeed, high levels of dietary LO apparently inhibited HUFA synthesis in enterocytes. This was explained as being an artifact of the *in vitro* assay system rather than actual enzymic inhibition. Thus the hypothesis was that there was competition between the exogenously added [1-^{14}C]18:3n-3 substrate and the dietary 18:3n-3 absorbed (as "free" fatty acid) by enterocytes which resulted in the unexpected lower activities. This certainly requires further study and indeed an experiment was performed in summer 2004 in which intracellular free fatty acid levels were to be measured but this experiment failed due to the low metabolic activity of trout enterocytes at this time of the year (see Chapter 7 Discussion). This seasonal effect is another area for further research. Irrespective of these mechanistic questions, it was found that providing high levels of fatty acid substrate for n-3HUFA synthesis by replacing FO with 18:3n-3 rich LO in salmon diets did not enable the fish to maintain tissue n-3HUFA levels. Moreover, fatty acid oxidation activity in enterocytes increased in fish fed dietary LO in comparison to hepatocytes where there was no dietary effect on oxidation of 18:3n-3. The fact that oxidation in enterocytes increased whereas desaturation activity generally decreased was also interpreted as possible competition between the two fatty acid metabolic pathways. In addition though, the data confirmed that there may be differences between enterocytes and hepatocytes in the mechanisms for regulation of fatty acid oxidation.

Despite the above result in enterocytes due to high levels of LO in the diet, it was clear that a source of 18:3n-3 is certainly required in any fish oil replacement. It had been shown previously that linseed oil, as part of an oil blend together with rapeseed oil, did not inhibit HUFA synthesis in enterocytes (Chapter 4). Consequently, the final dietary trial final investigated the effects of a dietary formulation containing blended vegetable oils, based on the information accumulated in previous trials in this

project and others reported in the literature. One factor considered in the development of this oil blend was the relative amounts of specific fatty acids that would enable high conversion of 18:3n-3 to DHA and EPA in salmon. So, through quantitative calculations of the amount of fatty acids contained in individual vegetable oils, it was possible to design a blend that partially mimicked the fatty acid composition of fish oil. Furthermore, the dietary trial was carried out throughout the entire growth cycle of salmon to be able to detect variations in HUFA synthesis and β-oxidation within different seasons or developmental stages of the fish. In summary, the formulation of the diet provided enterocytes with the appropriate requirements to induce increased desaturation/elongation of the exogenously added [1-^{14}C]18:3n-3 compared to fish fed the FO diet. Thus the VO blend increased HUFA synthesis in both enterocytes and hepatocytes. In addition, the proportion of substrate [1-^{14}C]18:3n-3 that was β-oxidised in enterocytes from salmon fed the blended VO diet was lower than that observed in the previous trials. The pattern of fatty acid oxidation was generally similar in enterocytes and hepatocytes. The oil blend used in this trial was therefore a good substitute for fish oil that can be used by the salmon industry with the minimum effect of compromised quality of the product. The trial also showed seasonal/environmental regulation of HUFA synthesis in enterocytes with a peak in activity around the point of seawater transfer that was significantly enhanced by feeding the VO blend. This was also observed with hepatocytes and again showed that regulation of HUFA synthesis in liver and intestine was possibly via similar mechanisms.

The information gathered in this research work, from the development and adoption of the isolation method to the final dietary trial is important for understanding the intracellular metabolism of dietary fatty acids in intestine/enterocytes in salmonids. Although the information is concise, the overall understanding of these metabolic

pathways in this organ/tissue is incomplete and will require further research. In the present work, it was not possible due to time and financial constraints to evaluate other potential fates of dietary fatty acids in intestinal cells such as incorporation into cellular lipids and secretion of lipids through lipoprotein production. Comprehensive fatty acid budget studies have been recently performed in hepatocytes (Stubhaug *et al.*, 2005). This study showed diet had a clear effect on the overall lipid metabolism, with replacing 75% of the fish oil with vegetable oil resulting in decreased uptake of all fatty acids and reduced incorporation of fatty acids into cellular lipids, but increased β-oxidation activity, and higher recovery in products of desaturation and elongation of [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3 (Stubhaug *et al.*, 2005). These authors also reported that the highest recovery of radioactivity in the cells was found in triacylglycerols, and of the phospholipids the highest recovery was found in phosphatidylcholine. A similar study in enterocytes will help to elucidate the way intestinal cells utilise different fatty acid substrates, the mechanisms of transport through membranes and, importantly, it may provide evidence regarding the role of HUFA synthesis in enterocytes; specifically if the HUFA synthesised by enterocytes is for their own cellular requirements (membrane synthesis) or, alternatively, if it is exported and can contribute to overall HUFA content of the fish. Recently, Bell and Dick (2004) give more information regarding production of DHA in trout. The authors measured DHA synthesis *in vivo*, and a mass balance calculation showed that even on diets tottaly lacking n-3HUFA rainbow trout were unable to synthesise sufficient 22:6n-3 from 18:3n-3 to retain their body concentration of this fatty acid.

The fact that the fatty acid substrate in the present work was highly catabolised suggest that β-oxidation of 18:3n-3 is maximal under all conditions. In a series of *in vivo* experiments (Bell *et al.*, 2001b, 2003c; 2004) the great majority of dietary D_5-

18:3n-3 is catabolised and is therefore unavailable for PUFA synthesis. As the authors enunciate, this is well established in mammals where it has been shown that both 18:2n-6 and 18:3n-3 are largely catabolised, even under conditions of extreme essential fatty acid deficiency, and the resulting acetate used for *de novo* synthesis of non-essential fatty acids (Cunnane, 1996). It therefore appears that there are no metabolic controls in place to spare precursor C18 PUFA for synthesis of functionally essential C20 and C22 PUFA even under conditions of EFA limitation (Bell and Dick, 2004).

Another interesting area of research currently under investigation in the laboratories in Stirling is the mechanisms by which dietary fatty acids affect HUFA synthetic activity and, specifically, the effects of fatty acids on the expression of fatty acid desaturase genes. There are some studies that report the effects of dietary fatty acids on desaturase gene expression in mammals (Cho *et al.*, 1999a, 1999b) and also in trout (Seiliez *et al.*, 2001). PUFA can potentially affect gene transcription by a number of direct and indirect mechanisms (Jump *et al.*, 1999) and are known to bind and directly influence the activities of a variety of transcription factors including peroxisome proliferator activated receptors α (PPARα), which in turn have been shown to be regulators of many genes involved in lipid homeostatic processes (Jump, 2002). In rodents, peroxisomal proliferators, which also activate PPARs, are known to up-regulate fatty acyl desaturation and oxidation (Grønn *et al.*, 1992). PPAR genes have been cloned from a variety of fish species including Atlantic salmon, plaice, sea bream and sea bass (Leaver *et al.*, 2004, 2005; Boukouvala *et al.*, 2004) and the peroxisomal proliferator, clofibrate, increased the desaturation of 20:5n-3 in rainbow trout (Tocher and Sargent, 1993). However, these data do not exclude the possibility that fatty acids may also influence desaturation and oxidation more directly at a membrane level through alterations in fluidity or membrane microenvironments (Zheng *et al.*, 2005b).

Molecular and genetic studies have been carried out to identify the mechanism of gene expression of desaturation and elongation enzymes in salmon (Zheng *et al.*, 2004, 2005a, 2005b). So far, the studies on nutritional regulation of desaturase gene expression have focussed on liver, but it is known that intestine is a site of high expression of Δ6, Δ5 desaturases and PUFA elongase in salmon, and that dietary VO increases the expression of the desaturase genes (Zheng et al., 2005a). Therefore, exploration of desaturase and elongase gene expression in enterocytes is certainly an area that requires further research.

Whether PPARs are involved in the regulation of fatty acid desaturase genes or not is still under investigation, but they are certainly involved in the regulation of fatty acid oxidation (Smith, 2002). It was interesting that the regulation of fatty acid oxidation in hepatocytes and enterocytes observed in this project appeared to be different as salmon liver expressed high levels of PPARα and lower levels of PPARγ, whereas intestine expressed high amounts of PPARγ and low amounts of PPARα (Leaver and Tocher, personal communication). Activation of PPARα switches on suites of genes involved in fatty acid oxidation whereas PPARγ (also expressed highly in adipose tissue) switches on genes involved in deposition of fatty acids (Smith, 2002; Walczak and Tontonoz, 2002). Thus, this difference in PPAR subtype may account for the differences observed in fatty acid oxidation observed between enterocytes and hepatocytes.

Future research in desaturation and elongation activity in enterocytes of other freshwater or marine fish, will result in a better understanding of the role of this tissue in the metabolic pathways of HUFA synthesis. There is much less known about the capacity to produce HUFA in potential aquaculture species including Atlantic cod (*Gadus morhua*) or Atlantic halibut (*Hippoglossus hippoglossus*), or other marine white

fish rich in HUFA than in salmonids. In addition, comparative genetic studies in these species and salmonids may elucidate the differences in HUFA synthetic capacity and offer insights to how HUFA synthesis could be potentially maximised in farmed fish species when fed diets containing high levels of VO.

Smoltification and seawater transfer of Atlantic salmon cause not only morphological and physiological transformations, but also resulted in important changes regarding fatty acid metabolism in both, enterocytes and hepatocytes. Moreover, seasonal effects on the metabolic activity of enterocytes in rainbow trout were clearly observed in the present study and proved a not insignificant problem. The mechanism underpinning the seasonal effect in trout is completely unknown. Therefore, seasonal effects on HUFA synthesis in salmonids are areas that require considerably more research both to identify and quantify as well as discovering the regulatory mechanisms. The environmental cues that have been discussed at least superficially in the present study have been daylength and water temperature, however salinity is another environmental factor that is completely unstudied in this respect. Thus, estimation of desaturation and elongation activities in fish cultured under a range of different salinities would possibly give some insight to its role, if any, in the regulation of HUFA synthesis in salmonids e.g. declining HUFA synthesis after seawater transfer in salmon. Investigating sea run trout may be of interest in this respect. However, Atlantic salmon are anadromous fish, which means that they hatch in fresh water, but then spend most of their life in the sea before returning to fresh water to spawn. In contrast, most eels are catadromous fish, which means that they live most of they life in freshwater and only return to the sea to spawn. Both, Atlantic salmon (Tocher *et al.*, 2003a) and European eel (*Anguilla anguilla*) (Kissil *et al.*, 1987) have been proved to have the capacity to desaturate and elongate dietary linoleic acid; therefore, a comparison of fatty acid

desaturation/elongation in enterocytes and hepatocytes, between anadromous and catadromous species may be an interesting experiment that could lead to a better understanding of the regulation of HUFA synthesis in fish. Moreover, a comparison between anadromous and catadromous fish in fatty acid desaturase and elongase gene expression in enterocytes and hepatocytes would probably be useful for the general understanding of fish fatty acyl metabolism.

In conclusion, the present study has provided new insights to the metabolism of dietary fatty acids in intestinal tissue of salmonid fish. As always the case, in addition to elucidating some aspects, the new discoveries have also highlighted the areas we know little about and led to further questions that require answers. Therefore, there is still much to learn about intracellular fatty acid metabolism in intestinal cells in fish that will require a considerable amount of further research.

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APPENDIX

Published Articles

Tocher, D.R., J. Fonseca-Madrigal, J.G. Bell, J.R. Dick, R.J. Henderson and J.R. Sargent. 2002. **Effects of diets containing linseed oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes in Atlantic salmon (*Salmo salar*).** *Fish Physiol. Biochem.* 26, 157-170.

Tocher, D.R., J.G. Bell, F. McGhee, J.R. Dick and J. Fonseca-Madrigal. 2003. **Effects of dietary lipid level and vegetable oil on fatty acid metabolism in Atlantic salmon (*Salmo salar* L.) over the whole production cycle.** *Fish Physiol. Biochem.* 29, 193–209.

Tocher, D.R., J. Fonseca-Madrigal, J.R. Dick, W-K. Ng, J.G. Bell and P.J. Campbell. 2004. **Effects of water temperature and diets containing palm oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes of rainbow trout (*Oncoryhnchus mykiss*).** *Comp. Biochem. Physiol.,* 137B, 49-63.

Fonseca-Madrigal J., V. Karalazos, P.J. Campbell, J.C. Bell and D.R. Tocher. 2005. **Influence of dietary palm oil on growth, tissue fatty acid compositions, and fatty acid metabolism in liver and intestine in rainbow trout (*Oncorhynchus mykiss*).** *Aquaculture Nutrition* 11, 241-250.

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Effects of water temperature and diets containing palm oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes of rainbow trout (*Oncorhynchus mykiss*)

Douglas R. Tocher[a,*], Jorge Fonseca-Madrigal[a], James R. Dick[a], Wing-Keong Ng[b], J. Gordon Bell[a], Patrick J. Campbell[c]

[a]*Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK*
[b]*Fish Nutrition Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia*
[c]*BioMar Ltd., Grangemouth Docks, Grangemouth FK3 8UL, Scotland, UK*

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Abstract

Food grade fisheries have reached their sustainable limits while aquaculture production has increased to meet consumer demands. However, for growth in aquaculture to continue and utilise sustainable, feeding ingredients, alternatives to fish oil (FO), the predominant lipid component of fish diets, must be developed. Therefore, there is currently considerable interest in the regulation of fatty acid metabolism in fish in order to determine strategies for the best use of plant oils in diets for commercially important cultured fish species. Plant oils are characteristically rich in C_{18} polyunsaturated fatty acids (PUFA) but devoid of C_{20} and C_{22} highly unsaturated fatty acids (HUFA) found in FO. The fatty acyl desaturase enzyme activities involved in the biosynthesis of HUFA from PUFA are known to be under nutritional regulation and can be increased in fish fed diets rich in plant oils. However, fatty acid desaturase activity is also known to be modulated by water temperature in fish. The present study aimed to investigate the interaction between water temperature and diet in the regulation of fatty acid metabolism in rainbow trout. Trout, acclimatized to 7, 11 or 15 °C, were fed for 4 weeks on diets in which the FO was replaced in a graded manner by palm oil. At the end of the trial, fatty acyl desaturation/elongation and β-oxidation activities were determined in isolated hepatocytes and intestinal enterocytes using [1-^{14}C]18:3n-3 as substrate, and samples of liver were collected for analysis of lipid and fatty acid composition. The most obvious effect of temperature was that fatty acid desaturation/elongation and β-oxidation were reduced in both hepatocytes and intestinal enterocytes from fish maintained at the highest water temperature (15 °C). There were differences between the two tissues with the highest desaturation/elongation and β-oxidation activities tending to be in fish held at 11 °C in the case of hepatocytes, but 7 °C in enterocytes. Correlations between fatty acid metabolism and dietary palm oil were most clearly observed in desaturation/elongation activities in both hepatocytes and enterocytes at 11 °C. The highest β-oxidation activities were generally observed in fish fed FO alone in both hepatocytes and enterocytes with palm oil having differential effects in the two cell types.

Keywords: Desaturation; Enterocytes; Hepatocytes; β-Oxidation; Palm oil; Polyunsaturated fatty acids; Rainbow trout

Abbreviations: BHT, butylated hydroxytoluene; CPO, crude palm oil; FAF-BSA, fatty acid-free bovine serum albumin; FO, fish oil; HBSS, Hanks balanced salt solution; HUFA, highly unsaturated fatty acids (carbon chain length $\geqslant C_{20}$ with $\geqslant 3$ double bonds)

*Corresponding author. Tel.: +44-1786-467996; fax: +44-1786-472133.
E-mail address: d.r.tocher@stir.ac.uk (D.R. Tocher).

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1. Introduction

The polyunsaturated fatty acids (PUFA), linoleate (18:2n-6) and linolenate (18:3n-3) cannot be synthesized de novo by animals, including fish, and so are termed essential fatty acids (EFA) (Holman, 1986). However, in fish, the C_{18} PUFA in themselves have no essential functions and their essentiality actually derives from their conversion to the functionally active highly unsaturated fatty acids (HUFA) eicosapentaenoate (20:5n-3), docosahexaenoate (22:6n-3) and arachidonate (20:4n-6) (Sargent et al., 1995, 1999). Fish vary in their ability to perform these conversions and thus qualitative and quantitative EFA requirements vary between species, but in salmonid fish, including rainbow trout (*Oncorhynchus mykiss*), 18:3n-3 and 18:2n-6 at a combined level of approximately 1% of the diet can satisfy EFA requirements (Sargent et al., 2002). However, the commercial culture of salmon and trout has traditionally used fish meal and fish oil (FO), rich in n-3HUFA, as protein and lipid sources for dry extruded feeds (Sargent and Tacon, 1999). This practice has been both scientifically sound, as salmonids are piscivorous in the wild, and commercially sound, as FO has been relatively cheap and readily available. The fatty acid composition of salmon and trout grown on diets containing FO are very similar to wild fish, being high in the n-3HUFA that are beneficial in the human diet (Ackman, 1980; Henderson and Tocher, 1987; Bell et al., 2001, 2002).

Due to over-exploitation of wild fisheries, an increasing proportion of fish for human consumption is provided by aquaculture which is expanding at over 10% per year (Tidwell and Allan, 2002). As a consequence, demand for FOs is increasing rapidly and current estimates suggest that aquaculture feeds will consume approximately 90% of the world FO supplies by 2010 (Barlow, 2000). If aquaculture is to continue to expand and supply more of the global demand for fish, alternatives to FO must be found, and the only sustainable alternatives are plant (vegetable) oils which are rich in C_{18} PUFA but devoid of the n-3HUFA abundant in FOs (Sargent et al., 2002). However, culture of salmonids on diets containing high levels of vegetable oils considerably alters the fatty acid composition of the flesh, resulting in high levels of the C_{18} PUFA and much reduced levels of n-3HUFA (Bell et al., 2001, 2002). Therefore, there is currently considerable interest in the regulation of the HUFA biosynthetic pathways in fish in order to determine how the conversion of C_{18} PUFA to HUFA can be improved in commercially important cultured fish species (Sargent et al., 2002; Tocher, 2003). For instance, the fatty acyl desaturase enzyme activities involved in the HUFA biosynthesis pathway are known to be under nutritional regulation in mammals (Brenner, 1981) and this has also been demonstrated in fish (Sargent et al., 2002). Similarly, fatty acid desaturase activity is known to be modulated by water temperature in fish (Ninno et al., 1974; De Torrengo and Brenner, 1976; Schuenke and Wodtke, 1983; Hagar and Hazel, 1985; Wodtke and Cossins, 1991).

The present study aimed to investigate the interaction between water temperature and diet in the regulation of fatty acid metabolism in rainbow trout. Palm oil was chosen as the vegetable oil to replace FO for several reasons. Firstly, palm oil production is predicted to exceed soybean oil production within the next 10 years making it the most abundant vegetable oil in the world (Gunstone, 2001). Secondly, palm oil has some advantages compared to other vegetable oils that may enable it to have a potential use in the diets of a wide range of fish species (Ng, 2002a,b). Finally, the use of crude palm oil (CPO) in the diets of Atlantic salmon and rainbow trout has given growth and feed utilisation efficiency comparable to fish fed equivalent levels of marine FOs (Torstensen et al., 2000; Rosenlund et al., 2001; Bell et al., 2002; Caballero et al., 2002). Rainbow trout, acclimatized to 7, 11 or 15 °C, were fed for 4 weeks on diets in which the FO was replaced in a graded manner by CPO. At the end of the trial, fatty acyl desaturation/elongation and oxidation activities were determined in isolated hepatocytes and intestinal enterocytes using [1-^{14}C]18:3n-3 as substrate, and samples of liver were collected for analysis of lipid and fatty acid composition.

2. Materials and methods

2.1. Experimental diets

Practical extruded diets (3-mm diameter) containing 47% crude protein and 22.5% crude lipid were formulated (BioMar Ltd, Scotland, UK) using the same basal ingredients and varying only in the content of CPO that was added at the expense of marine FO (Table 1). CPO was fully melted and thoroughly mixed with the FO before

Table 1
Ingredients and formulation (g per 100 g diet) of experimental diets

Component	Diet			
	P0	P25	P50	P100
Fishmeal[a]	34.3	34.3	34.3	34.3
Hi Pro Soya[b]	12.7	12.7	12.7	12.7
Wheat gluten[b]	10.0	10.0	10.0	10.0
Corn gluten[c]	10.0	10.0	10.0	10.0
Wheat[d]	10.0	10.0	10.0	10.0
Marine oil[a]	20.0	15.0	10.0	0.0
Palm oil[e]	0.0	5.0	10.0	20.0
Methionine	0.21	0.21	0.21	0.21
Lysine	0.79	0.79	0.79	0.79
Micronutrients[f]	2.41	2.41	2.41	2.41
Vitamin E[g]	0.015	0.011	0.0076	0.0
Ytrium oxide	0.02	0.02	0.02	0.02

[a] Norsemeal Ltd, London, UK.
[b] Cargill, Swinderbury, UK.
[c] Cerestar UK Ltd, Manchester, UK.
[d] J.D. Martin, Tranent, UK.
[e] United Plantations Bhd, Jenderata Estate, Teluk Intan, Malaysia.
[f] Vitamins, minerals and astaxanthin (Carophyl pink®), BioMar A/S, Brande Denmark.
[g] Roche, Basel, Switzerland.

the oils or oil mixtures were used to coat the extruded pellets to give the four diets with CPO replacing 0% (P0), 25% (P25), 50% (P50) and 100% (P100) of the FO. The fatty acid compositions of the resultant diets are shown in Table 2. The diets were designed to meet all the known nutritional requirements of salmonid fish including n-3 EFA (US National Research Council, 1993).

2.2. Experimental fish

Rainbow trout, with a mean initial body mass of approximately 45 g, were obtained from a local fish hatchery and stocked into three 1000-l tanks on arrival at the Institute of Aquaculture (University of Stirling, Scotland). Over a 2-week period, the water temperature of two of the tanks was gradually increased to 11 ± 1 and 15 ± 1 °C, respectively, while the third tank was maintained at the ambient water temperature of 7 ± 1 °C. The indoor tanks were subjected to a photoperiod regime of 12-h light:12-h dark. All fish were fed a commercial trout pellet during this acclimation period. After 2 weeks, each group of temperature-acclimatized fish was randomly distributed into four circular tanks of 100-l capacity (at 40 fish/tank) supplied with flow-through water of the same acclimation temperature at 1 l min^{-1}. Fish maintained at each temperature grouping were fed one of the four experimental diets for 4 weeks before sampling. Fish were fed to satiety three times daily.

2.3. Lipid extraction and fatty acid analyses

Fish were killed by a blow to the head and intact livers dissected from three fish per dietary treatment at each temperature and immediately frozen in liquid nitrogen. Total lipid contents of livers and diet samples were determined gravimetrically after extraction by homogenization in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, basically according to Folch et al. (1957). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transesterification using 2 ml of 1% H_2SO_4 in methanol plus 1 ml

Table 2
Fatty acid composition (% of total fatty acids) of experimental diets

Fatty acid	Diet			
	P0	P25	P50	P100
14:0	6.4	4.6	3.7	1.6
16:0	18.6	26.7	30.6	37.9
18:0	3.4	3.8	4.2	4.2
Total saturated[a]	29.3	35.8	39.1	44.1
16:1n-7	6.7	4.5	3.5	1.0
18:1n-9	11.1	20.2	24.2	35.9
18:1n-7	2.5	1.9	1.8	0.6
20:1n-9	5.4	3.5	2.8	0.4
22:1n-11	7.2	4.5	2.5	0.4
Total monounsaturated[b]	34.7	35.6	35.5	38.5
18:2n-6	5.9	8.1	9.1	11.8
20:4n-6	0.6	0.4	0.3	0.2
Total n-6 PUFA[c]	7.2	9.0	9.9	12.1
18:3n-3	1.3	1.0	0.9	0.6
18:4n-3	2.9	1.9	1.4	0.2
20:5n-3	9.2	6.0	4.7	1.2
22:5n-3	1.2	0.8	0.7	0.3
22:6n-3	11.3	7.9	6.4	2.7
Total n-3 PUFA[d]	26.9	18.4	14.6	5.1
Total PUFA[e]	36.0	28.5	25.4	17.4
n-3/n-6	3.7	2.0	1.5	0.4

[a] Total includes 15:0 and 20:0.
[b] Total includes 16:1n-9, 20:1n-7, 22:1n-9 and 24:1.
[c] Total includes 18:3n-6, 20:2n-6 and 22:5n-6.
[d] Total includes 20:3n-3 and 20:4n-3.
[e] Total includes C_{16} PUFA; PUFA, polyunsaturated fatty acids.

toluene as described by Christie (1982) and FAME extracted and purified as described previously (Tocher and Harvie, 1988). FAME were separated and quantified by gas–liquid chromatography (Fisons GC8600, Fisons Ltd, Crawley, UK) using a 30 m×0.32 mm capillary column (CP wax 52CB; Chrompak Ltd, London, UK). Hydrogen was used as carrier gas and temperature programming was from 50 to 180 °C at 40 °C min^{-1} and then to 225 °C at 2 °C min^{-1}. Individual methyl esters were identified by comparison to known standards and by reference to published data (Ackman, 1980).

2.4. Preparation of isolated hepatocytes and caecal enterocytes

Intact livers were dissected from three fish per dietary treatment, the gall bladder removed carefully and the main blood vessels trimmed. The liver was perfused via the hepatic vein with solution A (calcium and magnesium-free Hanks balanced salt solution (HBSS)+10 mM HEPES+1 mM EDTA) to clear blood from the tissue using a syringe fitted with a 21-g needle. The liver was chopped finely with scissors and approximately 0.5 g of chopped liver was taken and incubated with 20 ml of solution A containing 0.1% (w/v) collagenase in a 25 ml 'Reacti-flask' in a shaking water bath at 20 °C for 45 min. The digested liver tissue was filtered through 100-μm nylon gauze and the cells were collected by centrifugation at 300×*g* for 2 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. The washing was repeated with a further 20 ml of solution A without FAF-BSA. The hepatocytes were resuspended in 10 ml of Medium 199 containing 10 mM HEPES and 2 mM glutamine. One-hundred microlitres of cell suspension was mixed with 400 μl of Trypan Blue and hepatocytes were counted and their viability assessed using a haemocytometer. With relatively minor modification, the above method has been used successfully to isolate enterocyte-enriched preparations from various regions of the intestine including pyloric caecae (Tocher et al., 2002). Briefly, the intestinal tract was removed and pyloric caecae dissected, cleaned of adhering adipose tissue and lumenal contents rinsed away with solution A. The caecae were chopped finely and incubated with 0.1% (w/v) collagenase as above. The digested intestinal tissues were filtered through 100-μm nylon gauze and the cells collected, washed, resuspended in medium (as above), and viability checked as for hepatocytes. The enriched-enterocyte preparation was predominantly enterocytes although some secretory cells were present. The viability was >95% at isolation and decreased by less than 10% over the period of the incubation. One-hundred microlitres of the hepatocyte and enterocyte suspensions were retained for protein determination according to the method of Lowry et al. (1951) after incubation with 0.4 ml of 0.25% (w/v) SDS/1 M NaOH for 45 min at 60 °C.

2.5. Incubation of hepatocyte and enterocyte preparations with [1-^{14}C]18:3n-3

Six millilitres of each hepatocyte or enterocyte suspension were dispensed into a 25-cm^2 tissue culture flask. Hepatocytes and enterocytes were incubated with 0.3 μCi (~1 μM) [1-^{14}C] 18:3n-3 and added as a complex with FAF-BSA in phosphate buffered saline prepared as described previously (Ghioni et al., 1997). After addition of isotope, the flasks were incubated at 20 °C for 2 h. After incubation, the cell layer was dislodged by gentle rocking and the cell suspension transferred to glass conical test tubes and 1 ml of each suspension withdrawn into a 2-ml microcentrifuge tube for β-oxidation assay as described below. The cell suspensions remaining in the glass conical centrifuge tubes were used for the desaturation/elongation assay as described below.

2.6. Assay of hepatocyte and enterocyte fatty acyl desaturation/elongation activities

The cell suspensions were centrifuged at 500×*g* for 2 min, the supernatant discarded and the hepatocyte or enterocyte 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 s before extraction of total lipid using ice-cold chloroform/methanol (2:1, v/v) containing 0.01% (w/v) 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 isohexane containing 0.01% BHT and applied as 2.5-cm streaks to thin-layer chromatography (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, GA) 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.

2.7. Assay of hepatocyte and enterocyte fatty acyl oxidation activities

The assay of fatty acid oxidation in intact hepatocytes has been demonstrated in rats (Frøyland et al., 1996; Madsen et al., 1998) and requires the determination of acid-soluble radioactivity as described in detail previously (Frøyland et al., 2000; Torstensen et al., 2000). Briefly, 1 ml of hepatocyte or enterocyte suspension was homogenized with a hand-held tissue disrupter (Ultra-Turrax T8/S8N-5G probe, IKA-Werke GmbH & Co., Staufen, Germany) and centrifuged at 10 000×*g* for 10 min in a microcentrifuge. Five-hundred microlitres of the supernatant was taken into a clean 2 ml microcentrifuge tube and 100 μl of ice-cold 6% FAF-BSA solution in water was added. After mixing thoroughly, the protein was precipitated by the addition of 1.0 ml of ice-cold 4 M perchloric acid ($HClO_4$). After vortexing, the tubes were centrifuged at 10 000×g for 10 min in a microcentrifuge. Five-hundred microlitres of the supernatant was carefully transferred to a scintillation vial, 4 ml of scintillant added and radioactivity in the acid-soluble fraction determined as described above for desaturation/elongation assay.

2.8. Materials

[1-^{14}C]18:3n-3 (50–55 mCi mmol^{-1}) was obtained from NEN (NEN Life Science Products-UK Ltd, Stevenage, UK). HBSS, Medium 199, HEPES buffer, glutamine, collagenase (type IV), FAF-BSA, BHT, silver nitrate and perchloric acid were obtained from Sigma Chemical Co. (Poole, UK). TLC plates, 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.

2.9. Statistical analysis

All data are presented as means ± S.D. ($n=3$) and all statistical analyses were performed using PRISM 3 (Graphpad Software Inc., San Diego, USA). The effects of dietary CPO on liver fatty acid composition, and fatty acid desaturation and β-oxidation at each specific temperature were analysed by one-way ANOVA followed, where appropriate, by Tukey's post-test to determine significant differences between individual treatments. The combined effects of both water temperature and dietary CPO and their interactions were determined by two-way ANOVA. Percentage data, and data which were identified as non-homogeneous (Bartlett's test), were subjected to either arcsine or log transformation before analysis. Differences were regarded as significant when $P<0.05$ (Zar, 1984).

3. Results

3.1. Dietary fatty acid compositions

The control diet (P0), formulated with 100% FO, contained approximately 29% total saturates, mainly 16:0, almost 35% total monounsaturated fatty acids with approximately one-third as 18:1n-9 and one-third as the long chain monoenes 20:1 and 22:1, 7% n-6 fatty acids predominantly 18:2n-6 and 27% n-3 fatty acids predominantly n-3HUFA, 20:5n-3 and 22:6n-3 in approximately equal amounts (Table 2). Inclusion of graded amounts of CPO resulted in graded increased percentages of 16:0, 18:1n-9 and 18:2n-6 with concomitant decreased proportions of n-3HUFA, total PUFA and long chain monoenes. Thus, in the diet formulated with 100% CPO (P100), the levels of 16:0, 18:1n-9 and 18:2n-6 had risen to 38, 36 and 12% of total fatty acids, respectively, whereas 20:5n-3 and 22:6n-3 totalled only 4% (Table 2).

3.2. Effects of diet on liver lipid content and fatty acid composition

The lipid content and fatty acid compositions of livers from trout held at 7, 11 or 15 °C are shown in Tables 3–5, respectively. Neither dietary palm

Table 3
Total lipid content (percentage of wet wt.) and fatty acid composition (percentage of total fatty acids) of total lipid from liver of rainbow trout fed graded amounts of palm oil at a water temperature of 7 °C

Fatty acid	P0	P25	P50	P100
Lipid content	2.9±0.6[b]	2.9±0.2[b]	2.6±0.7[b]	4.3±0.5[a]
14:0	1.6±0.3	1.6±0.3	1.2±0.3	1.8±0.3
16:0	20.0±0.6	19.4±0.2	20.8±2.1	19.2±1.3
18:0	4.2±0.7	4.9±0.4	4.8±0.8	5.2±0.1
Total saturated[a]	26.2±0.6	26.3±0.2	27.2±1.7	26.6±0.9
16:1n-7	3.8±1.7	4.1±1.3	3.4±2.0	5.3±0.1
18:1n-9	11.9±3.1[b]	15.4±3.7[ab]	15.9±5.6[ab]	24.5±0.7[a]
18:1n-7	2.4±0.2	1.9±0.2	1.9±0.5	1.9±0.4
20:1n-9	2.9±0.3	2.3±0.6	2.2±0.7	3.3±0.6
22:1	1.7±0.3[b]	1.3±0.4[b]	1.0±0.4[b]	2.8±0.3[a]
24:1n-9	1.2±0.1[a]	1.0±0.1[ab]	1.0±0.2[ab]	0.7±0.0[b]
Total monoenes[b]	25.1±4.4[b]	26.9±4.4[b]	26.4±5.7[b]	39.8±1.3[a]
18:2n-6	2.6±0.1[b]	3.5±0.1[ab]	4.0±1.5[ab]	4.8±0.1[a]
20:3n-6	0.3±0.2	0.8±0.1	1.2±1.0	1.1±0.4
20:4n-6	1.7±0.5	1.7±0.5	2.2±0.9	1.4±0.4
Total n-6 PUFA[c]	5.3±0.1[b]	7.3±0.5[ab]	8.5±3.8[ab]	8.7±1.0[a]
20:4n-3	0.7±0.1[a]	0.4±0.1[ab]	0.4±0.2[ab]	0.3±0.1[b]
20:5n-3	6.6±0.6[a]	5.0±0.6[ab]	4.8±1.7[ab]	2.5±0.6[b]
22:5n-3	1.7±0.0[a]	1.4±0.2[a]	1.4±0.3[a]	0.9±0.1[b]
22:6n-3	33.5±4.4[a]	32.0±3.7[a]	30.6±3.6[a]	20.0±0.2[b]
Total n-3 PUFA[d]	43.3±5.0[a]	39.4±4.1[a]	37.6±5.6[a]	24.4±0.8[b]
Total n-9 PUFA	0.1±0.1	0.2±0.1	0.4±0.2	0.5±0.2
Total PUFA	48.7±4.8[a]	46.8±4.5[a]	46.5±4.3[a]	33.6±0.7[b]
n-3/n-6	8.1±1.1[a]	5.4±0.3[ab]	5.2±2.7[ab]	2.8±0.4[b]

Results are means±S.D. ($n=3$). Significance of differences between means were determined by one-way ANOVA followed, where appropriate, by Tukey's multiple comparison test as described in Section 2. Values within a row with a different superscript letter are significantly different ($P<0.05$). PUFA, polyunsaturated fatty acids.

[a] Totals include 15:0 and 20:0 present at up to 0.3%.

[b] Totals include 16:1n-9, 20:1n-11 and 20:1n-7 present at up to 0.5%.

[c] Totals include 18:3n-6, 20:2n-6, 22:4n-6 and 22:5n-6 present at up to 0.5%.

[d] Totals include 18:3n-3 and 18:4n-3 present at up to 0.4%.

oil inclusion nor temperature had any significant effect on the lipid content of the trout livers (Table 6). There was a significant interaction between the two parameters showing that effects on lipid content were not consistent across the temperature range or range of palm oil inclusion. The principal fatty acids of liver total lipid from fish fed diet P0 (100% FO) were, in rank order, 22:6n-3>16:0>18:1n-9>20:5n-3=18:0>16:1n-7 (Tables 3–5). The graded change in fatty acid compositions of the diets in response to increasing CPO content, described above, was partly reflected in the liver fatty acid compositions with graded increased percentages of 18:1n-9 and 18:2n-6, and decreased proportions of n-3HUFA but, in contrast, liver 16:0 levels were not greatly increased by dietary CPO (Tables 3–5). The effects of palm oil inclusion on liver 18:1n-9, 18:2n-6, 20:5n-3, 22:6n-3, total monoene, n-6 and n-3PUFA levels and the n-3/n-6PUFA ratio were highly significant (Table 6). Temperature had no effect on the levels of any of these fatty acids other than on 20:5n-3 and the n-3/n-6PUFA ratio which both appeared to be higher at the lowest temperature. The level of 16:0 was also affected by temperature, but this effect varied with palm oil inclusion resulting in significant interaction, the only fatty acid to show such interaction (Table 6).

3.3. Effects of diet on hepatocyte and enterocyte fatty acid desaturation/elongation activities

Dietary CPO had significant effects on the total desaturation of [1-^{14}C]18:3n-3, measured as the

Table 4
Total lipid content (% of wet wt.) and fatty acid composition (% of total fatty acids) of total lipid from liver of rainbow trout fed graded amounts of palm oil at a water temperature of 11 °C

Fatty acid	P0	P25	P50	P100
Lipid content	3.8±0.5	3.6±0.5	2.8±0.3	3.4±0.4
14:0	2.1±0.0[a]	1.9±0.6[ab]	1.3±0.2[ab]	1.1±0.3[b]
16:0	17.3±1.0[b]	18.5±0.9[b]	20.7±0.4[a]	17.7±0.6[b]
18:0	6.3±0.3	5.2±1.0	5.5±1.0	6.5±0.9
Total saturated[a]	26.0±1.2	26.0±1.3	27.9±0.6	25.7±0.3
16:1n-7	4.2±1.0	4.0±1.1	2.8±0.6	2.6±0.9
18:1n-9	14.5±2.0[b]	16.4±4.4[ab]	14.5±2.7[b]	24.4±4.7[a]
18:1n-7	2.2±0.2	2.1±0.5	1.7±0.1	1.7±0.1
20:1n-9	2.9±0.4	3.5±1.9	1.9±0.3	3.3±0.3
22:1	1.6±0.1	2.6±2.3	0.7±0.1	0.9±0.8
24:1n-9	1.1±0.3	0.9±0.2	0.9±0.2	1.0±0.1
Total monoenes[b]	27.8±2.0	30.8±6.3	23.4±2.7	34.9±5.8
18:2n-6	3.1±0.1[b]	3.7±0.5[b]	3.4±0.4[b]	5.3±0.5[a]
20:2n-6	0.6±0.2[b]	0.5±0.1[b]	0.6±0.2[b]	1.3±0.2[a]
20:3n-6	0.7±0.0[b]	0.8±0.4[b]	1.0±0.2[b]	1.6±0.1[a]
20:4n-6	1.9±0.3[b]	1.9±0.5[b]	2.1±0.3[ab]	3.5±0.9[a]
22:5n-6	0.5±0.0[b]	0.4±0.2[b]	0.5±0.2[b]	1.0±0.1[a]
Total n-6 PUFA[c]	6.9±0.4[b]	7.5±0.8[b]	7.8±0.7[b]	13.1±0.8[a]
20:4n-3	0.6±0.1[a]	0.5±0.2[a]	0.3±0.0[ab]	0.1±0.1[b]
20:5n-3	4.9±0.3[a]	4.7±0.6[a]	4.4±1.8[a]	1.4±0.2[b]
22:5n-3	1.1±0.1[a]	1.1±0.2[a]	1.1±0.1[a]	0.5±0.1[b]
22:6n-3	31.8±3.3[ab]	28.5±4.7[ab]	34.6±1.8[a]	23.3±5.3[b]
Total n-3 PUFA[d]	39.2±2.9[ab]	35.6±4.8[ab]	40.7±3.6[a]	25.5±5.6[b]
Total n-9 PUFA	0.1±0.1[b]	0.2±0.0[b]	0.2±0.1[b]	0.8±0.1[a]
Total PUFA	46.1±3.2	43.3±5.1	48.7±3.0	39.4±6.1
n-3/n-6	5.7±0.2[a]	4.8±0.6[b]	5.3±0.9[a]	1.9±0.3[b]

Results are means±S.D. ($n=3$). Significance of differences between means were determined by one-way ANOVA followed, where appropriate, by Tukey's multiple comparison test as described in Section 2. Values within a row with a different superscript letter are significantly different ($P<0.05$). PUFA, polyunsaturated fatty acids.

[a] Totals include 15:0 and 20:0 present at up to 0.3%.

[b] Totals include 16:1n-9, 20:1n-11 and 20:1n-7 present at up to 0.7%.

[c] Totals include 18:3n-6 and 22:4n-6 present at up to 0.2%.

[d] Totals include 18:3n-3 and 18:4n-3 present at up to 0.4%.

summed radioactivity recovered as desaturated fatty acid products (18:4, 20:4, 20:5, 22:5 and 22:6), in hepatocytes, but the effects were dependent on water temperature as indicated by the significant interaction (Table 7). Fatty acid desaturation in hepatocytes increased in a graded manner with dietary CPO inclusion at 11 °C, and similarly, CPO also increased desaturation at 7 °C but had little effect at 15 °C (Fig. 1). Temperature had a significant effect (Table 7) with generally lower activities in hepatocytes from fish held at the highest water temperature (Fig. 1).

Increasing dietary CPO gave a graded increase in fatty acid desaturation in caecal enterocytes from fish held at 11 °C, and generally increased activity in fish maintained at 15 °C but dietary CPO decreased fatty acid desaturation in fish maintained at the lowest water temperature (Fig. 2). Thus, dietary CPO had no significant effect on fatty acid desaturation in caecal enterocytes when analysed by two-way ANOVA because of the significant interaction with temperature (Table 7). The effect of temperature on fatty acid desaturation in caecal enterocytes was highly significant (Table 7) and a general pattern of decreasing activity with increasing water temperature was observed (Fig. 2). The total desaturation of [1-^{14}C]18:3n-3 in hepatocytes generally exceeded that in enterocytes other than that in fish fed P0 diet at 7 °C (Figs. 1 and 2).

Table 5
Total lipid content (% of wet wt.) and fatty acid composition (% of total fatty acids) of total lipid from liver of rainbow trout fed graded amounts of palm oil at a water temperature of 15 °C

Fatty acid	P0	P25	P50	P100
Lipid content	3.3±0.0	3.8±0.7	3.5±0.2	3.0±0.9
14:0	2.6±0.5[a]	1.8±0.6[ab]	1.8±0.4[ab]	1.0±0.2[b]
16:0	17.7±0.3[b]	19.5±1.2[a]	19.3±0.7[a]	19.9±0.9[a]
18:0	5.7±1.4	6.3±0.6	5.4±1.2	6.7±0.4
Total saturated[a]	26.6±1.3	28.0±1.1	27.0±0.3	27.9±1.2
16:1n-7	3.6±1.1	3.7±0.7	3.2±0.4	2.4±1.0
18:1n-9	12.0±2.1[b]	17.4±2.8[ab]	17.7±3.5[ab]	21.0±5.9[a]
18:1n-7	2.5±0.3	2.2±0.3	2.1±0.2	1.7±0.4
20:1n-9	4.2±0.9	3.7±1.5	4.3±0.5	3.0±0.4
22:1	2.5±1.5	1.8±1.1	2.4±1.1	0.5±0.1
24:1n-9	1.3±0.2	1.1±0.0	1.0±0.1	0.9±0.2
Total monoenes[b]	27.4±5.9	31.0±5.4	31.9±3.2	30.3±7.5
18:2n-6	3.4±0.8[a]	3.6±0.7[ab]	4.3±0.2[ab]	5.0±0.3[a]
20:2n-6	0.9±0.2	0.8±0.2	1.0±0.1	1.3±0.2
20:3n-6	0.3±0.1[c]	0.6±0.1[bc]	1.0±0.3[ab]	1.4±0.2[a]
20:4n-6	2.0±0.8	1.6±0.4	1.6±0.2	3.0±1.5
22:5n-6	0.4±0.1	0.5±0.1	0.5±0.0	0.8±0.3
Total n-6 PUFA[c]	7.1±0.4[b]	7.4±0.5[b]	8.5±0.5[b]	11.7±1.9[a]
20:4n-3	0.7+0.1[a]	0.3±0.1[bc]	0.4+0.1[b]	0.1+0.0[c]
20:5n-3	5.4±1.1[a]	3.3±0.1[b]	3.2±0.7[b]	2.1±0.2[b]
22:5n-3	1.4±0.1[a]	1.0±0.2[b]	0.9±0.2[bc]	0.6±0.0[c]
22:6n-3	30.2±4.4	28.1±5.0	27.2±2.4	26.5±4.8
Total n-3 PUFA[d]	38.8±4.8	33.4±4.8	32.4±3.2	29.6±4.5
Total n-9 PUFA	0.1±0.0[c]	0.3±0.0[b]	0.2±0.0[bc]	0.5±0.0[a]
Total PUFA	46.1±4.9	41.1±1.7	41.1±3.4	41.8±6.4
n-3/n-6	5.5±0.7	4.6±0.8	3.8±0.3	2.5±0.0

Results are means±S.D. ($n=3$). Significance of differences between means were determined by one-way ANOVA followed, where appropriate, by Tukey's multiple comparison test as described in Section 2. Values within a row with a different superscript letter are significantly different ($P<0.05$). PUFA, polyunsaturated fatty acids.

[a] Totals include 15:0 and 20:0 present at up to 0.4%.

[b] Totals include 16:1n-9, 20:1n-11 and 20:1n-7 present at up to 0.5%.

[c] Totals include 18:3n-6 and 22:4n-6 present at up to 0.2%.

[d] Totals include 18:3n-3 and 18:4n-3 present at up to 0.5%.

3.4. Effects of diet on hepatocyte and enterocyte fatty acid oxidation activities

Water temperature had a significant effect on fatty acid β-oxidation in hepatocytes as measured by the production of acid-soluble radioactivity from [1-^{14}C]18:3n-3 (Table 7), with lower activity in fish kept at 15 °C compared to fish maintained at the lower temperatures (Fig. 3). The effect of water temperature was independent of dietary CPO (Table 7). CPO inclusion had a slight, but statistically significant, effect on hepatocyte β-oxidation, although the precise nature of this effect was not entirely clear (Table 7). It appears that increasing dietary CPO inclusion resulted in increased β-oxidation activity although never to a level greater than the activity in fish fed the 100% FO diet (P0) and, as a result, no statistically significant differences were obtained upon one-way ANOVA (Fig. 3).

In enterocytes, the production of acid-soluble products from [1-^{14}C]18:3n-3 was also affected by water temperature and, as with hepatocytes, β-oxidation decreased as water temperature increased (Table 7, Fig. 4). Enterocyte β-oxidation was also significantly affected by dietary CPO but this effect was dependent on water temperature as indicated by the highly significant interaction (Table 7). Dietary CPO tended to decrease β-oxidation in enterocytes at 7 and 15 °C but this trend was not apparent at 11 °C (Fig. 4). The production of acid-soluble products from [1-

^{14}C]18:3n-3 was higher in enterocytes than that in hepatocytes irrespective of dietary treatment or water temperature (Figs. 3 and 4).

4. Discussion

On the basis of liver fatty acid compositions, temperature had no significant effect on the incorporation and deposition of palm oil-derived fatty acids in rainbow trout. The proportions of 18:1n-9 and 18:2n-6 increased in total lipid in a graded manner, irrespective of water temperature, reaching equivalent levels in all three temperature treatments. It was also noteworthy that these changes occurred after only a 4-week feeding period. Interestingly, the level of 16:0 was not similarly increased in liver. This is consistent with data from a previous study in rainbow trout which showed that 16:0 did not increase in liver total lipid in fish fed a diet containing palm oil (Caballero et al., 2002). Although palm oil substituted only 40% of the added oil in the earlier trial on trout, it was performed over a period of 9 weeks (Caballero et al., 2002). In a longer trial of 21 weeks, the amount of 16:0 in liver total lipid only increased from 4.2 to 6.8 mg g^{-1} tissue in Atlantic salmon fed a diet in which all the added oil was palm oil (Torstensen et al., 2000). In contrast, the levels of 16:0 in fish fed diets containing palm oil were significantly increased in the fillets of rainbow trout (Caballero et al., 2002) and the fillet (Bell et al., 2002) and belly flap of salmon (Torstensen et al., 2000). However, it was important for the objectives of the present trial to note that there was no apparent or obvious effect of temperature on the uptake and deposition in the liver of dietary fatty acids. More details of the effect of temperature on the apparent digestibility of lipids and fatty acids in the present trial are given elsewhere (Ng et al., in press).

The primary aim of this study was to determine if there was interaction between water temperature and nutritional factors, specifically dietary CPO, in the regulation of fatty acid metabolism in trout, and this was achieved. The results clearly showed that both water temperature and diet had significant

Table 7
Significance of effects of dietary palm oil and water temperature on fatty acid desaturation/elongation and β-oxidation in hepatocytes and enterocytes

Cells/assay	Treatment	*F*-value	Significance (*P* value)
Hepatocytes			
Desaturation	Palm oil	58.70	<0.0001
	Temperature	31.18	<0.0001
	Interaction	16.90	<0.0001
β-Oxidation	Palm oil	5.23	0.0064
	Temperature	40.21	<0.0001
	Interaction	0.57	0.7517
Enterocytes			
Desaturation	Palm oil	2.67	0.0645
	Temperature	89.83	<0.0001
	Interaction	18.00	<0.0001
β-oxidation	Palm oil	37.49	<0.0001
	Temperature	108.93	<0.0001
	Interaction	46.97	<0.0001

Data from Figs. 1–4 were analysed by two-way ANOVA as described in Section 2.

Table 6
Significance of effects of dietary palm oil and water temperature on fatty acid composition of total lipid from liver

	Palm oil		Temperature		Interaction	
Fatty acid	*F* value	Significance	*F* value	Significance	*F* value	Significance
Lipid content	2.15	0.1199	0.73	0.4914	3.71	0.0095
16:0	6.05	0.0032	5.26	0.0128	2.77	0.0344
Total saturated	1.81	0.1717	3.21	0.0580	1.61	0.1883
18:1n-9	12.61	<0.0001	0.07	0.9354	0.67	0.6711
Total monoenes	5.35	0.0058	0.11	0.8962	1.99	0.1070
18:2n-6	18.17	<0.0001	1.05	0.3639	0.92	0.4969
20:4n-6	2.99	0.0511	2.23	0.1288	2.06	0.0971
Total n-6 PUFA	21.20	<0.0001	3.86	0.0351	2.17	0.0814
20:5n-3	25.88	<0.0001	6.07	0.0074	1.27	0.3094
22:6n-3	12.87	<0.0001	0.44	0.6500	1.75	0.1534
Total n-3 PUFA	16.98	<0.0001	1.13	0.3400	1.77	0.1473
n-3/n-6 PUFA	26.63	<0.0001	5.61	0.0100	1.52	0.2153

Data from Tables 3–5 were analysed by two-way ANOVA as described in Section 2. PUFA, polyunsaturated fatty acids.

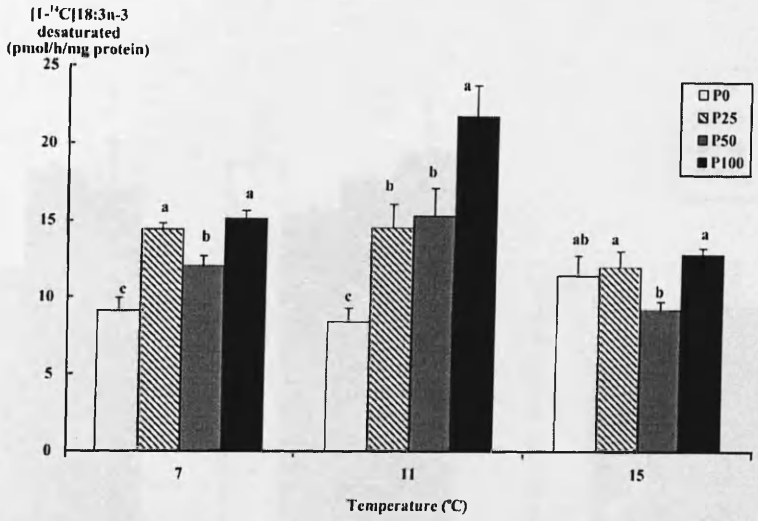

Fig. 1. Total fatty acid desaturation/elongation activity in hepatocytes after feeding the experimental diets for 4 weeks. Results are means ± S.D. ($n=3$) and represent the rate of conversion (pmol h^{-1} mg $protein^{-1}$) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Columns for a specific temperature with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post-test ($P<0.05$).

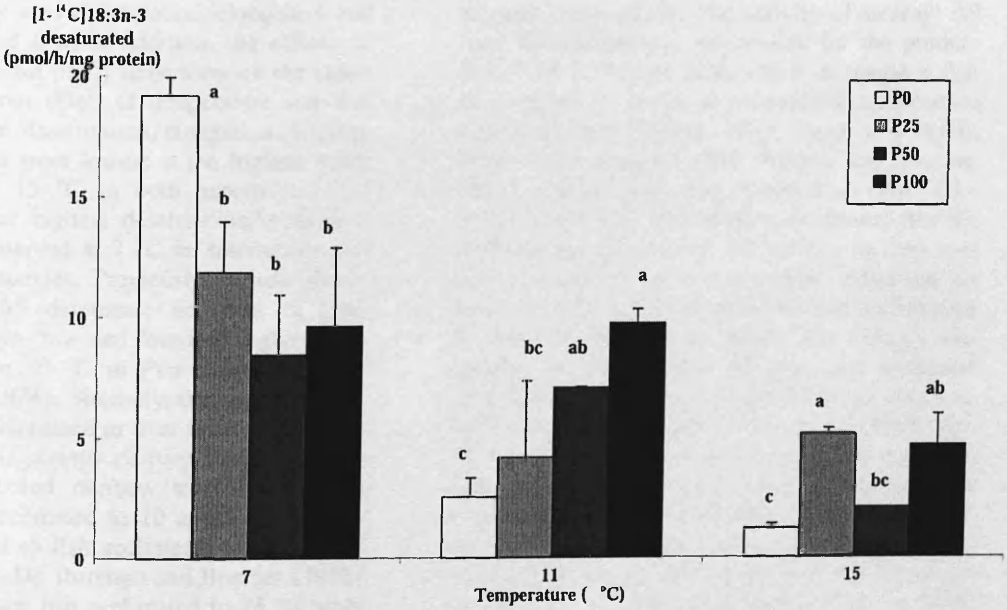

Fig. 2. Total fatty acid desaturation/elongation activity in pyloric caecal enterocytes after feeding the experimental diets for 4 weeks. Results are means ± S.D. ($n=3$) and represent the rate of conversion (pmol h^{-1} mg $protein^{-1}$) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Columns for a specific temperature with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post-test ($P<0.05$).

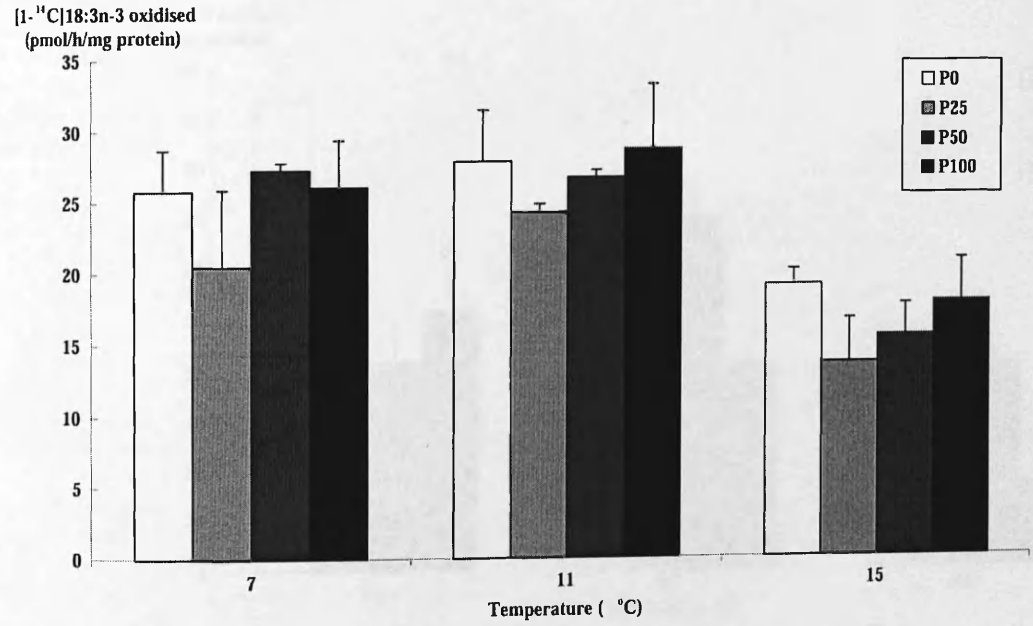

Fig. 3. Fatty acid β-oxidation activity in hepatocytes after feeding the experimental diets for 4 weeks. Results are means ± S.D. (n = 3) and represent the rate of oxidation (pmol h^{-1} mg $protein^{-1}$) of [1-^{14}C]18:3n-3 to acid-soluble products. There were no significant differences among dietary treatments at a specific temperature.

effects on fatty acid desaturation/elongation and β-oxidation, and that, in addition, the effects of either were at least partly dependent on the other. The most obvious effect of temperature was that activities of the desaturation/elongation pathway and β-oxidation were lowest at the highest water temperature of 15 °C in both hepatocytes and enterocytes. The highest desaturation/elongation activity was observed at 7 °C in enterocytes and 11 °C in hepatocytes. Previously, it was shown that Δ6 and Δ5 desaturase activities of liver microsomes were two- and four-fold higher at 16 °C compared to 30 °C in *Pimelodus maculatus* (Ninno et al., 1974). Similarly, the activity of Δ6 desaturase was increased in liver microsomes from common carp (*Cyprinus carpio*) (Schuenke and Wodtke, 1983) and rainbow trout (Hagar and Hazel, 1985) acclimated to 10 and 5 °C, respectively, compared to fish acclimated to 30 and 20 °C, respectively. De Torrengo and Brenner (1976) showed that when fish acclimated to 28 °C were shifted to 18 °C, Δ6 desaturase activity was initially decreased 1 day after transfer, probably due to a kinetic effect, but subsequently increased 1 week after transfer, presumably due to induction of gene transcription. The activity of stearoyl Δ9 fatty acid desaturase, responsible for the production of 18:1n-9 from 18:0, is also increased in fish in response to lower environmental temperature (Schuenke and Wodtke, 1983; Hagar and Hazel, 1985; Kayama et al., 1986; Wodtke and Cossins, 1991). The Δ9 gene was cloned from carp (Tiku et al., 1996) and subsequent work showed that the cold-induced increase in Δ9 activity in carp was due to increased gene expression, indicating an important role for this enzyme in cold acclimation in fish (Trueman et al., 2000). Interestingly, the specific activity of carp Δ9 was also increased isothermally by addition of 5% fetal calf serum to the culture medium (Macartney et al., 1996). The increased activity was associated with increased transcript levels although it was not determined whether this was due to increased transcript stability or increased transcription. (Macartney et al., 1996). However, it showed that fish Δ9 fatty acid desaturation activity could be regulated by both temperature and nutrition.

Recently, we measured fatty acid oxidation and desaturation pathways in a single, combined assay in isolated hepatocytes from Atlantic salmon using

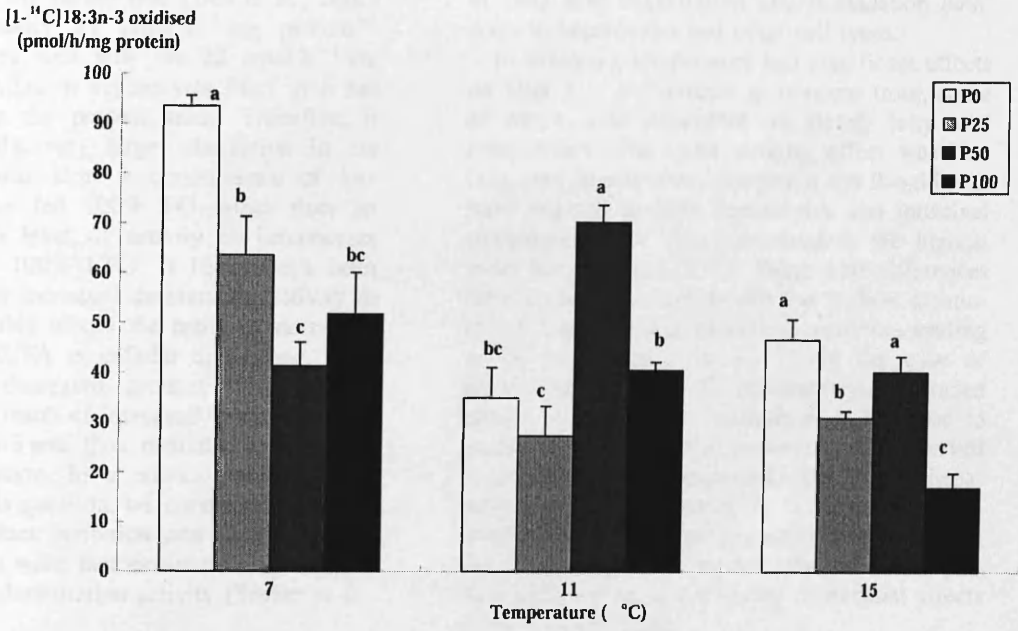

Fig. 4. Fatty acid β-oxidation activity in pyloric caecal enterocytes after feeding the experimental diets for 4 weeks. Results are means ± S.D. ($n=3$) and represent the rate of oxidation (pmol h^{-1} mg $protein^{-1}$) of [1-^{14}C]18:3n-3 to acid-soluble products. Columns for a specific temperature with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post-test ($P<0.05$).

[1-^{14}C]18:3n-3 as substrate (Tocher et al., 2002). The same assay system was used in this study and the results showed that, in both hepatocytes and enterocytes, more radioactivity was recovered in acid-soluble products than that was recovered in desaturated products, indicating that a greater proportion of the exogenously added [1-^{14}C]18:3n-3 was being oxidised. The fatty acid oxidation activity was generally higher in enterocytes compared to hepatocytes as observed in the earlier study (Tocher et al., 2002). As with desaturation/elongation, less 18:3n-3 was β-oxidised at 15 °C compared to 11 or 7 °C, but the physiological explanation for this is not obvious in a poikilothermic animal. There are surprisingly few data in the literature on β-oxidation of fatty acids in fish, particularly in hepatocytes or intestinal tissue (Small and Connock, 1981; Sidell et al., 1995; Frøyland et al., 1998). The effect of temperature has not been studied, but it is perhaps noteworthy that liver is a site of significant β-oxidation in Antarctic fish with peroxisomal β-oxidation possibly accounting for up to 30% of total hepatic activity (Crockett and Sidell, 1993), whereas, in contrast, liver is not regarded as a major site of β-oxidation activity in salmon (Frøyland et al., 2000).

A previous study with Atlantic salmon showed that graded increases in dietary palm oil in post-smolts gave similarly graded increases in fatty acid desaturation and elongation in isolated hepatocytes (Bell et al., 2002). In this study on salmon in seawater, replacement of 100% of FO with CPO resulted in a 10-fold increase in hepatocyte desaturation activity, which was much greater than that reported in several other studies in salmonids in both freshwater and seawater, in which increases of 2.4- to 2.8-fold were observed in fish fed diets in which 100% of the FO was replaced by various vegetable oils (Bell et al., 1997, 2001; Tocher et al., 1997, 2000, 2001, 2002). The present trial with trout in freshwater compares well with the latter studies, with hepatocyte desaturation activity at 11 °C in fish fed P100 being 2.6-fold greater than that in fish fed P0. Thus, the 10-fold stimulation of activity in the earlier trial with palm oil in salmon was not repeated in the present trial with trout although the latter trial was of much shorter duration. However, the total desaturation activity recorded in hepatocytes from salmon fed

100% CPO in the earlier trial (Bell et al., 2002) was approximately 21 pmol h^{-1} mg protein^{-1} which compares well with the 22 pmol h^{-1} mg protein^{-1} recorded in hepatocytes from trout fed 100% CPO in the present study. Therefore, it appears that the very large stimulation in the salmon trial was more a consequence of low activity in fish fed 100% FO rather than an unusually high level of activity in hepatocytes from fish fed 100% CPO. It has always been unclear whether increased desaturation activity in fish fed vegetable oils is the result of decreased levels of n-3HUFA in cellular lipids, and, thus, mediated by decreased product inhibition, or whether it is a result of increased levels of 18:2n-6 and/or 18:3n-3 and, thus, mediated by increased substrate provision. In a previous study, which investigated this question, we concluded that both decreased product inhibition and increased substrate provision were factors determining hepatocyte fatty acid desaturation activity (Tocher et al., 2003).

The data obtained from enterocytes may possibly be relevant to the question above. In a previous trial on salmon in which fatty acid metabolism in enterocytes was investigated, increasing vegetable oil, in the form of linseed oil, resulted in decreased desaturation activity (Tocher et al., 2002). The mechanism for this apparent reduced activity was unclear, but it was suggested that the assay may be compromised by high levels of 18:3n-3 in the enterocytes. Therefore, it was interesting that enterocyte fatty acid desaturation was not inhibited and indeed was stimulated, at least at 11 and 15 °C, in the present trial in which CPO, containing negligible 18:3n-3, replaced FO. Similarly, the results of the β-oxidation assay gave contrasting results in enterocytes in the two trials. In the earlier trial on salmon, the β-oxidation activity in caecal enterocytes tended to be increased in fish fed linseed oil compared to FO whereas, in the present study, dietary CPO tended to reduce enterocyte β-oxidation activity. In contrast, hepatocyte β-oxidation activity was generally unaffected by dietary oil in both trials although the total β-oxidation activity was much higher in the trout hepatocytes in the present trial, irrespective of water temperature and dietary oil, compared to the salmon hepatocytes studied previously (Tocher et al., 2002). The results from these trials clearly demonstrate that further work is required to investigate fatty acid metabolism in intestinal cells, and the interaction of fatty acid desaturation and β-oxidation pathways in hepatocytes and other cell types.

In summary, temperature had significant effects on fatty acid metabolism in rainbow trout, some of which were dependent on dietary fatty acid composition. The most striking effect was that fatty acid desaturation/elongation and β-oxidation were reduced in both hepatocytes and intestinal enterocytes from fish maintained at the highest water temperature (15 °C). There were differences between the two tissues with the highest desaturation/elongation and β-oxidation activities tending to be in fish held at 11 °C in the case of hepatocytes, but 7 °C in enterocytes. Graded effects on fatty acid metabolism in response to graded dietary palm oil were most clearly observed in desaturation/elongation activities in both hepatocytes and enterocytes at 11 °C. The highest β-oxidation activities were generally observed in fish fed FO alone in both hepatocytes and enterocytes with palm oil inclusion having differential effects in the two cell types.

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Influence of dietary palm oil on growth, tissue fatty acid compositions, and fatty acid metabolism in liver and intestine in rainbow trout (*Oncorhynchus mykiss*)

J. FONSECA-MADRIGAL[1], V. KARALAZOS[1], P.J. CAMPBELL[2], J.G. BELL[1] & D.R. TOCHER[1]
[1] *Institute of Aquaculture, University of Stirling, Stirling;* [2] *BioMar Ltd, Grangemouth Docks, Grangemouth, UK*

Abstract

This study aimed to investigate the effects of dietary crude palm oil (CPO) on fatty acid metabolism in liver and intestine of rainbow trout. Triplicate groups of rainbow trout for 10 weeks at 13 °C were fed on diets in which CPO replaced fish oil (FO) in a graded manner (0–100%). At the end of the trial, fatty acid compositions of flesh, liver and pyloric caeca were determined and highly unsaturated fatty acid (HUFA) synthesis and fatty acid oxidation were estimated in isolated hepatocytes and caecal enterocytes using [1-^{14}C]18:3n-3 as substrate. Growth performance and feed efficiency were unaffected by dietary CPO. Fatty acid compositions of selected tissues reflected the dietary fatty acid composition with increasing CPO resulting in increased proportions of 18:1n-9 and 18:2n-6 and decreased proportions of n-3HUFA, 20:5n-3 and 22:6n-3. Palmitic acid, 16:0, was also increased in flesh and pyloric caeca, but not in liver. The capacity of HUFA synthesis from 18:3n-3 increased by up to threefold in both hepatocytes and enterocytes in response to graded increases in dietary CPO. In contrast, oxidation of 18:3n-3 was unaffected by dietary CPO in hepatocytes and reduced by high levels of dietary CPO in enterocytes. The results of this study suggest that CPO can be used at least to partially replace FO in diets for rainbow trout in terms of permitting similar growth and feed conversion, and having no major detrimental effects on lipid and fatty acid metabolism, although flesh fatty acid compositions are significantly affected at an inclusion level above 50%, with n-3HUFA reduced by up to 40%.

KEY WORDS: β-oxidation, desaturation, enterocytes, hepatocytes, palm oil, polyunsaturated fatty acids, rainbow trout

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Correspondence: J. Fonseca-Madrigal, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK. E-mail: jorge.fonseca-madrigal@stir.ac.uk

Introduction

Commercial diets for salmon and trout traditionally use fishmeal and fish oil (FO), rich in the n-3 highly unsaturated fatty acids (HUFA), eicosapentaenoate (20:5n-3, EPA) and docosahexaenoate (22:6n-3, DHA), as protein and lipid sources (Sargent & Tacon 1999). The fatty acid compositions of salmon and trout grown on diets containing FO are high in n-3HUFA that are beneficial in the human diet (Ackman 1980; Henderson & Tocher 1987; Bell *et al.* 2001a, 2002). Demand for FO has been increasing and estimates suggest that aquaculture feeds could consume 90% of world supplies by 2010 (Barlow 2000), so for aquaculture to continue to expand, alternatives to FO must be found. The only sustainable alternatives to FO are vegetable oils which are rich in C_{18} polyunsaturated fatty acids (PUFA) such as linoleate (18:2n-6) and linolenate (18:3n-3), but devoid of n-3HUFA (Sargent *et al.* 2002). The conversion of C_{18} PUFA to HUFA requires sequential steps of fatty acyl chain desaturation and elongation (Cook 1996). Although salmonid fish have the capability to produce EPA and DHA from 18:3n-3, the desaturation/elongation pathway does not convert 18:3n-3 to EPA and DHA at high rates (Tocher 2003). As a consequence, there is considerable interest in the regulation of the HUFA biosynthetic pathways in fish in order to determine whether the conversion of C_{18} PUFA to HUFA can be enhanced (Sargent *et al.* 2002; Tocher 2003).

Apart from providing essential PUFA (Sargent *et al.* 1995), dietary lipids are also a major source of energy in salmonid diets (Sargent *et al.* 1989; Frøyland *et al.* 1998).

Studies investigating mitochondrial β-oxidation suggested that saturated and monounsaturated fatty acids are preferred over PUFA for energy production in fish (Henderson 1996). Thus, in rainbow trout (*Oncorhynchus mykiss*) liver, 22:1n-11 and 16:0 were the best substrates for mitochondrial β-oxidation while in red muscle 16:0, 16:1, 18:1n-9 and 18:2n-6 were the preferred substrates (Henderson & Sargent 1985; Kiessling & Kiessling 1993). Therefore, when selecting potential vegetable oils for substituting FO in fish diets, energy availability as well as PUFA content must be considered. To minimize any reduction in growth rate, and nutritional quality in terms of the health benefits of farmed fish to human consumers, potential substitutes for FO should avoid excessive deposition of 18:2n-6, retain high levels of n-3HUFA and provide sufficient energy in the form of saturated and monounsaturated fatty acids (Bell *et al.* 2002). Crude palm oil (CPO) is a potential candidate in that it has relatively low levels of 18:2n-6 and an abundance of 16:0 and 18:1n-9 (Ng 2002a,b). Palm oil production is also predicted to exceed soybean oil production within the next few years to become the most abundant vegetable oil in the world (Gunstone 2001). Furthermore, the use of CPO in the diets of Atlantic salmon and rainbow trout has given growth and feed utilization efficiency comparable with fish fed equivalent levels of marine FO (Torstensen *et al.* 2000; Rosenlund *et al.* 2001; Bell *et al.* 2002; Caballero *et al.* 2002).

In an earlier trial, we investigated the effects of water temperature on the digestion of fatty acids in trout fed CPO (Ng *et al.* 2003). Although this was a short trial of only 4 weeks, some effects of CPO on fatty acid metabolism were observed, although the effects were complicated by the considerable influence of growth temperature on enzymic activity (Tocher *et al.* 2004). Furthermore the previous study did not report the important outcomes of feeding CPO on growth, feed efficiency and flesh fatty acid composition. The present study investigates the effects of dietary CPO on fatty acid metabolism in rainbow trout in a fully replicated trial enabling growth, feed efficiency and final flesh fatty acid compositions to be related to the changes in lipid and fatty acid metabolism. Triplicate groups of rainbow trout were fed for 10 weeks on diets in which CPO replaced FO at levels of 0, 25, 50 and 100%. At the end of the trial, fatty acyl desaturation/elongation and oxidation activities were determined in isolated hepatocytes and caecal enterocytes using [1-^{14}C]18:3n-3 as substrate, and samples of flesh, liver and pyloric caeca were collected for analysis of fatty acid composition.

Materials and methods

Experimental fish and diets

Rainbow trout, with a mean initial body weight of about 27 g, were obtained from a local fish hatchery (Almondbank, Perthshire, UK), and stocked randomly (at 40 fish/tank) into 12 circular tanks of 100-L capacity on arrival at the Institute of Aquaculture (University of Stirling, UK). The tanks were supplied with 1 L min^{-1} flow-through water at 13 °C and fish were subjected to a photoperiod regime of 12-h light : 12-h dark. The fish were fed commercial trout pellet during an initial 1-week acclimatization period. After this, randomly assigned triplicate tanks of fish were fed to satiety three times daily with one of four experimental diets for 10 weeks. The diets were formulated to meet all the known nutritional requirements of salmonid fish (U.S. National Research Council 1993). Extruded diets (3 mm diameter), containing approximately 472 g kg^{-1} crude protein and approximately 224 g kg^{-1} crude lipid, were manufactured (BioMar A/S., Brande, Denmark) with varying contents of CPO added at the expense of marine FO (Table 1). CPO was thoroughly

Table 1 Ingredients, formulation (g kg^{-1} of diet) and proximate composition of diets containing graded amounts of palm oil

	Diet			
	P0	P25	P50	P100
Component				
Fishmeal[1]	343	343	343	343
Hi Pro Soya[2]	127	127	127	127
Wheat gluten[2]	100	100	100	100
Corn gluten[3]	100	100	100	100
Wheat[4]	100	100	100	100
Marine oil[1]	200	150	100	0
Palm oil[5]	0	50	100	200
Methionine	2.1	2.1	2.1	2.1
Lysine	7.9	7.9	7.9	7.9
Micronutrients[6]	24.1	24.1	24.1	24.1
Vitamin E[7]	0.15	0.11	0.076	0.0
Ytrium oxide	0.2	0.2	0.2	0.2
Composition				
Moisture	65	82	72	69
Crude protein	479	465	478	467
Crude lipid	223	215	225	233
Ash	71	71	70	70
Gross energy (kJ g^{-1})	22.8	23.6	23.8	23.6

[1] Norsemeal Ltd, London, UK.
[2] Cargill, Swinderbury, UK.
[3] Cerestar UK Ltd, Manchester, UK.
[4] J.D. Martin, Tranent, UK.
[5] United Plantations Bhd, Jenderata Estate, Teluk Intan, Malaysia.
[6] Vitamins, minerals and astaxanthin (Carophyll pink®), BioMar A/S, Brande, Denmark.
[7] Roche, Basel, Switzerland.

Table 2 Fatty acid composition (% of total fatty acids) of experimental diets containing increasing levels of palm oil

	Diet			
Fatty acid	P0	P25	P50	P100
14:0	6.4	4.6	3.7	1.6
16:0	18.6	26.7	30.6	37.9
18:0	3.4	3.8	4.2	4.2
Total saturated[1]	29.3	35.8	39.1	44.1
16:1n-7	6.7	4.5	3.5	1.0
18:1n-9	11.1	20.2	24.2	35.9
18:1n-7	2.5	1.9	1.8	0.6
20:1n-9	5.4	3.5	2.8	0.4
22:1n-11	7.2	4.5	2.5	0.4
Total monounsaturated[2]	34.7	35.6	35.5	38.5
18:2n-6	5.9	8.1	9.1	11.8
20:4n-6	0.6	0.4	0.3	0.2
Total n-6PUFA[3]	7.2	9.0	9.9	12.1
18:3n-3	1.3	1.0	0.9	0.6
18:4n-3	2.9	1.9	1.4	0.2
20:5n-3	9.2	6.0	4.7	1.2
22:5n-3	1.2	0.8	0.7	0.3
22:6n-3	11.3	7.9	6.4	2.7
Total n-3PUFA[4]	26.9	18.4	14.6	5.1
Total PUFA[5]	36.0	28.5	25.4	17.4
n-3/n-6	3.7	2.0	1.5	0.4

[1] Total includes 15:0 and 20:0.
[2] Total includes 16:1n-9, 20:1n-7, 22:1n-9 and 24:1.
[3] Total includes 18:3n-6, 20:2n-6 and 22:5n-6.
[4] Total includes 20:3n-3 and 20:4n-3.
[5] Total includes C_{16} PUFA; PUFA, polyunsaturated fatty acids.

mixed with the FO before the oil mixtures were used to coat the extruded pellets. The four diets included CPO at 0, 50, 100 and 200 g kg^{-1} of the diet, replacing 0% (P0), 25% (P25), 50% (P50) and 100% (P100) of the added FO respectively. The fatty acid compositions of the resultant diets are shown in Table 2. The experiment was conducted in accordance with British Home Office guidelines regarding research on experimental animals.

Lipid extraction and fatty acid analyses

After 10 weeks, four fish per dietary treatment (one per tank replicate with an additional fish taken randomly from one of the tanks) were killed by a blow to the head, livers were dissected out and immediately frozen in liquid nitrogen. The intestinal tract was also removed, pyloric caeca dissected out, trimmed of any adhering adipose tissue, and any digesta gently squeezed out before the caeca were frozen in liquid nitrogen. For flesh samples, Norwegian quality cuts were taken, and skinned and de-boned before being frozen in liquid nitrogen. Total lipids of flesh, livers, pyloric caeca and diet samples were extracted by homogenization in chloroform/methanol (2 : 1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, according to Folch *et al.* (1957). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification as described by Christie (1982) and FAME extracted and purified as described previously (Tocher & Harvie 1988). FAME were separated and quantified by gas-liquid chromatography (Fisons GC8600; Fisons Ltd, Crawley, UK) using a 30 m × 0.32 mm capillary column (CP wax 52CB; Chrompak Ltd, London, UK). Hydrogen was used as carrier gas and temperature programming was from 50 °C to 180 °C at 40 °C min^{-1} and then to 225 °C at 2 °C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman 1980).

Preparation of isolated hepatocytes and caecal enterocytes

Four fish from each dietary treatment (as above) were killed by a blow to the head and the livers and intestinal tracts immediately dissected out. The gall bladder was removed, the main blood vessels trimmed, and the liver perfused via the hepatic vein with calcium and magnesium-free Hanks balanced salt solution (HBSS) + 10 mM HEPES + 1 mM EDTA (solution A) to clear blood from the tissue. The liver was chopped finely and about 0.5 g taken and incubated with 20 mL solution A containing 0.1% (w/v) collagenase in a shaking water bath at 20 °C for 45 min. The tissue was filtered through 100 μm nylon gauze and hepatocytes collected by centrifugation at 300 × *g* for 2 min. The cell pellet was washed with 20 mL solution A containing 1% w/v fatty acid-free bovine serum albumin (FAF-BSA) and re-centrifuged. The washing was repeated with a further 20 mL solution A without FAF-BSA. The hepatocytes were resuspended in 10 mL Medium 199 containing 10 mM HEPES and 2 mM glutamine. One hundred microlitre of cell suspension was mixed with 400 μL Trypan blue, hepatocytes counted and viability assessed using a haemocytometer. With minor modification, the same method was used to isolate enterocyte-enriched preparations from pyloric caeca. Briefly, pyloric caeca were dissected, cleaned of adhering adipose tissue and lumenal contents rinsed away with solution A. The caeca were chopped finely and incubated with 0.1% (w/v) collagenase. The digested caeca were filtered through 100 μm nylon gauze and the cells collected, washed, resuspended in medium (as above) and viability checked as for hepatocytes. The preparation comprised predominantly enterocytes. Viability was >95% at isolation and decreased by <5% over the period of the incubation. One hundred microlitre of the

hepatocyte and enterocyte suspensions were retained for protein determination according to the method of Lowry *et al.* (1951) after incubation with 0.4 mL of 0.25% (w/v) SDS/1 M NaOH for 45 min at 60 °C.

Incubation of hepatocyte and enterocyte preparations with [1-^{14}C]18:3n-3

Six millilitre of each hepatocyte or caecal enterocyte suspension were dispensed into 25 cm^2 tissue culture flasks and incubated at 20 °C for 2 h with 0.3 μCi (approximately 1 μM) [1-^{14}C]18:3n-3, added as a complex with FAF-BSA in phosphate buffered saline prepared as described previously (Ghioni *et al.* 1997). After incubation, the cell suspensions were transferred to glass conical test tubes and 1 mL of each suspension withdrawn into a 2 mL microcentrifuge tube for β-oxidation assay. The remaining cell suspensions were used for the desaturation/elongation assay.

Assay of hepatocyte and enterocyte fatty acyl desaturation/elongation activities

The cell suspensions were centrifuged at 500 × *g* for 2 min, the supernatant discarded and the hepatocyte or enterocyte cell pellets washed with 5 mL ice-cold HBSS/FAF-BSA. The supernatant was discarded and total lipid extracted using ice-cold chloroform/methanol (2 : 1, v/v) containing 0.01% (w/v) BHT as described previously (Tocher *et al.* 1988). Total lipid was transmethylated and FAME prepared as described above. The methyl esters were redissolved in 100 μL isohexane containing 0.01% BHT and applied as 2.5 cm streaks to thin-layer chromatography (TLC) plates impregnated by spraying with 2 g silver nitrate in 20 mL acetonitrile and preactivated at 110 °C for 30 min. Plates were fully developed in toluene/acetonitrile (95 : 5, v/v) (Wilson & Sargent 1992) and autoradiography 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, GA, USA) and radioactivity determined in a TRI-CARB 2000CA scintillation counter (United Technologies Packard, Pangbourne UK). Results were corrected for counting efficiency and quenching of ^{14}C.

Assay of hepatocyte and enterocyte fatty acyl oxidation activities

Fatty acid oxidation by hepatocytes and enterocytes was assessed as described previously (Frøyland *et al.* 2000; Torstensen *et al.* 2000). Briefly, 1 mL of hepatocyte or enterocyte suspension was homogenized with a hand-held tissue disrupter (Ultra-Turrax T8/S8N-5G probe; IKA-Werke GmbH & Co., Slaufen, Germany) and centrifuged at 10 000 × *g* for 10 min. Five hundred microlitre of supernatant was taken and 100 μL ice-cold 6% aqueous FAF-BSA solution added. After mixing thoroughly, the protein was precipitated by addition of 1 mL ice-cold 4 M perchloric acid and the tubes centrifuged at 10 000 × *g* for 10 min. Five hundred microlitre of the supernatant was transferred to a scintillation vial, 4 mL of scintillant added and radioactivity in the acid-soluble fraction determined.

Materials

[1-^{14}C]18:3n-3 (50–55 mCi mmol^{-1}) was obtained from NEN (DuPont (UK) Ltd, Stevenage, UK). HBSS, Medium 199, HEPES buffer, glutamine, collagenase (type IV), FAF-BSA, BHT, silver nitrate and perchloric acid were obtained from Sigma Chemical Co. (Poole, UK). TLC plates, 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, UK).

Statistical analysis

All the data are presented as mean ± SD (n = 3 or 4) and all statistical analyses were performed using Prism 3 (Graphpad Software Inc., San Diego, CA, USA). The effects of dietary CPO on tissue fatty acid compositions, HUFA synthesis and β-oxidation were analysed by one-way ANOVA followed, where appropriate, by Tukey's *posthoc* test to determine significant differences between individual treatments. Percentage data and data which were identified as nonhomogeneous (Bartlett's test) were subjected to arcsine transformation before analysis. Differences were regarded as significant when $P < 0.05$ (Zar 1984).

Results

Diet P0, contained over 29% total saturates with almost 19% 16:0, approximately 35% total monoenes with around one-third each as 18:1n-9 and the long chain monoenes 20:1 and 22:1, 7% n-6PUFA predominantly 18:2n-6, and 27% n-3PUFA, with over 20% as the n-3HUFA, EPA and DHA (Table 2). The replacement of increasing proportions of FO with CPO in the diets resulted in increased proportions of 16:0, 18:1n-9 and 18:2n-6 and decreased proportions of

Table 3 Growth and performance of rainbow trout (*Oncorhynchus mykiss*) fed experimental diets containing increasing levels of palm oil for 10 weeks

Parameter/diet	P0	P25	P50	P100
Initial weight (g)	27.2 ± 0.7	26.6 ± 0.5	27.8 ± 0.4	27.3 ± 1.1
Final weight (g)	76.1 ± 4.5	81.0 ± 2.9	76.7 ± 7.1	70.1 ± 8.5
Final length (cm)	17.5 ± 0.4	17.9 ± 0.5	17.3 ± 0.6	16.8 ± 0.4
Weight gain %[1]	180.2 ± 21.9	204.7 ± 16.6	175.8 ± 24.8	156.8 ± 26.8
SGR[2]	1.47 ± 0.11	1.59 ± 0.08	1.45 ± 0.13	1.34 ± 0.15
FCR[3]	1.50 ± 0.21	1.44 ± 0.12	1.79 ± 0.62	1.68 ± 0.36
Mortalities	1.0 ± 1.7	0.0 ± 0.0	1.7 ± 0.6	1.0 ± 0.0

Values are given as mean ± SD ($n = 3$).

[1] Weight gain = (final weight − initial weight)/(initial weight) × 100.

[2] Specific growth rate (SGR) = [Ln (final weight) − Ln (initial weight)]/(days) × 100.

[3] Feed conversion ratio (FCR) = feed offered/weight gain. There were no significant differences ($P > 0.05$) in initial weight, final weight or length of fish either among dietary treatments or between replicate tanks within the same treatment.

n-3HUFA, total PUFA and long chain monoenes. Therefore, the diet formulated with 100% CPO (P100), showed levels of 16:0, 18:1n-9 and 18:2n-6 of 38, 36 and 12% respectively, whereas the combined level of EPA and DHA was reduced to under 4% (Table 2).

Final weights and lengths were not different, either among dietary treatments or between replicate tanks from the same treatment (Table 3). Feeding diets containing CPO at 25% of added oil, had apparently positive effects on mean final weights, SGR and FCR compared with fish fed FO (0% PO) although the effects were not statistically significant. Mortalities over the experimental period were <5% for all treatments. The changes in fatty acid composition of the diets in response to increasing replacement of FO with CPO, described above, were reflected in the flesh fatty acid compositions (Table 4). That is, as dietary CPO increased the flesh had increased proportions of 18:1n-9, 18:2n-6 and 16:0, and decreased proportions of n-3HUFA (Table 4). Similarly, increasing replacement of FO with CPO was reflected in the liver fatty acid compositions, with graded increased proportions of 18:1n-9 and 18:2n-6, but not 16:0, and decreased proportions of n-3HUFA (Table 5). Changes in fatty acid compositions of pyloric caeca in response to increasing dietary CPO content were as described for flesh (Table 5).

Increasing levels of dietary CPO resulted in increased HUFA synthesis in both hepatocytes and enterocytes as assessed by the recovery of radioactivity in the desaturation products (18:4, 20:4, 20:5, 22:5 and 22:6) of [1-^{14}C]18:3n-3 (Fig. 1). HUFA synthesis in fish fed the P100 diet was 2.9- and 2.8-fold higher in hepatocytes and enterocytes, respectively, than in fish fed 100% FO (P0). The response of HUFA synthesis to graded increases in dietary CPO was most pronounced in hepatocytes with most products of [1-^{14}C]18:3n-3 desaturation and elongation being increased in a stepwise manner (Fig. 2). The rate at which [1-^{14}C]18:3n-3 was oxidized was two to threefold higher in enterocytes than in hepatocytes (Fig. 3). The rate of oxidation of [1-^{14}C]18:3n-3 in hepatocytes was not significantly affected by dietary CPO whereas in caecal enterocytes the rate of oxidation was significantly lower in cells from fish fed the P75 and P100 diets than in cells from fish fed diets with lower CPO (Fig. 3).

Discussion

A number of earlier studies have utilized vegetable oils in feed formulations for salmonids at replacement levels of up to 100%, and no changes in growth rates and feed conversion have been observed (Bell *et al.* 2001a, 2002; Caballero *et al.* 2002; Torstensen *et al.* 2004). The results of this study are consistent with these findings. Feeding diets containing CPO, in the range of 25–100% of added oil, had no significant effect on growth rate and feed conversion ratio, compared with fish fed 100% FO (P0). Fish fed 100% CPO (P100) had slightly lower mean final weights than those fed the other three diets, although the differences were not significant. A similar marginal reduction in final weight was observed in other trials in which 100% of dietary FO was replaced by vegetable oil, specifically rapeseed oil, in salmon (Bell *et al.* 2001a; Torstensen *et al.* 2004). In the present study, the lower final weight of fish fed 100% CPO could be explained by the lower digestibility of the saturated, especially 16:0 and monounsaturated fatty acids in diets containing 100% CPO compared with those containing FO (Ng *et al.* 2003). It is well established that the saturated and monounsaturated fatty acids are preferred over PUFA for energy production in fish (Henderson 1996).

The fatty acid compositions of tissue lipids of salmonids are readily influenced by the fatty acid composition of dietary lipid and linear correlations exist between individual fatty acids in tissue total lipid and their concentrations in dietary

Table 4 Fatty acid compositions (percentage of total fatty acids by weight) of total lipid of flesh from rainbow trout fed diets containing palm oil

Fatty acid	P0	P25	P50	P100
14:0	4.2 ± 0.2^{a}	3.0 ± 0.3^{b}	2.7 ± 0.2^{b}	1.4 ± 0.1^{c}
16:0	22.4 ± 0.3^{b}	24.5 ± 1.9^{a,b}	24.2 ± 1.3^{a,b}	26.5 ± 1.3^{a}
18:0	4.3 ± 0.1^{a}	4.1 ± 0.2^{a,b}	3.8 ± 0.3^{b}	3.9 ± 0.2^{a,b}
Total saturated[1]	31.8 ± 0.5	32.3 ± 2.6	31.2 ± 1.9	32.3 ± 1.7
16:1n-7	5.2 ± 0.1^{a}	4.2 ± 0.3^{b}	4.0 ± 0.3^{b}	2.3 ± 0.3^{c}
18:1n-9	11.6 ± 0.7^{d}	16.4 ± 0.9^{c}	20.2 ± 1.9^{b}	26.9 ± 1.6^{a}
18:1n-7	2.4 ± 0.1^{a}	2.1 ± 0.1^{b}	2.0 ± 0.0^{b}	1.5 ± 0.2^{c}
20:1n-9	3.6 ± 0.4^{a}	2.8 ± 0.3^{b}	2.6 ± 0.1^{b}	2.0 ± 0.1^{c}
22:1	3.5 ± 0.3^{a}	2.5 ± 0.2^{b}	2.3 ± 0.2^{b}	1.0 ± 0.2^{c}
24:1n-9	0.7 ± 0.0^{a}	0.7 ± 0.1^{a}	0.6 ± 0.0^{a}	0.5 ± 0.0^{b}
Total monoenes[2]	27.2 ± 1.7^{c}	28.9 ± 1.6^{b,c}	32.0 ± 2.4^{a,b}	34.6 ± 1.2^{a}
18:2n-6	4.5 ± 0.2^{d}	5.7 ± 0.2^{c}	6.8 ± 0.4^{b}	9.0 ± 0.4^{a}
20:2n-6	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.7 ± 0.1^{a}
20:3n-6	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.3 ± 0.0^{b}	0.6 ± 0.1^{a}
20:4n-6	0.7 ± 0.0^{b}	0.7 ± 0.0^{b}	0.6 ± 0.0^{b}	0.9 ± 0.1^{a}
Total n-6 PUFA[3]	6.1 ± 0.2^{c}	7.5 ± 0.3^{b}	8.5 ± 0.5^{b}	11.9 ± 0.7^{a}
18:3n-3	0.8 ± 0.0^{a}	0.7 ± 0.0^{b}	0.7 ± 0.0^{b}	0.4 ± 0.0^{c}
18:4n-3	1.1 ± 0.0^{a}	0.8 ± 0.0^{b}	0.7 ± 0.0^{b,c}	0.4 ± 0.2^{c}
20:4n-3	0.9 ± 0.1^{a}	0.7 ± 0.0^{b}	0.7 ± 0.0^{b}	0.3 ± 0.1^{c}
20:5n-3	5.9 ± 0.2^{a}	4.7 ± 0.3^{a,b}	4.2 ± 0.8^{b}	2.1 ± 0.5^{c}
22:5n-3	1.5 ± 0.0^{a}	1.2 ± 0.1^{b}	1.0 ± 0.0^{c}	0.5 ± 0.1^{d}
22:6n-3	24.5 ± 1.5^{a}	23.3 ± 1.7^{a}	21.0 ± 2.8^{a,b}	17.5 ± 2.5^{b}
Total n-3 PUFA[4]	34.9 ± 1.3^{a}	31.3 ± 1.5^{a}	28.2 ± 3.7^{a,b}	21.2 ± 3.3^{b}
Total PUFA	41.0 ± 1.1^{a}	38.8 ± 1.8^{a,b}	36.8 ± 3.2^{a,b}	33.1 ± 2.6^{b}
(n-3)/(n-6)	5.8 ± 0.5^{a}	4.2 ± 0.1^{b}	3.4 ± 0.7^{b}	1.8 ± 0.4^{c}

Results are given as mean ± SD (n = 3). Values within a row with a different superscript letter are significantly different ($P < 0.05$).
PUFA, polyunsaturated fatty acids.
[1] Totals include 15:0 present at at up to 0.5%.
[2] Totals include 16:1n-9 and 20:1n-7 present at up to 0.5%.
[3] Totals include 18:3n-6, 22:4n-6 and 22:5n-6, present at up to 0.5%.
[4] Totals include 20:3n-3 present at up to 0.2%.

lipid (Torstensen *et al.* 2000; Bell *et al.* 2001a, 2002, 2003a,b; Tocher *et al.* 2003). In the present study, dietary CPO inclusion was reflected in the fatty acid compositions of total lipid of all the tissues. The changes in liver fatty acid composition were similar to those reported in an earlier trial in which trout were fed similar diets over a period of 4 weeks at three different temperatures (Ng *et al.* 2003; Tocher *et al.* 2004). The magnitude of the changes in the present trial, run at 13 °C, were similar to the fish grown at 11 °C in the earlier trial. In the present trial the initial size of the fish was half that used in the earlier trial (27 g versus 45 g), and the duration of the trial was 2.5 × longer (10 weeks versus 4 weeks). Therefore, it was particularly noteworthy that the data were so similar between the trials suggesting that the dietary influence on the tissue lipids had reached an equilibrium after only 4 weeks in fish <50 g. Similar effects of dietary CPO were observed in both flesh and pyloric caeca, although 16:0 levels in these tissues were also increased by increased dietary inclusion of CPO, whereas this was not observed in liver lipid, either in this trial or in the previous trial (Tocher *et al.* 2004). The level of 16:0 was increased, although only modestly compared with 18:1n-9 and 18:2n-6, in the flesh of salmon fed incremental dietary CPO (Bell *et al.* 2002). These data suggest that liver metabolism is predisposed to treat 16:0 differently to most other fatty acids and that its level in liver is more tightly controlled than in other tissues, although all tissues appear to heavily oxidize 16:0 for energy. The difference in metabolism between liver and the other tissues may be related to lipid class compositions with liver having less triacylglycerol and more phospholipid than muscle and caeca (Henderson & Tocher 1987; Sargent *et al.* 1989; Tocher 1995). Palmitic acid is an important component of phospholipid, especially in the *sn*-2 position of phosphatidylcholine and, to a lesser extent, phosphatidylethanolamine (Henderson & Tocher 1987; Tocher 1995). Thus, the levels of 16:0 incorporated in tissues may be related to the level of phospholipid, or the phospholipid/triacylglycerol ratio, in specific tissues.

Several previous studies have shown that HUFA synthesis, as measured by the desaturation and elongation of 18:3n-3, is increased in salmonids, including trout fed diets in which dietary FO is replaced with vegetable oils (Tocher *et al.* 1997,

Table 5 Fatty acid compositions (percentage of total fatty acids by weight) of total lipid of liver and pyloric caeca from rainbow trout fed diets containing palm oil

	Liver				Pyloric caeca			
Fatty acid	P0	P25	P50	P100	P0	P25	P50	P100
14:0	2.0 ± 0.3	2.1 ± 0.9	1.6 ± 0.0	1.0 ± 0.1	5.0 ± 0.5^{a}	3.8 ± 0.3^{b}	3.2 ± 0.2^{b}	1.6 ± 0.1^{c}
16:0	23.3 ± 1.6	20.7 ± 1.3	21.2 ± 2.1	21.6 ± 1.6	20.7 ± 3.7^{b}	25.9 ± 1.7^{ab}	27.2 ± 3.0^{a}	27.5 ± 1.3^{a}
18:0	6.6 ± 0.5	5.6 ± 0.2	6.3 ± 0.6	6.6 ± 1.2	4.2 ± 0.8	4.7 ± 0.6	5.0 ± 0.6	4.3 ± 0.2
Total saturated[1]	32.5 ± 1.5	28.8 ± 2.5	29.5 ± 2.8	29.4 ± 1.5	30.5 ± 5.1	34.9 ± 2.3	35.6 ± 3.8	33.6 ± 1.5
16:1n-7	3.8 ± 1.4	4.6 ± 1.5	3.9 ± 0.1	2.6 ± 0.7	7.4 ± 0.7^{a}	5.2 ± 0.1^{b}	4.4 ± 0.1^{b}	2.6 ± 0.5^{c}
18:1n-9	12.1 ± 3.2^{b}	21.1 ± 2.0^{a,b}	22.0 ± 3.4^{a}	24.6 ± 5.6^{a}	16.2 ± 1.4^{d}	24.8 ± 0.7^{c}	27.9 ± 0.6^{b}	38.1 ± 1.5^{a}
18:1n-7	2.1 ± 0.3 ^{a,b}	2.4 ± 0.1^{a}	2.0 ± 0.2^{a,b}	1.7 ± 0.2^{b}	3.3 ± 0.3^{a}	2.9 ± 0.1^{a}	2.3 ± 0.1^{b}	1.7 ± 0.1^{c}
20:1n-9	2.6 ± 0.8	3.8 ± 0.8	3.4 ± 0.6	3.9 ± 1.1	7.0 ± 0.7^{a}	4.8 ± 0.6^{b}	3.9 ± 0.0^{b,c}	2. 8 ± 0.1^{c}
22:1n-11	1.0 ± 0.5	1.7 ± 1.2	0.9 ± 0.5	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1n-9	1.0 ± 0.1^{a}	0.9 ± 0.1^{a,b}	0.9 ± 0.1^{a,b}	0.8 ± 0.1^{b}	1.0 ± 0.0^{a}	0.8 ± 0.1^{a,b}	0.7 ± 0.1^{b}	0.5 ± 0.0^{c}
Total monoenes[2]	22.6 ± 6.2	34.8 ± 3.4	33.2 ± 4.8	34.2 ± 7.4	35.6 ± 2.8^{a}	39.3 ± 0.6^{a}	39.8 ± 0.4^{a}	46.2 ± 2.1^{b}
18:2n-6	2.6 ± 0.3^{b}	4.1 ± 1.6^{a,b}	3.8 ± 0.2^{a,b}	5.5 ± 0.4^{a}	7.1 ± 0.6^{b}	8.3 ± 1 .3^{a,b}	8.1 ± 1.3^{a,b}	10.4 ± 1.1^{a}
20:2n-6	0.6 ± 0.1^{b}	0.8 ± 0.1^{b}	0.8 ± 0.1^{b}	1.6 ± 0.1^{a}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.6 ± 0.1^{a}
20:3n-6	0.3 ± 0.0^{b}	0.5 ± 0.2^{b}	0.7 ± 0.3^{b}	1.5 ± 0.2^{a}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.3 ± 0.0^{b}	0.4 ± 0.1^{a}
20:4n-6	2.1 ± 0.6	1.5 ± 0.5	1.6 ± 0.1	2.3 ± 0.3	0.7 ± 0.0^{a}	0.6 ± 0.1^{a,b}	0.5 ± 0.0^{a,b}	0.5 ± 0.1^{b}
Total n-6PUFA[3]	6.0 ± 0.1^{b}	7.5 ± 1.1^{b}	7.5 ± 0.4^{b}	12.3 ± 0.7^{a}	8.6 ± 0.6^{b}	9.7 ± 1.2^{a,b}	9.6 ± 1.4^{a,b}	12.2 ± 1.3^{a}
18:3n-3	0.4 ± 0.0	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.0	1.3 ± 0.1^{a}	0.8 ± 0.1^{b}	0.7 ± 0.1^{b}	0.4 ± 0.1^{c}
18:4n-3	0.3 ± 0.1	0.4 ± 0.3	0.2 ± 0.1	0.0 ± 0.0	2.0 ± 0.3^{a}	1.0 ± 0.2^{b}	0.7 ± 0.1^{b,c}	0.3 ± 0.1^{c}
20:4n-3	0.5 ± 0.0^{a}	0.3 ± 0.2^{a}	0.2 ± 0.0^{a}	0.0 ± 0.0^{b}	1.1 ± 0.1^{a}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b}	0.2 ± 0.1^{c}
20:5n-3	5.0 ± 1.4^{a}	2.9 ± 0.4^{a,b}	2.3 ± 0.3^{b}	1.0 ± 0.7^{b}	5.4 ± 1.0^{a}	2.8 ± 0.6^{b}	2.3 ± 0.5^{b,c}	0.8 ± 0.2^{c}
22:5n-3	1.4 ± 0.1^{a}	0.9 ± 0.1^{b}	0.8 ± 0.1^{b,c}	0.4 ± 0.3^{c}	1.3 ± 0.2^{a}	0.6 ± 0.1^{b}	0.6 ± 0.1^{b,c}	0.2 ± 0.0^{c}
22:6n-3	31.2 ± 3.8	24.0 ± 6.4	26.0 ± 2.2	22.3 ± 5.3	14.2 ± 2.5^{a}	9.7 ± 0.9^{b}	9.6 ± 1.3^{b}	5.8 ± 1.6^{b}
Total n-3PUFA[4]	38.9 ± 4.6^{a}	28.9 ± 5.7^{a,b}	29.8 ± 1.9^{a,b}	24.0 ± 6.3^{b}	25.3 ± 3.8^{a}	15.6 ± 1.4^{b}	14.4 ± 2.3^{b}	7.7 ± 2.0^{c}
Total PUFA	44.9 ± 4.7	36.4 ± 5.3	37.3 ± 2.2	36.3 ± 6.9	33.9 ± 1.1^{a}	25.9 ± 2.2^{b}	24.6 ± 3.5^{b}	20.2 ± 3.3^{b}
n3/n6	6.4 ± 0.7^{a}	3.9 ± 1.1^{b}	3.9 ± 0.1^{b}	1.9 ± 0.4^{c}	2.9 ± 0.5^{a}	1.6 ± 0.2^{b}	1.5 ± 0.0^{b}	0.6 ± 0.1^{c}

Results are given as mean ± SD ($n = 4$). Values within a row for a particular tissue with a different superscript letter are significantly different ($P < 0.05$).

PUFA, polyunsaturated fatty acids.

[1] Totals include 15:0 present at up to 0.5%.

[2] Totals include 16:1n-9 and 20:1n-7 present at up to 0.5%.

[3] Totals include 18:3n-6, 22:4n-6 and 22:5n-6, present at up to 0.5%.

[4] Totals include 20:3n-3 present at up to 0.2%.

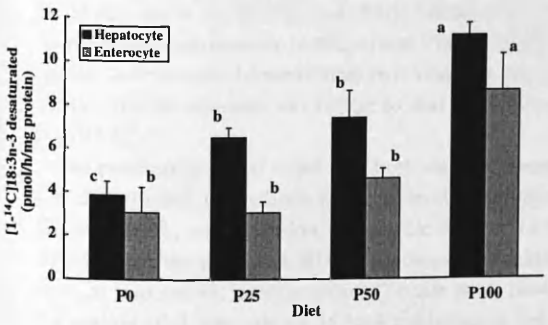

Figure 1 Total fatty acid desaturation/elongation activity in hepatocytes and caecal enterocytes after feeding the experimental diets for 10 weeks. Results are given as mean ± SD ($n = 4$) and represent the rate of conversion (pmol h^{-1} mg $protein^{-1}$) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Columns assigned to a specific cell type with different superscript letters are significantly different.

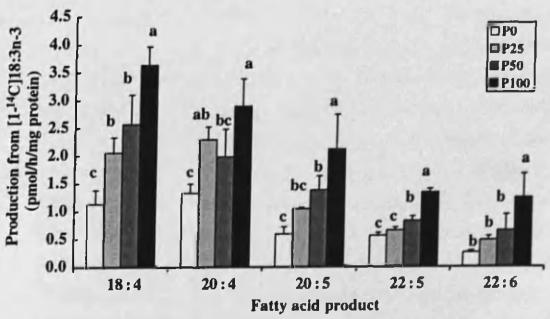

Figure 2 Individual fatty acid products of the desaturation and elongation of [1-^{14}C]18:3n-3 in hepatocytes. Results are given as mean ± SD ($n = 4$) and represent the rate of production (pmol h^{-1} mg $protein^{-1}$) of individual fatty acids as determined by the recovery of radioactivity in each fatty acid fraction. Columns referring to a specific fatty acid having different superscript letters are significantly different.

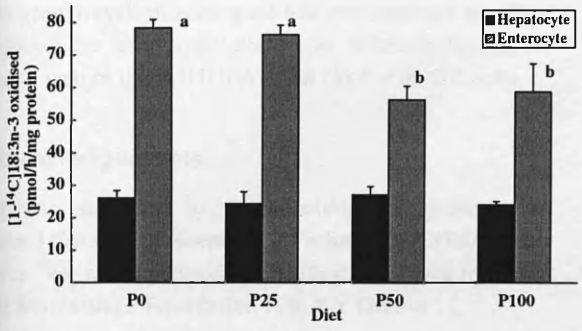

Figure 3 Fatty acid oxidation activity in hepatocytes and caecal enterocytes after feeding the experimental diets for 10 weeks. Results are given as mean ± SD ($n = 4$) and represent the rate of oxidation (pmol h^{-1} mg $protein^{-1}$) of [1-^{14}C]18:3n-3 to acid soluble products. Columns assigned to a specific cell type with different superscript letters are significantly different.

2000, 2001). In some trials, increments of dietary vegetable oil have been reflected in incremental increases in the activity of the HUFA biosynthetic pathway. Specifically, we have shown that increments in dietary CPO, rapeseed oil and linseed oil all resulted in incrementally increased HUFA synthesis in salmon (Bell *et al.* 2001a, 2002; Tocher *et al.* 2002). A similar increase in HUFA synthetic activity in isolated caecal enterocytes has been reported in salmon fed linseed oil and trout fed CPO (Tocher *et al.* 2002, 2004). Therefore, the results of the present trial are entirely consistent with these data. In a previous trial investigating dietary CPO in trout, the effects of temperature significantly impacted on the effects of diet, and HUFA synthetic activity only correlated with dietary CPO at a growth temperature of 11 °C and not at 15 °C (Ng *et al.* 2003; Tocher *et al.* 2004). As the growth temperature in the present trial, at 13 °C, was chosen to be exactly between these two values, it was noteworthy that the response was similar to that at 11 °C rather than 15 °C.

The previous trial had noted that both water temperature and diet affected the balance between desaturation/elongation to HUFA, and oxidation, as possible fates of 18:3n-3, but also that the effects on HUFA synthesis and oxidation were, at least partly, interdependent (Tocher *et al.* 2004). In the present trial, the rate of 18:3n-3 oxidation in fish fed 100% FO (P0) was around sevenfold and 25-fold greater than the rate of HUFA synthesis from 18:3n-3 in hepatocytes and enterocytes respectively. However, increasing dietary CPO shifted this balance by increasing the rate of HUFA synthesis while either not affecting oxidation, as in hepatocytes, or decreasing it, as in enterocytes. As a consequence, in fish fed 100% CPO (P100), the amount by which oxidation of 18:3n-3 exceeded HUFA synthesis was reduced to only two- and sevenfold in hepatocytes and enterocytes respectively. Thus, although oxidation was still the primary fate of 18:3n-3 in both cell types, there was a three- to fourfold shift towards utilization of 18:3n-3 for HUFA synthesis rather than for energy via β-oxidation in response to dietary CPO.

These data are generally consistent with those from previous trials which have shown that more radioactivity from [1-^{14}C]18:3n-3 was recovered in acid soluble (oxidation) products than was recovered in desaturated products, in both hepatocytes and enterocytes, indicating that a greater proportion of the exogenously added [1-^{14}C]18:3n-3 was being oxidized (Tocher *et al.* 2002, 2004). The data are consistent with *in vivo* experiments with stable isotopes that have shown that the majority of dietary 18:3n-3 was catabolized in trout, as is the case in many other species including humans (Bell *et al.* 2001b). There are relatively few data in the literature on the effects of diet on β-oxidation of fatty acids in fish, particularly in hepatocytes or intestinal tissue (Small & Connock 1981; Crockett & Sidell 1993; Sidell *et al.* 1995; Frøyland *et al.* 2000; Nordgarden *et al.* 2003). However, different dietary oils including CPO did not significantly affect β-oxidation capacity in red muscle in Atlantic salmon (Torstensen *et al.* 2000). In previous trials, we have noted that oxidation of [1-^{14}C]18:3n-3 was not increased in hepatocytes by dietary linseed oil in salmon or by dietary CPO in trout (Tocher *et al.* 2002, 2004). However, the oxidation of [1-^{14}C]18:3n-3 in caecal enterocytes tended to be increased in salmon fed linseed oil compared with FO (Tocher *et al.* 2002). In contrast, in the present study, as in our previous study, dietary CPO tended to reduce oxidation of [1-^{14}C]18:3n-3 in isolated enterocytes (Tocher *et al.* 2004).

The main objective of the present study was to determine the effects of dietary CPO on growth performance, and lipid and fatty acid metabolism in rainbow trout. The results showed that the inclusion of CPO in the diet of rainbow trout had significant effects on tissue fatty acid compositions and also on fatty acid desaturation/elongation and oxidation, but without major detrimental effects on growth. In conclusion, this study suggests that CPO can be used as an effective substitute for FO in rainbow trout in terms of permitting similar growth and feed conversion, and having no apparent detrimental effects on fish health. However, inclusion of CPO at levels above 50% resulted in significant reductions in n-3HUFA in the tissues, which is particularly important in the case of flesh, such that the nutritional benefit to the consumer could be reduced. However, this does not necessarily preclude the use of high levels of CPO in dietary formulations for rainbow trout. Such diets could be used for the majority

of the growth cycle providing the fish were returned to a FO-containing diet at an appropriate time before marketing, in order to restore the n-3HUFA levels (Bell *et al.* 2003a,b).

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APPENDIX

Published Articles

Tocher, D.R., J. Fonseca-Madrigal, J.G. Bell, J.R. Dick, R.J. Henderson and J.R. Sargent. 2002. **Effects of diets containing linseed oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes in Atlantic salmon (*Salmo salar*).** *Fish Physiol. Biochem.* 26, 157-170.

Tocher, D.R., J.G. Bell, F. McGhee, J.R. Dick and J. Fonseca-Madrigal. 2003. **Effects of dietary lipid level and vegetable oil on fatty acid metabolism in Atlantic salmon (*Salmo salar* L.) over the whole production cycle.** *Fish Physiol. Biochem.* 29, 193–209.

Tocher, D.R., J. Fonseca-Madrigal, J.R. Dick, W-K. Ng, J.G. Bell and P.J. Campbell. 2004. **Effects of water temperature and diets containing palm oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes of rainbow trout (*Oncoryhnchus mykiss*).** *Comp. Biochem. Physiol.*, 137B, 49-63.

Fonseca-Madrigal J., V. Karalazos, P.J. Campbell, J.C. Bell and D.R. Tocher. 2005. **Influence of dietary palm oil on growth, tissue fatty acid compositions, and fatty acid metabolism in liver and intestine in rainbow trout (*Oncorhynchus mykiss*).** *Aquaculture Nutrition* 11, 241-250.

Fish Physiology and Biochemistry **26:** 157–170, 2002.

Effects of diets containing linseed oil on fatty acid desaturation and oxidation in hepatocytes and intestinal enterocytes in Atlantic salmon (*Salmo salar*)

Douglas R. Tocher, Jorge Fonseca-Madrigal, J. Gordon Bell, James R. Dick, R. James Henderson and John R. Sargent
Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland
(Phone: +44-1786-467996; Fax.: +44-1786-472133; E-mail: d.r.tocher@stir.ac.uk)

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Abstract

We hypothesized that replacing fish oil with 18:3n-3-rich linseed oil may enable salmon to maintain the levels of tissue n-3HUFA levels through a combination of increased desaturation activity and increased substrate fatty acid provision. To this end we investigated desaturation/elongation of [1-^{14}C18:3n-3 in hepatocytes and intestinal enterocytes, and determined the extent to which 18:3n-3 was oxidized and desaturated by measuring both simultaneously in a combined assay. Salmon smolts were stocked randomly into five seawater pens and fed for 40 weeks on diets in which the fish oil was replaced in a graded manner by linseed oil. At the end of the trial, fatty acyl desaturation/elongation and oxidation activities were determined in isolated hepatocytes and intestinal enterocytes using [1-^{14}C]18:3n-3 as substrate, and samples of liver and intestinal tissue were collected for analysis of lipid and fatty acid composition. The results showed that, despite increased desaturation of [1-^{14}C]18:3n-3 in hepatocytes, provision of dietary 18:3n-3 did not prevent the decrease in tissue n-3HUFA in fish fed linseed oil. Intestinal enterocytes were a site of significant fatty acid desaturation but, in contrast to hepatocytes, the activity was not increased by feeding linseed oil and was generally lower in fish fed linseed oil compared to fish fed only fish oil. In contrast, oxidation of [1-^{14}C]18:3n-3 in enterocytes was generally increased in fish fed linseed oil compared to fish fed the diet containing only fish oil. However, oxidation of [1-^{14}C]18:3n-3 in hepatocytes was 4- to 8-fold lower than in enterocytes and was not affected by diet. Furthermore, oxidation of [1-^{14}C]18:3n-3 in enterocytes exceeded desaturation irrespective of dietary treatment, whereas similar amounts of [1-^{14}C]18:3n-3 were desaturated and oxidized in hepatocytes from fish fed only fish oil and desaturation exceeded oxidation by 3-fold in fish fed the diet containing 100% linseed oil. The molecular mechanisms underpinning these results were discussed.

Abbreviations: BHT – butylated hydroxytoluene; FAF-BSA – fatty acid-free bovine serum albumin; FO – fish oil; HBSS – Hanks balanced salt solution; HUFA – highly unsaturated fatty acids (carbon chain length ≥ C_{20} with ≥ 3 double bonds); LO – linseed oil.

Introduction

The polyunsaturated fatty acids (PUFA), linoleate (18:2n-6) and linolenate (18:3n-3) cannot be synthesized *de novo* by animals, including fish and, therefore, are termed essential fatty acids (EFA) (Holman 1986). Qualitative and quantitative EFA requirements vary between species and, although salmonids require both 18:3n-3 and 18:2n-6 at a combined level of around 1% of the diet, their essentiality actually derives from their desaturation and elongation to the functionally active highly unsaturated fatty acids (HUFA) eicosapentaenoate (20:5n-3), docosahexaenoate (22:6n-3) and arachidonate (20:4n-6) (Sargent et al. 1995, 1999).

The fatty acyl desaturase enzyme activities are known to be under nutritional regulation in mammals (Brenner 1981) and this has also been demonstrated in fish (Tocher et al. 1996; Sargent et al. 2002).

Exploitation and, indeed, over-exploitation of wild fisheries has meant that an increasing proportion of fish for human consumption is provided by aquaculture which is expanding at over 10% per year (Tidwell and Allan 2002). Paradoxically, diets for aquaculture, including salmonid (salmon, trout and char) culture, have been based traditionally on fish meals and oils as the predominant protein and lipid sources (Sargent and Tacon 1999). As a consequence, demand for fish oils is rapidly increasing and current estimates suggest aquaculture feeds will consume more than 85% of world fish oil supplies by 2010 and so, if aquaculture is to continue to expand and supply more of the global demand for fish, alternatives to fish oil must be found (Barlow 2000). The only sustainable alternative to fish oils are plant (vegetable) oils which are rich in C_{18} PUFA but devoid of the n-3HUFA abundant in fish oils (Sargent et al. 2002). Therefore, there is currently considerable interest in the regulation of the HUFA biosynthetic pathways in fish to determine the effectiveness with which the fatty acids in vegetable oils can be utilized by commercially important cultured fish species including salmon (Sargent et al. 2002).

In previous trials we have shown that replacing fish oil with rapeseed oil (rich in 18:2n-6 and 18:1n-9) or palm oil (rich in 16:0 and 18:1n-9) in the diets of Atlantic salmon resulted in increased activity of the fatty acyl desaturation/elongation pathway in isolated hepatocytes (Bell et al. 2001a, 2002). Although the diets in both these trials were based on fish meal, which supplied sufficient 20:5n-3 and 22:6n-3 to satisfy the EFA requirements, the fatty acid compositions of tissues, including liver and muscle, were characterized by significantly decreased levels of n-3HUFA, particularly when vegetable oil replaced 100% of the fish oil, which compromised the nutritional quality of the flesh for the human consumer (Bell et al. 2001a, 2002). However, palm oil contains virtually no 18:3n-3 and rapeseed oil contains only moderate levels (Padley et al. 1986). It appeared, therefore, that despite increased desaturation/elongation activity in the liver, the levels of dietary 18:3n-3 provided may have limited the ability of salmon to maintain levels of tissue n-3HUFA in the flesh. Few plant oils contain high levels of 18:3n-3, a notable exception being linseed oil which can contain up to 56% 18:3n-3 with an 18:3n-3:18:2n-6 ratio of over 3 in selected strains (Padley et al. 1986). The primary hypothesis we aimed to test in the present study was that replacing fish oil with 18:3n-3-rich linseed oil in salmon diets would enable the fish to maintain tissue n-3HUFA levels through a combination of increased desaturation/elongation activity and increased substrate fatty acid provision. Other *in vivo* studies using stable isotopes (deuterium-labeled 18:3n3) had shown that dietary 18:3n-3 was readily and substantially oxidized (Bell et al. 2001b) and that intestine was a tissue with hitherto unappreciated fatty acyl desaturation activity (Bell et al. 2003). Therefore, in addition, we aimed to investigate desaturation/elongation of 18:3n-3 in intestinal enterocytes. Thirdly, we aimed to determine the extent to which the 18:3n-3 tracer was oxidized and compare that with the amount desaturated/elongated by measuring both simultaneously in a combined assay in both hepatocytes and enterocytes.

Table 1. Formulation (g/kg) of feed

Component	FO	LO25	LO50	LO75	LO100
Fismeal[1]	338	338	338	338	338
Maize gluten[2]	200	200	200	200	200
Soya (Hi Pro)[3]	100	100	100	100	100
Fish oil[4]	258	193.5	129	64.5	0
Linseed oil[5]	0	64.5	129	193.5	258
Micronutrients[6]	25	25	25	25	25

[1] Scandinavian LT-fish meal (Nordsildmel, Norway).
[2] Cargill/ADM, Decatur, Illinois.
[3] Soybean meal (Denofa, Fredrikstad, Norway).
[4] Capelin oil (Nordsildmel, Norway) supplemented with 200 ppm BHT.
[5] Crude E.C.C. linseed oil (N.V. Oliefabriek Lictervelde, Belgium) supplemented with 500 ppm Ronoxan A (Roche, Switzerland).
[6] Vitamin, mineral and carotenoid pigment premix formulated to Nutreco specification (Farmix, Trouw Nutrition, the Netherlands).

Salmon smolts were stocked randomly into five seawater pens and, after acclimatization for three weeks, were fed for 40 weeks on diets in which the fish oil (FO) was replaced in a graded manner by linseed oil (LO). At the end of the trial, fatty acyl desaturation/elongation and oxidation activities were determined in isolated hepatocytes and intestinal enterocytes using [1-^{14}C]18:3n-3 as substrate, and samples of liver and intestinal tissue were collected for analysis of lipid and fatty acid composition.

Table 2. Proximate (% of weight of feed) and fatty acid compositions (% of total fatty acids by weight) of the experimental diets

	FO	LO25	LO50	LO75	LO100
Protein	44.1	44.1	44.2	44.4	43.6
Lipid	28.9	28.9	30.2	29.5	31.1
Ash	6.5	6.9	7.2	7.5	7.6
Moisture	5.9	6.3	5.7	6.1	5.6
Fatty acid					
14:0	6.3	4.7	3.4	2.0	0.4
16:0	12.1	10.6	9.3	8.1	6.9
18:0	1.1	1.7	2.1	2.7	3.1
Total saturates[1]	19.9	17.2	15.1	13.0	10.5
16:1n-7	8.1	6.1	4.2	2.3	0.5
18:1n-9	11.9	13.6	15.1	16.0	17.0
18:1n-7	3.3	2.6	2.2	1.6	1.0
20:1n-9	17.9	13.1	9.0	5.0	1.1
22:1n-11	13.3	10.1	7.1	4.3	1.1
22:1n-9	2.1	1.5	1.0	0.5	0.1
Total monoenes[2]	58.4	48.4	39.6	30.5	21.1
18:2n-6	4.2	7.4	9.8	12.3	15.1
20:4n-6	0.2	0.2	0.1	0.1	0.1
Total n-6PUFA[3]	5.0	8.0	10.2	12.6	15.2
18:3n-3	0.9	14.0	25.6	37.8	50.4
18:4n-3	2.9	2.1	1.6	0.9	0.2
20:5n-3	5.9	4.6	3.5	2.2	1.0
22:6n-3	5.0	4.0	3.4	2.4	1.5
Total n-3PUFA[4]	15.7	25.6	34.6	43.7	53.3
Total PUFA	21.7	34.4	45.3	56.5	68.5

[1]Totals include 15:0, present at up to 0.3%;
[2]Totals include 16:1n–7, 20:1n-11,20:1n-7 and 24:1, present at up to 0.5%;
[3]Totals include 18:3n–6, 20:2n-6 and 20:3n-6, present at up to 0.2%;
[4]Totals include 20:3n-3, 20:4n-3 and 22:5n–3, present at up to 0.4%. PUFA, polyunsaturated fatty acids.

Materials and methods

Animals and diets

In June 2001, Atlantic salmon post-smolts were randomly assigned to 5 cages (5 m × 5 m; 600 fish per cage), with the mean initial weight across the five cages being 127 ± 3 g (range 123 to 130 g). The smolts were fed one of five diets, consisting of a control diet containing FO alone and four diets in which the FO was replaced in a graded manner by linseed oil (LO). Specifically, the five diets were 100% FO (FO), 100%LO (LO100) and FO/LO in ratios of 3:1 (LO25), 1:1 (LO50) and 1:3 (LO75). The experimental diets were prepared by the Nutreco Aquaculture Research Centre, Stavanger, Norway and were fed by hand. The formulation, proximate analyses and fatty acid compositions of the diets (6 mm pellet) are shown in Tables 1 and 2. All diets were formulated to satisfy the nutritional requirements of salmonid fish (US National Research Council 1993). The fish were fed the experimental diets for 40 weeks with the experiment terminating in March 2002. The final weights of the fish were 1.78 ± 0.40 kg (FO), 1.89 ± 0.34 kg (LO25), 1.90 ± 0.33 kg (LO50), 1.87 ± 0.35 kg (LO75) and 1.87 ± 0.33 kg (LO100) and there were no significant differences between different dietary treatments (ANOVA, $p = 0.1555$, $n = 87$). Fish were not fed during the 24 h prior to sampling.

Lipid extraction and lipid class composition

Intact livers were dissected from 18 fish (pooled into 6 samples of 3 livers each) per dietary treatment at each sampling point and immediately frozen in liquid nitrogen. For intestinal samples, four fish were sampled per dietary treatment. Pyloric caecae and portions of the midgut immediately posterior to the caecal regions were dissected from the intestinal tract, adhering adipose tissue removed and any residual lumenal contents gently and carefully extruded before the cleaned caecae and midgut sections were rapidly frozen in liquid nitrogen. Total lipid contents of livers, intestinal tissues and diet samples were determined gravimetrically after extraction by homogenization in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, basically according to Folch et al. (1957). Separation of lipid classes was performed by high-performance thin-layer chromatography (HPTLC). Approximately 10 μg of lipid extract was loaded as a 2 mm streak and the plate developed to two-thirds distance with methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.). After desiccation, the plate was fully developed with isohexane/diethyl ether/acetic acid (85:15:1, by vol.). The classes were quantified by charring at 160 °C for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid, followed by calibrated densitometry using a Shimadzu CS-9000 dual-wavelength flying spot scanner and DR-13 recorder (Henderson and Tocher 1992).

Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transesterification using 2 ml of 1% H_2SO_4 in methanol plus 1 ml toluene as described by Christie (1982) and FAME extracted and purified as described previously (Tocher and Harvie 1988). FAME were separated and quantified by gas-liquid chromatography (Fisons GC8600, Fisons Ltd., Crawley, U.K.) using a 30m × 0.32 mm capillary column (CP wax 52CB; Chrompak Ltd., London, U.K). Hydrogen was used as carrier gas and temperature programming was from 50 °C to 180 °C at 40 °C/min and then to 225 °C at 2 °C/min. Individual methyl esters were identified by comparison to known standards and by reference to published data (Ackman 1980).

Preparation of isolated hepatocytes

Fish were killed by a blow to the head and the livers dissected immediately. The gall bladder was removed carefully and the main blood vessels trimmed. The liver was perfused via the hepatic vein with solution A (calcium and magnesium-free Hanks balanced salt solution (HBSS) + 10 mM HEPES + 1 mM EDTA) to clear blood from the tissue using a syringe fitted with a 23 g needle. The liver was chopped finely with scissors and about 1 g of chopped liver was taken and incubated with 20 ml of solution A containing 0.1% (w/v) collagenase in a 25 ml "Reacti-flask" in a shaking water bath at 20 °C for 45 min. The digested liver tissue was filtered through 100 μm nylon gauze and the cells collected by centrifugation at 300 × *g* for 2 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. The washing was repeated with a further 20 ml of solution A without FAF-BSA. The hepatocytes were resuspended in 10 ml of Medium 199 containing 10 mM HEPES and 2 mM glutamine. 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 cell suspension was retained for protein determination.

Preparation of isolated enterocytes

The methodology for preparing hepatocytes as above was specifically developed for use in the field and has been successfully used in several previous trials (Bell et al. 1997, 2001a, 2002a; Tocher et al. 1997, 2000, 2001). Preliminary laboratory-based trials had shown that this method, with minor modifications, could be used successfully to isolate enterocyte-enriched preparations from various regions of the intestine including pyloric caecae. Briefly, the intestinal tract was removed and pyloric caecae dissected, cleaned of adhering adipose tissue, slit open and lumenal contents rinsed away with solution A. Similarly, a 1–2 cm portion of the mid gut, immediately posterior to the caecal-containing region, was dissected, cleaned and rinsed as for the caecae. The caecae and midgut samples were chopped finely with scissors and incubated with 20 ml of solution A containing 0.1% (w/v) collagenase in a 25 ml "Reacti-flask" in a shaking water bath at 20 °C for 45 min. The digested intestinal tissues were filtered through 100 μm nylon gauze and the cells collected and washed exactly as described for hepatocytes. The enterocytes were resuspended in 10 ml of Medium 199 containing 10 mM HEPES, 2 mM glutamine and viability checked as for hepatocytes. The enriched enterocyte preparation was very predominantly enterocytes although there were some secretory cells present. The viability was routinely > 95% at isolation and generally decreased by only 5–10% over the period of the incubation. One hundred μl of the cell suspension was retained for protein determination.

Incubation of hepatocyte and enterocyte preparations with [1-^{14}C]18:3n-3

Six ml of each hepatocyte or enterocyte suspension were dispensed into a 25 cm^2 tissue culture flask. Hepatocytes and enterocytes were incubated with 0.3 μCi (~1 μm) [1-^{14}C] 18:3n-3, added as a complex with FAF-BSA in phosphate buffered saline prepared as described previously (Ghioni et al. 1997). After addition of isotope the flasks were incubated at 20 °C for 2 h. After incubation, the cell layer was dislodged by gentle rocking and the cell suspension transferred to glass conical test tubes and 1 ml of each suspension withdrawn into a 2 ml microcentrifuge tube for β-oxidation assay as described below. The cell suspensions remaining in the glass conical centrifuge tubes were used for the desaturation/elongation assay as described below.

Assay of hepatocyte and enterocyte fatty acyl desaturation/elongation activities

The cell suspensions were centrifuged at 500 g for 2 min, the supernatant discarded and the hepatocyte

or enterocyte 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) 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 isohexane 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, U.K.). Results were corrected for counting efficiency and quenching of ^{14}C under exactly these conditions.

Assay of hepatocyte and enterocyte fatty acyl oxidation activities

The assay of fatty acid oxidation in intact hepatocytes has been demonstrated in rats (Frøyland et al. 1996; Madsen et al. 1998) and requires the determination of acid-soluble radioactivity as described in detail previously (Frøyland et al. 2000; Torstensen et al. 2000). Briefly, the 1 ml of hepatocyte or enterocyte suspension was homogenized with a hand-held tissue disrupter (Ultra-Turrax T8/S8N-5G probe, IKA-Werke GmbH & Co., Slaufen, Germany) and centrifuged at 2000 × *g* for 10 min in a microcentrifuge. Five hundred μl of the supernatant was taken into a clean 2 ml microcentrifuge and 100 μl of ice-cold 6% FAF-BSA solution in water was added. After mixing thoroughly, the protein was precipitated by the addition of 1.0 ml of ice-cold 4M perchloric acid ($HClO_4$). After vortexing, the tubes were centrifuged at 5000 g for 10 min in a microcentrifuge. Five hundred μl of the supernatant was carefully transferred to a scintillation vial, 4 ml of scintillant added and radioactivity in the acid-soluble fraction determined as described above for desaturation/elongation assay.

Protein determination

Protein concentration in isolated hepatocyte and enterocyte suspensions was determined according to the method of Lowry et al. (1951) after incubation with 0.4 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, U.K.). HBSS, Medium 199, HEPES buffer, glutamine, collagenase (type IV), FAF-BSA, BHT, silver nitrate and perchloric acid were obtained from Sigma Chemical Co. (Poole, U.K.). Thin-layer chromatography (TLC) plates, 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

All the data are presented as means ± SD (n = 3, 4 or 6 as stated). The relationships between dietary fatty acid contents and growth, liver total lipid and neutral lipid contents and liver fatty acid compositions, and between hepatocyte fatty acyl desaturation activity and both dietary and liver fatty acid compositions were determined by regression analyses (Prism 3, Graphpad Software, Inc., San Diego, USA). Some data such as growth (final weight), and the effects of diet on fatty acid desaturation and oxidation were also analyzed by one-way ANOVA followed where appropriate by Tukey's post test (Figures 1 & 3) or the student's t-test (Figure 2) to determine significant differences between individual treatments. Percentage data and data which were identified as non-homogeneous (Bartlett's test) were subjected to either arcsine or log transformation before analysis. Differences were regarded as significant when $P < 0.05$ (Zar 1984).

Results

Dietary fatty acid compositions

The control diet (FO), formulated with 100% FO, contained approximately 20% total saturates, mainly 16:0, almost 60% total monounsaturated fatty acids over half of which were the long chain monoenes, 20:1 and 22:1, 5% n-6 fatty acids predominantly 18:2n-6,

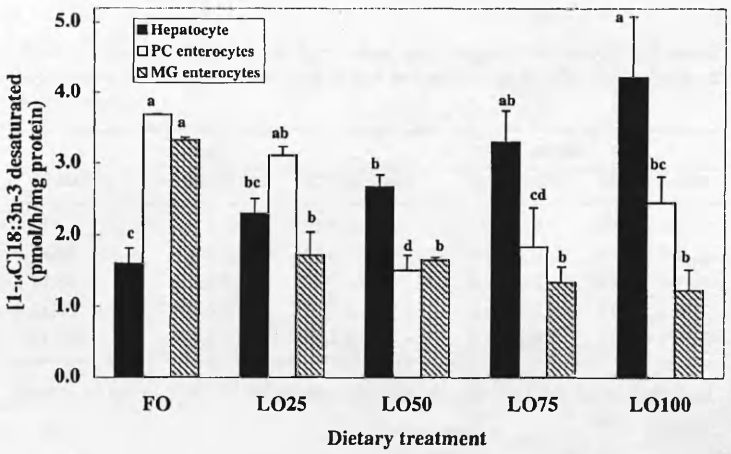

Figure 1. Total fatty acid desaturation/elongation activity in hepatocytes and enterocytes isolated from pyloric caecae (PC) and mid gut (MG) after feeding the experimental diets for 40 weeks. Results are means ± S.D. (n = 3) and represent the rate of conversion (pmol.h^{-1}.mg protein^{-1}) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Columns for a specific a tissue with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$). FO, fish oil; LO, linseed oil.

and 16% n-3 fatty acids, predominantly the n-3HUFA, 20:5n-3 and 22:6n-3 in approximately equal amounts, and less than 1% 18:3n-3 (Table 2). Inclusion of graded amounts of LO resulted in graded increased percentages of 18:3n-3, 18:2n-6 and 18:1n-9 with concomitant decreased proportions of n-3HUFA, 16:0, total saturates, 20:1, 22:1 and total monoenes. Thus, in the diet formulated with 100% LO (LO100), the levels of 18:3n-3 and 18:2n-6 had risen to 50% and 15% of total fatty acids, respectively, whereas 20:5n-3 and 22:6n-3 totalled only 2.5%, and total saturates and monoenes were reduced to 10% and 21%, respectively (Table 2).

Effects of diet on liver and pyloric caecal total and neutral lipid contents

The total lipid contents of both liver and pyloric caecae increased with increasing dietary inclusion of LO (Table 3). Regression analyses showed significant positive correlations (slopes) between dietary 18:3n-3 content and total lipid content of the tissues with r^2 values greater than 0.8 (Table 6). The proportion of neutral lipids also generally increased in liver lipids with increasing dietary LO and regression analyses showed there was a significant positive correlation with a degree of fit similar to total lipid (Tables 3 and 6). The proportion of neutral lipids in pyloric caecal lipids was higher than in liver and did not show a significant positive correlation with dietary LO (18:3n-3) although there was an upward trend (Tables 3 and 6). The midgut total and neutral lipid contents were not significantly different to the caecal compositions and so were not presented.

Effects of diet on liver and pyloric caecal fatty acid compositions

The principal fatty acids of liver total lipid from fish fed diet FO were, in rank order, 22:6n-3 (24%), 16:0 (18%), 18:1n-9 (12%), 20:5n-3 (10%) and 20:1n-9 (7%) (Table 4). The graded change in fatty acid compositions of the diets in response to increasing LO content, described above, was reflected in the liver fatty acid compositions with graded increased percentages of 18:3n-3, 18:2n-6 and 18:1n-9 and decreased proportions of n-3HUFA, 16:0, total saturates, 20:1, 22:1 and total monoenes. In addition, however, there were increased proportions of 18:4n-3, 20:3n-3, 20:4n-3 and 20:2n-6, and decreased proportions of 20:4n-6 and 22:5n-3 (Table 4). In the livers of fish fed diet LO100, the levels of 18:3n-3 and 18:2n-6 had risen to almost 33% and 12% of total fatty acids, respectively, whereas 20:5n-3 and 22:6n-3 were reduced to around 3% and 6%, respectively. These effects were highly significant as shown by regression analyses with positive correlations (slopes) between dietary 18:3n-3 and liver 18:3n-3 and negative correlations between dietary 18:3n-3 and liver 20:5n-3 and 22:6n-3 levels (Table 6). Similar highly significant

Table 3. Effects of dietary oil on lipid content (percentage of wet weight) and neutral lipid content (percentage of total lipid of liver and pyloric caecae from Atlantic salmon *Salmo salar* L.)

Treatment	Liver		Pyloric caecae	
	lipid content	neutral lipids	lipid content	neutral lipids
FO	3.8 ± 0.3	53.6 ± 2.9	4.0 ± 0.4	79.3 ± 3.7
LO25	4.4 ± 0.3	52.9 ± 2.0	4.8 ± 1.0	81.6 ± 1.4
LO50	4.5 ± 0.5	59.7 ± 2.4	4.6 ± 0.9	81.0 ± 2.3
LO75	5.2 ± 0.5	59.3 ± 3.0	5.4 ± 1.0	84.1 ± 3.0
LO100	7.3 ± 1.5	73.1 ± 5.2	5.5 ± 1.2	82.9 ± 1.7

Results are means ± SD. (n = 6 for liver and 4 for caecae). FO, fish oil; LO, linseed oil.

Table 4. Fatty acid compositions (percentage of total fatty acids by weight) of total lipid of liver from Atlantic salmon (*Salmo salar* L.) fed the experimental diets for 40 weeks.

	FO	LO25	LO50	LO75	LO100
14:0	2.8 ± 0.2	2.2 ± 0.2	1.7 ± 0.1	1.1 ± 0.0	0.5 ± 0.0
16:0	17.9 ± 1.1	15.0 ± 1.6	13.3 ± 1.0	11.3 ± 1.1	7.3 ± 0.8
18:0	3.1 ± 0.2	3.8 ± 0.2	4.1 ± 0.2	4.6 ± 0.1	4.9 ± 0.2
Total saturated[1]	24.1 ± 1.2	21.3 ± 1.8	19.3 ± 1.0	17.0 ± 1.1	12.7 ± 1.0
16:1n-7	3.5 ± 0.2	3.0 ± 0.2	2.4 ± 0.2	1.5 ± 0.1	0.7 ± 0.1
18:1n-9	11.9 ± 1.1	14.7 ± 0.8	14.1 ± 1.5	15.7 ± 1.3	19.1 ± 2.4
18:1n-7	3.5 ± 0.1	3.0 ± 0.1	2.3 ± 0.1	1.7 ± 0.1	1.2 ± 0.1
20:1n-9	6.8 ± 1.2	6.1 ± 0.5	4.2 ± 0.7	3.0 ± 0.2	1.4 ± 0.3
22:1n-11	2.1 ± 0.4	1.9 ± 0.3	1.6 ± 0.5	1.0 ± 0.1	0.5 ± 0.1
24:1n-9	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.4 ± 0.1
Total monoenes[2]	30.2 ± 3.1	31.1 ± 1.9	26.4 ± 3.0	24.5 ± 1.9	23.9 ± 2.9
18:2n-6	2.6 ± 0.1	4.4 ± 0.1	6.0 ± 0.3	8.0 ± 0.2	11.6 ± 0.6
20:2n-6	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
20:4n-6	1.4 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.3 ± 0.1
Total n-6 PUFA[3]	4.9 ± 0.2	6.6 ± 0.2	8.0 ± 0.3	10.0 ± 0.2	13.3 ± 0.7
18:3n-3	0.5 ± 0.2	6.8 ± 0.5	14.4 ± 0.6	20.3 ± 1.1	32.6 ± 1.2
18:4n-3	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	1.1 ± 0.2
20:3n-3	0.1 ± 0.0	0.8 ± 0.1	1.6 ± 0.2	2.5 ± 0.2	3.8 ± 0.4
20:4n-3	1.6 ± 0.1	1.9 ± 0.1	2.4 ± 0.2	3.0 ± 0.1	3.1 ± 0.4
20:5n-3	10.0 ± 0.4	8.5 ± 0.5	7.6 ± 0.7	6.4 ± 0.8	3.1 ± 1.1
22:5n-3	3.7 ± 0.4	2.8 ± 0.2	2.3 ± 0.3	1.6 ± 0.1	0.8 ± 0.3
22:6n-3	24.4 ± 1.6	19.7 ± 1.5	17.2 ± 1.7	13.7 ± 1.4	5.6 ± 1.6
Total n-3 PUFA	40.6 ± 2.1	40.8 ± 2.0	46.0 ± 2.4	48.3 ± 1.1	50.0 ± 3.3
Total PUFA	45.7 ± 2.0	47.6 ± 1.9	54.3 ± 2.2	58.5 ± 0.9	63.4 ± 2.6

Results are means ± SD (n = 6).
[1]Includes 15:0 present at up to 0.3%;
[2]Includes 16:1n-9, 20:1n-7, 20:1n-11 and 22:1n-9 each present at up to 0.4%;
[3]Includes 18:3n-6, 20:3n-6 and 22:5n-6 present at up to 0.4%; FO, fish oil; LO, linseed oil; PUFA, polyunsaturated fatty acids.

Table 5. Fatty acid compositions (percentage of total fatty acids by weight) of total lipid of pyloric caeca from Atlantic salmon (*Salmo salar* L.) fed the experimental diets for 40 weeks

	FO	LO25	LO50	LO75	LO100
14:0	4.0 ± 0.5	3.4 ± 0.2	2.5 ± 0.2	1.5 ± 0.1	0.5 ± 0.0
16:0	16.1 ± 0.6	13.6 ± 0.8	13.0 ± 1.0	10.7 ± 0.6	9.7 ± 0.5
18:0	3.5 ± 0.3	3.5 ± 0.3	4.4 ± 0.4	4.4 ± 0.2	5.0 ± 0.3
Total saturated[1]	24.0 ± 0.5	20.8 ± 1.0	20.1 ± 1.4	16.7 ± 0.7	15.3 ± 0.8
16:1n-7	5.5 ± 0.5	4.5 ± 0.2	3.0 ± 0.2	1.9 ± 0.1	0.6 ± 0.0
18:1n-9	14.4 ± 1.0	15.2 ± 0.4	15.8 ± 0.6	16.2 ± 0.6	17.3 ± 0.2
18:1n-7	3.9 ± 0.2	3.3 ± 0.1	2.6 ± 0.1	1.8 ± 0.0	1.2 ± 0.0
20:1n-9	13.8 ± 1.5	11.3 ± 0.9	7.9 ± 0.8	4.6 ± 0.5	1.4 ± 0.1
22:1n-11	7.9 ± 1.3	7.4 ± 0.6	4.9 ± 0.7	3.2 ± 0.5	1.1 ± 0.1
24:1n-9	0.9 ± 0.0	0.8 ± 0.0	0.7 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
Total monoenes[2]	47.1 ± 4.0	43.0 ± 1.8	35.4 ± 2.2	28.4 ± 1.8	22.0 ± 0.4
18:2n-6	3.3 ± 0.2	5.9 ± 0.2	7.3 ± 0.6	10.5 ± 0.4	12.7 ± 0.4
20:2n-6	0.5 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
20:4n-6	1.1 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
Total n-6 PUFA[3]	5.2 ± 0.2	7.8 ± 0.2	9.5 ± 0.5	12.1 ± 0.4	14.1 ± 0.3
18:3n-3	0.7 ± 0.2	8.9 ± 0.6	13.5 ± 1.5	27.1 ± 1.5	34.6 ± 1.9
18:4n-3	0.8 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	0.8 ± 0.1	1.3 ± 0.1
20:3n-3	0.1 ± 0.1	0.8 ± 0.1	1.7 ± 0.2	2.5 ± 0.5	2.4 ± 0.2
20:4n-3	1.0 ± 0.1	1.4 ± 0.2	1.9 ± 0.0	1.7 ± 0.3	1.9 ± 0.2
20:5n-3	5.3 ± 1.2	4.9 ± 0.3	5.1 ± 0.9	3.3 ± 0.3	2.7 ± 0.5
22:5n-3	2.1 ± 0.5	1.8 ± 0.2	1.9 ± 0.3	1.0 ± 0.1	0.7 ± 0.1
22:6n-3	13.4 ± 2.5	9.4 ± 1.1	9.6 ± 1.9	6.2 ± 1.2	4.9 ± 0.7
Total n-3 PUFA	23.5 ± 3.6	28.2 ± 1.8	34.7 ± 1.7	42.7 ± 1.4	48.4 ± 1.0
Total PUFA	28.8 ± 3.5	36.0 ± 1.7	44.3 ± 1.6	54.7 ± 1.2	62.5 ± 0.9

Results are means ± SD (n = 4).
[1]Includes 15:0 present at up to 0.3%;
[2]Includes 16:1n-9, 20:1n-7, 20:1n-11 and 22:1n-9 each present at up to 0.4%;
[3]Includes 18:3n-6, 20:3n-6 and 22:5n-6 present in at up to 0.4%. FO, fish oil; LO, linseed oil; PUFA, polyunsaturated fatty acids.

Table 6. Correlation (regression) analyses (r^2, slope values and significance) for dietary 18:3n-3 and liver and caecal total and neutral lipid contents, percentages of liver and caecal 18:3n-3, 20:5n-3 and 22:6n-3, and fatty acyl desaturation and β-oxidation activities in hepatocytes and enterocyte from caecae and mid gut

	Liver			Caecae		
Fatty	r^2	slope	significance	r^2	slope	significance
Total lipid content	0.83	0.064 ± 0.017	0.0315	0.86	0.029 ± 0.007	0.0222
Neutral lipid content	0.78	0.369 ± 0.113	0.0466	0.70	0.079 ± 0.030	0.0756
18:3n-3	0.98	0.633 ± 0.051	0.0011	0.98	0.700 ± 0.059	0.0013
20:5n-3	0.94	−0.130 ± 0.080	0.0070	0.83	−0.055 ± 0.014	0.0306
22:6n-3	0.96	−0.356 ± 0.043	0.0036	0.93	−0.165 ± 0.026	0.0077
Total desaturation	0.98	0.051 ± 0.004	0.0010	0.43	−0.030 ± 0.020	0.2265
(mid-gut)				0.74	−0.038 ± 0.013	0.0630
β-Oxidation	0.06	−0.003 ± 0.006	0.6946	0.41	0.058 ± 0.040	0.2433
(mid gut)				0.30	0.038 ± 0.034	0.3387

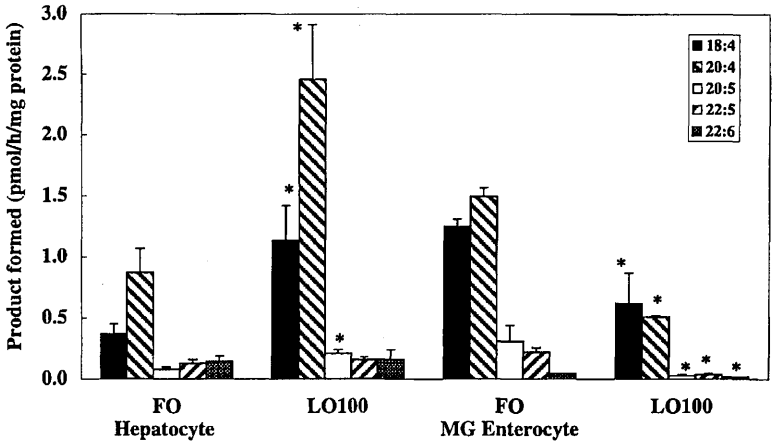

Figure 2. Individual fatty acid products of the desaturation of [1-^{14}C]18:3n-3 in hepatocytes and enterocytes isolated from the mid gut (MG) from fish fed the FO and LO100 diets for 40 weeks. Results are means ± S.D. (n = 3) and represent the rate production (pmol.h^{-1}.mg protein^{-1}) of individual fatty acids as determined by the recovery of radioactivity in each fatty acid fraction. Mean values for radioactivity recovered as individual fatty acids in a specific tissue in fish fed the LO100 diets were significantly different to those for fish fed the FO diet where indicated (*) as determined by the student t-test ($P < 0.05$). FO, fish oil; LO, linseed oil.

correlations between dietary level and liver level can be derived for any of the major fatty acids.

In comparison with liver, the fatty acid composition of total lipid from pyloric caecae of fish fed the FO diet showed higher levels of monoenes, particularly 20:1 and 22:1 and lower levels of n-3HUFA, with levels of 20:5n-3 and 22:6n-3 only about 50% the levels observed in liver (Table 5). However, the effects of increased inclusion of dietary LO were identical to those described for liver with the levels of 18:3n-3 and 18:2n-6 increased to almost 35% and 13% of total fatty acids, respectively, and 20:5n-3 and 22:6n-3 reduced to around 3% and 5%, respectively, in fish fed the LO100 diet. As with liver, regression analyses showed these effects were highly significant (Table 6). The fatty acid compositions of the midgut samples were virtually identical to those for the caecae (data not shown).

Effects of diet on hepatocyte and intestinal enterocyte fatty acid desaturation/elongation activities

The total desaturation of [1-^{14}C]18:3n-3, measured as the summed radioactivity recovered as desaturated fatty acid products (18:4, 20:4, 20:5, 22:5 and 22:6), was 2-fold higher in enterocytes than hepatocytes in fish fed the diet containing FO (Figure 1). However, the desaturation of [1-^{14}C]18:3n-3 in hepatocytes was increased in a graded manner by increasing dietary LO, and regression analyses confirmed a significant positive correlation with a very high degree of confidence ($p = 0.001$ and $r^2 = 0.98$) between dietary 18:3n-3 levels and hepatocyte fatty acid desaturation (Figure 1. and Table 6). In contrast, total desaturation of [1-^{14}C]18:3n-3 in pyloric caecal (PC) enterocytes was significantly decreased in fish fed dietary LO at levels of 50% or more (Figure 1), but there was no direct correlation between dietary 18:3n-3 levels and caecal fatty acid desaturation activity (Table 6). In enterocytes isolated from the midgut (MG) region just posterior to the caecae, desaturation of [1-^{14}C]18:3n-3 tended to decrease with increasing dietary LO with the activity being significantly lower in fish fed all levels of LO compared to fish fed the diet containing 100% FO (Figure 1).

In both hepatocytes and enterocytes, the main products of 18:3n-3 desaturation were the Δ6 desaturated products, 18:4n-3 and 20:4n-3 (Figure 2). Furthermore, the major effect of feeding diets containing LO on the desaturation of 18:3n-3 in hepatocytes was to increase the amount of Δ6 desaturated products whereas recovery of radioactivity in 20:5n-3 was only slightly, but significantly, increased, and recoveries in 22:5n-3 and 22:6n-3 were unaffected. In MG enterocytes, the recovery of radioactivity in all products of 18:3n-3 desaturation was significantly reduced in fish fed the LO100 diet compared to fish fed the FO diet (Figure 2).

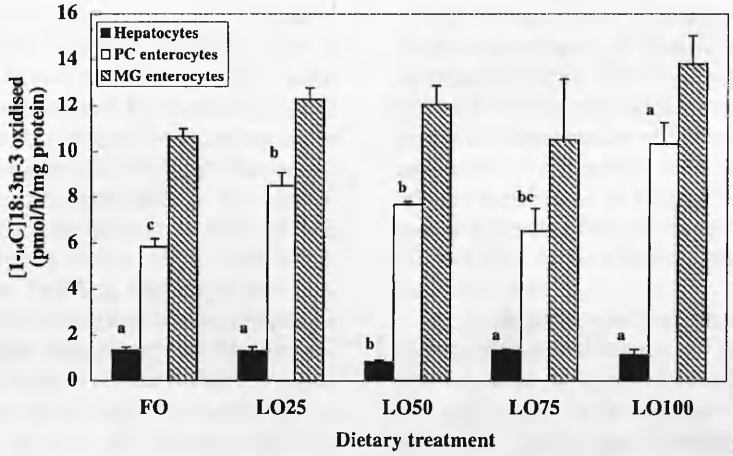

Figure 3. Fatty acid β-oxidation activity in hepatocytes and enterocytes isolated from pyloric caecae (PC) and mid gut (MG) after feeding the experimental diets for 40 weeks. Results are means ± S.D. (n = 3) and represent the rate of oxidation (pmol.h^{-1}.mg protein^{-1}) of [1-^{14}C]18:3n-3 to acid soluble products. Columns for a specific a tissue with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$). FO, fish oil; LO, linseed oil.

Effects of diet on hepatocyte and intestinal enterocyte fatty acid oxidation activities

The production of acid soluble products from [1-^{14}C]18:3n-3 was higher in enterocytes than in hepatocytes, and highest in MG enterocytes, irrespective of dietary treatment (Figure 3). In enterocytes, the production of acid-soluble products from [1-^{14}C]18:3n-3 was generally higher, significantly so in PC enterocytes, in fish fed diets containing LO compared to fish fed the FO diet (Figure 3), but there was no significant correlation with dietary 18:3n-3 levels (Table 6). There was no significant effect of dietary treatment on the production of acid-soluble products from [1-^{14}C]18:3n-3 in hepatocytes (Figure 3, Table 6).

Discussion

The primary hypothesis we aimed to test in the present study was that replacing FO with 18:3n-3-rich LO in salmon diets would enable the fish to maintain tissue n-3HUFA levels through a combination of increased desaturation/elongation activity and increased substrate fatty acid provision. The results show that this was not the case. Increasing dietary LO increased lipid accumulation, through increased neutral lipids, particularly in the liver. This also occurred in intestinal tissue although it was already higher in neutral lipid than liver. In previous trials, increasing inclusion of rapeseed oil or palm oil in the diet also increased the liver lipid levels in Atlantic salmon in seawater (Bell et al. 2001a, 2002). More importantly though, both liver and intestinal tissue showed very large increases in the proportions of 18:3n-3, and also 18:2n-6, with n-3HUFA decreasing significantly with increasing dietary LO, particularly at inclusion of > 50%. The greater effect on n-3HUFA was in liver where 20:5n-3 decreased over 3-fold and 22:6n-3 over 4–fold in fish fed the diet containing 100% LO. Previously, we observed that the level of 22:6n-3 in liver was halved in fish fed a diet containing 100% rapeseed oil compared to fish fed a 100% FO diet (Bell et al. 2001a). Therefore, supplying high amounts of 18:3n-3 in the diet in the form of LO did not alleviate the decrease in tissue n-3HUFA.

The decreased levels of tissue n-3HUFA occurred despite the fatty acid desaturation/elongation activity in liver being increased over 2.6-fold in fish fed the 100% LO diet compared to the control diet. Increasing hepatocyte fatty acid desaturation with increasing inclusion of vegetable oil in the diet has been observed in several previous studies. Similar experiments on salmon in seawater using graded levels of rapeseed oil and palm oil gave similar graded increases in hepatocyte fatty acid desaturation activity, with 100% replacement of FO giving increases of 2.7- (Bell et al. 2001a) and 10-fold (Bell et al. 2002), respectively. In freshwater, salmonid hepatocyte desaturation activities were up to 2.5-fold (Bell et al. 1997), 2.4-fold (Tocher et al. 2000) and 2.8-fold (Tocher et al. 2001) greater in fish fed vegetable oils compared to fish

fed FO. It is unclear whether the increased desaturation activity in fish fed vegetable oils is the result of decreasing levels of 20:5n-3 and 22:6n-3 in liver membrane lipids, and, thus, mediated by decreased product inhibition, or whether it is a result of increased levels of membrane 18:2n-6 and/or 18:3n-3 and, thus, mediated by increased substrate provision. In this respect, it is important to note that the correlations reported in Table 6 were between dietary 18:3n-3 levels and the various outcomes, including hepatocyte fatty acyl desaturation/elongation. However, similar correlations are obtained when you compare any of the other dietary fatty acids that vary in a graded manner across the diets, which includes the major fatty acids 18:2n-6, 20:5n-3, 22:6n-3, 18:1n-9, 16:0, 20:1n-9 and 22:1. Therefore, regression analyses of dietary 20:5n-3 or 22:6n-3 with hepatocyte desaturation also shows a significant correlation, albeit negative compared to the correlation with dietary 18:3n-3, but with a similar significance and r^2 value. Therefore, the present study cannot elucidate the mechanism, but it does demonstrate clearly that, irrespective of mechanism, the increased hepatic fatty acyl desaturation/elongation induced by feeding vegetable oils to salmon is ineffective in maintaining tissue levels of either 20:5n-3 or 22:6n-3, even in the presence of high levels of dietary 18:3n-3.

It is perhaps noteworthy that desaturation/elongation activity was measured by incubating cells with [1-^{14}C]18:3n-3 and determining the amount of radioactivity recovered as desaturated and elongated fatty acid products. Thus, desaturation of [1-^{14}C]18:3n-3 in hepatocytes and enterocytes was observed through the levels of radioactivity recovered mainly as 18:4n-3 and 20:4n-3 whereas recovery of radioactivity as 20:5n-3 and 22:6n-3 was much lower. Similarly, the effects of feeding LO are mainly manifested in changes in the recovery of radioactivity in the two main products 18:4n-3 and 20:4n-3. These data suggest that production of 20:5n-3 and, especially 22:6n-3, is low within the time-scale of the assay. That desaturation of 18:3n-3 was not sufficient to maintain tissue levels of 20:5n-3 and 22:6n-3 in fish fed increasing LO suggests that these steps are also low *in vivo*. Therefore, the reduced level of dietary n-3HUFA as dietary LO inclusion increased was the biggest factor in determining the tissue levels of 20:5n-3 and 22:6n-3. Consistent with this, the intermediates 18:4n-3 and 20:4n-3 were increased in both liver and intestinal tissue lipids with LO feeding. Interestingly, there was no evidence for desaturation of dietary 18:2n-6 as, in addition to declining 20:4n-6, there was no increase in the levels of the intermediates 18:3n-6 or 20:3n-6 in either liver or intestinal tissue. This is probably due to the excess of 18:3n-3 effectively blocking the Δ6 desaturase and preventing desaturation of 18:2n-6, an effect has been observed previously (Bell et al. 1993). The only indication of metabolism of 18:2n-6 in fish fed LO was the increased levels of the so-called "dead end" products, 20:2n-6 (and 20:3n-3 from 18:3n-3) in both liver and intestinal tissue.

A secondary aim in the present study was to determine if intestinal enterocytes were a site of significant fatty acid desaturation/elongation in salmon and this was proved to be the case. Indeed, enterocytes were more active than hepatocytes in desaturating and elongating the exogenously added [1-^{14}C]18:3n-3 substrate in fish fed 100% FO. However, in fish fed dietary LO, the intestinal enterocytes did not appear to have the capacity to increase their desaturation activity as with hepatocytes. In fact, fatty acid desaturation/elongation activity decreased in MG enterocytes proportionally with increasing dietary LO, and initially decreased (up to 50% LO inclusion) in PC enterocytes. This was surprising and different to the data obtained with hepatocytes in the present study and all previous studies on hepatocytes (Bell et al. 1997, 2001a, 2002; Tocher et al. 1997, 2000, 2001). One possible explanation is that fatty acyl desaturation and elongation in the two tissues serves different purposes with different regulatory mechanisms. Thus, the primary purpose of hepatic fatty acid desaturation may be to provide the body with sufficient long-chain HUFA for membrane biosynthesis and as such it is nutritionally induced when membrane HUFA declines as it does when feeding vegetable oil diets. In contrast, the fatty acyl desaturation activity in enterocytes may be simply to supply its own needs for HUFA for biomembrane synthesis. Being a tissue with a high turnover of cells/membranes, fatty acid desaturation/elongation may be relatively high and unaffected by diet. However, the activities in enterocytes were not maintained in the present study suggesting this is not a full explanation. A full explanation of the apparent difference in fatty acyl desaturation and elongation activities in hepatocytes and enterocytes and their nutritional regulation in liver and intestine *in vivo* will require considerably more research.

Our third aim in the present study was to determine the extent to which 18:3n-3 was oxidized in comparison with the amount desaturated/elongated simultaneously, in a combined assay, in both hep-

atocytes and enterocytes. Measurement of fatty acid oxidation in Atlantic salmon using isolated hepatocytes and [1-^{14}C]16:0, rather than labeled acyl CoA substrates as used in mitochondrial assays, has been described previously (Ji et al. 1996). In the present study we modified existing methods (Frøyland et al. 1995, 2000; Ji et al. 1996) slightly in order to determine fatty acid oxidation and the desaturation pathway in a single, combined assay. Our results showed that in hepatocytes from fish fed the FO diet, there was a similar amount of radioactivity recovered in acid soluble products as there was in desaturated products indicating that approximately equal amounts of the exogenously added [1-^{14}C]18:3n-3 were being oxidized and desaturated. In contrast, fatty acid oxidation activity was about 1.5- fold and 3-fold higher than desaturation activity in PC and MG enterocytes, respectively, in fish fed the FO diet. In addition, fatty acid oxidation activity was around 5- to 8-fold greater in intestinal enterocytes compared to hepatocytes in fish fed the FO diet. There are few data in the literature with which to compare the present data. Relatively high levels of fatty acid oxidation have been reported for red muscle and heart in trout, whereas liver, kidney and white muscle have only limited capacity to oxidize fatty acids (Henderson and Tocher 1987). Recently though, white muscle was shown to be a quantitatively important site of fatty acid catabolism in salmon, particularly juveniles, when expressed on a per tissue basis (Frøyland et al. 1998, 2000). However, liver was not a major site of mitochondrial or peroxisomal β-oxidation irrespective of how the data were presented (Frøyland et al. 2000). Certainly, the level of fatty acid oxidation appeared relatively low in the hepatocytes, but there are few data on the fatty acid oxidative capacity of fish intestinal tissues (Small and Connock 1981).

It terms of dietary effects, again there was a difference between hepatocytes and enterocytes in fatty acid oxidation in response to dietary LO. In hepatocytes, and in contrast to the desaturation pathway, there was no dietary effect on the oxidation of [1-^{14}C]18:3n-3. However, in enterocytes, fatty acid oxidation activity was generally increased in fish fed diets containing LO whereas desaturation activity generally decreased although there was no correlation with the dietary level of 18:3n-3. This was interesting in relation to the discussion above on the effects of diet on desaturation/elongation activities, because the apparent reciprocal manner in which the oxidation and desaturation assays varied in enterocytes suggested that there was the possibility of competition between the two pathways for available labeled fatty acid substrate. Whether this is the case and whether such competition between the two pathways for fatty acid could occur *in vivo* is unclear.

In rats, supplementation of the diet with 20:5n-3 and 22:6n-3 increased β-oxidation of PUFA, especially 18:2n-6, 18:3n-3 and 20:5n-3 (Grønn et al. 1992a). The results in rats suggested both mitochondrial and peroxisomal oxidation were increased but particularly peroxisomal with respect to n-3 PUFA. In Atlantic salmon, fatty acid oxidation is predominantly mitochondrial β-oxidation with peroxisomal β-oxidation accounting for less than 10% of total oxidation (Frøyland et al. 2000). Mitochondrial β-oxidation of [1-^{14}C]palmitoyl-CoA in red muscle of Atlantic salmon was not significantly affected by feeding vegetable oils namely, high oleic acid sunflower oil or palm oil, as 100% replacement of FO (capelin oil) for 21 weeks (Torstensen et al. 2000). In that study, the β-oxidation activity in liver homogenates, using [1-^{14}C]palmitoyl-CoA as substrate, was about 100-fold lower than in red muscle and 7-fold lower than in white muscle in fish fed capelin oil at the start of the trial, and no β-oxidation activity was detected in liver after feeding capelin oil or the vegetable oils (Torstensen et al. 2000). Studies in rat liver parenchymal cells and subcellular fractions suggested that 20:5n-3 can increase mitochondrial fatty acid oxidation, possibly by increasing transport of fatty acids into mitochondria as it increased the level of carnitine palmitoyltransferase-I mRNA (Madsen et al. 1999). The effect of 20:5n-3 on gene transcription may have been mediated through activation of peroxisome proliferator activated receptor α (PPARα) (Berge et al. 1999).

Although oxidation in intestinal enterocytes increased rather than decreased as the level of dietary 20:5n-3 decreased in the present study, the above suggests a possible molecular mechanism for the effects of diet on fatty acid desaturation and oxidation. Dietary fatty acids are known to affect the expression of fatty acid desaturase genes in both mammals (Cho et al. 1999a,b) and trout (Seiliez et al. 2001). PUFA can potentially affect gene transcription by a number of direct and indirect mechanisms (Jump et al. 1999) and are known to bind and directly influence the activities of a variety of transcription factors including PPARs, which in turn have been shown to be regulators of many genes involved in lipid homeostatic processes (Jump 2002). In rodents, peroxisomal

proliferators, which also activate PPARs, are known to up-regulate fatty acyl desaturation and oxidation (Grønn et al. 1992b). PPAR genes have been identified in Atlantic salmon and plaice (Ruyter et al. 1997; Leaver et al. 1998) and the peroxisomal proliferator, clofibrate, increased the desaturation of 20:5n-3 in rainbow trout (Tocher and Sargent 1993). However, these data do not exclude the possibility that fatty acids may also influence desaturation and oxidation more directly at a membrane level through alterations in fluidity or membrane microenvironments.

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Effects of dietary lipid level and vegetable oil on fatty acid metabolism in Atlantic salmon (*Salmo salar* L.) over the whole production cycle

D.R. Tocher, J.G. Bell, F. McGhee, J.R. Dick and J. Fonseca-Madrigal
Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland UK (Phone: +44-1786-467996; Fax: +44-1786-472133; E-mail: d.r.tocher@stir.ac.uk)

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Abstract

Changes in fatty acid metabolism in Atlantic salmon (*Salmo salar*) induced by vegetable oil (VO) replacement of fish oil (FO) and high dietary oil in aquaculture diets can have negative impacts on the nutritional quality of the product for the human consumer, including altered flesh fatty acid composition and lipid content. A dietary trial was designed to investigate the twin problems of FO replacement and high energy diets in salmon throughout the entire production cycle. Salmon were grown from first feeding to around 2 kg on diets in which FO was completely replaced by a 1:1 blend of linseed and rapeseed oils at low (14–17%) and high (25–35%) dietary oil levels. This paper reports specifically on the influence of diet on various aspects of fatty acid metabolism. Fatty acid compositions of liver, intestinal tissue and gill were altered by the diets with increased proportions of C_{18} polyunsaturated fatty acids and decreased proportions of n-3 highly unsaturated fatty acids (HUFA) in fish fed VO compared to fish fed FO. HUFA synthesis in hepatocytes and enterocytes was significantly higher in fish fed VO, whereas β-oxidation was unaltered by either dietary oil content or type. Over the entire production cycle, HUFA synthesis in hepatocytes showed a decreasing trend with age interrupted by a large peak in activity at seawater transfer. Gill cell prostaglandin (PG) production showed a possible seasonal trend, with peak activities in winter and low activities in summer and at seawater transfer. PG production in seawater was lower in fish fed the high oil diets with the lowest PG production generally observed in fish fed high VO. The changes in fatty acid metabolism induced by high dietary oil and VO replacement contribute to altered flesh lipid content and fatty acid compositions, and so merit continued investigation to minimize any negative impacts that sustainable, environmentally-friendly and cost-effective aquaculture diets could have in the future.

Abbreviations: FO – fish oil; HUFA – highly unsaturated fatty acids acids (carbon chain length $\geq C_{20}$ with ≥ 3 double bonds); LO – linseed oil; RO – rapeseed oil; VO – vegetable oil.

Introduction

Salmonid and marine fish culture presently relies heavily on available supplies of fish meal and fish oil which, because of increased exploitation pressure on strictly limited global feed grade fisheries, as well as the increasing ecological and ethical objections to exploiting a non-sustainable resource, places the industry in a vulnerable position (Sargent and Tacon 1999; Barlow 2000; Tidwell and Allan 2002). However, anadromous fish like Atlantic salmon (*Salmo salar*) have limited ability to convert (α-linolenic (18:3n-3) and linoleic (18:2n-6) acids, which are abundant in many vegetable oils, to their long-chain highly unsaturated fatty acid (HUFA) products which are essential physiological components of all cell membranes and organs (Tocher 2003). Therefore, replacement of dietary fish oil with high quality, n-3 polyunsaturated fatty acid (PUFA)-rich vegetable oils may have potential in salmonids culture (Sargent et al. 2002). The hypothesis which we wish to test is that salmon can be grown on diets containing appropri-

ate vegetable oils without deleterious effects on fish physiology or health or its value as an important nutritious rood. However, in addition, the use of high-energy feeds containing high percentages of oil, which have drastically reduced production times in the salmon industry, can have undesirable side effects including increased oil deposition in the flesh (adiposity), reduced pigment visualisation and reduced smoking performance, leading to processor and retailer rejection and consumer dissatisfaction (Sheehan et al. 1996; Johansen and Jobling 1998). Replacement of fish oil with vegetable oils may exacerbate this problem through the deposition of triglycerides as a result of feeding high levels of fatty acids not readily utilised by salmon, particularly in seawater (Bell 1998).

Several previous trials have investigated aspects of fish oil replacement in diets for Atlantic salmon. There have been trials investigating the replacement of fish oil with vegetable oil in the diets of salmon parr in freshwater (Bell et al. 1997; Tocher et al. 2000), and other trials that have looked at the effects of feeding vegetable oils to smolts in seawater (Bell et al. 2001a, 2002; Rosenlund et al. 2001; Torstensen et al. 2000; Tocher et al. 2002). A variety of different oils such as soybean (Hardy et al. 1987; Lie et al. 1993), sunflower (Bell et al. 1991, 1993), borage (Tocher et al. 1997), rapeseed (Tocher et al. 2000; Bell et al. 2001a), linseed (Tocher et al. 2000, 2002) and palm oil (Torstensen et al. 2000; Bell et al. 2002) have been investigated as well as oil blends (Bell et al. 2003a; Rosenlund et al. 2001; Jobling et al. 2002a,b; Tocher et al. 2003). The effects of dietary oil level have been investigated for a number of years (see Cowey 1993; Jobling 2001) with more recent studies including levels of oil that are routine in the salmon farming industry today (Jobling et al. 2002c). There has also been a trial looking at the effect of dietary oil level and vegetable oil replacement (Jobling et al. 2002a,b). However, most of the above trials have been of relatively short duration and none of the previous trials have been run throughout the entire 2 year production cycle of the salmon from first-feeding to harvest.

The present dietary trial was designed to investigate the twin problems of replacement of fish oil with alternative oils and high energy (oil) diets in Atlantic salmon culture. Salmon were grown throughout the entire production cycle, from first feeding to harvest size, on diets in which fish oil was replaced by a vegetable oil blend at a high and low dietary oil level. This paper specifically reports on the effects of the experimental diets on various aspects of fatty acid metabolism. Thus, the effects of feeding the diets for an entire two year growth cycle on fatty acid compositions of liver and intestinal tissue, and fatty acid desaturation/elongation and β-oxidation in hepatocytes and intestinal enterocytes were determined in fish of harvest size. In addition, the time course of hepatocyte fatty acid desaturation and elongation, and the production of prostaglandin in gills, throughout the trial, including the effects of seawater transfer, were determined.

Materials and methods

Animals and diets

In March 2000, Atlantic salmon fry were distributed randomly into 8 tanks (3 m × 3 m, depth 0.5 m) at a stocking level of 3000/tank, and weaned onto extruded feeds containing either fish oil (FO) or vegetable oil (VO), a 1:1 blend of rapeseed and linseed oils. Each oil was fed to duplicate tanks of fry/parr at either 14% (L) or 25% total oil (H), resulting in four dietary treatments in total, LFO (low fish oil), HFO (high fish oil), LVO (low vegetable oil) and HVO (high vegetable oil). Fish were fed the diets described above until sea water transfer in April 2001, at which point fish (average weight ~ 40 g) were transferred into 5 m × 5 m net pens at 600 fish/pen. The fish were fed the same diet in seawater as in freshwater although the dietary oil levels were increased to 17% in the low oil diets (LFO and LVO) and 30% (3 mm pellet) rising to 37% (6 and 9 mm pellets) in the high oil diets (HFO and HVO) in the seawater phase. The diets aimed to be practical and therefore were formulated according to current practices in the salmon feed industry and were manufactured by the major salmon feed producers (BioMar A/S, Brande, Denmark; Ewos Technology Centre, Livingston, Scotland; Skretting, Stavangar, Norway). All diets were formulated to satisfy the nutritional requirements of salmonid fish (U.S. National Research Council 1993). Diet formulations and proximate compositions are given in Table 1 and fatty acid compositions in Table 2. Fish were ongrown until June 2002 at which time they had reached around 2 kg. Final weights in kg were 1.44 ± 0.37 (LFO), 1.88±0.57 (HFO), 1.71±0.36 (L VO) and 2.02±0.57 (HVO) (all n = 80). Two-way ANOVA showed that growth was significantly affected by both oil type ($F = 14.68$, $P = 0.0002$) and oil level ($F = 49.11$, $P < 0.0001$) in an independent manner (interaction

F= 1.48, P= 0.2754), with higher growth in fish fed high oil level and vegetable oil. Water temperature throughout the trial averaged 10.5 ± 2.2°C (measured at 2 m), varying by season with lowest temperatures recorded in February (5.9 °C) and highest in August (14.4 °C).

At the end of the 2 year trial, HUFA synthesis and β-oxidation was determined in isolated hepatocytes and intestinal caecal enterocytes, and samples of liver and pyloric caecal collected for lipid and fatty acid analyses. In addition though, samples were collected throughout the time course of the trial at predetermined time intervals including immediately pre- and post- seawater transfer. At all these points, livers and gills were collected for fatty acid analyses and determination of hepatocyte HUFA synthesis and gill cell prostaglandin F production.

Lipid extraction and fatty acid analyses

Fish were not extensively starved prior to sampling and so were fed the day before but not on the day of sampling giving a time of sampling around 12–15 hours after last feeding. Livers, gills (at all time points) and pyloric caecae (at trial end) were dissected from 4 fish per dietary treatment and immediately frozen in liquid nitrogen. Total lipid contents of salmon tissues were determined gravimetrically after extraction by homogenization in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, basically according to Folch et al. (1957). Samples of total lipid (2 mg) were applied as 2 cm streaks to thin-layer chromatography plates, the plates developed fully with isohexane/diethyl ether/acetic acid (90:10:1, by vol.) as developing solvent, and the origin area corresponding to total polar lipids were scraped into stoppered glass test tubes. Fatty acid methyl esters (FAME) were prepared from total polar lipids by acid-catalyzed transesterification directly on the silica using 2 ml of 1% H_2SO_4 in methanol plus 1 ml toluene as described by Christie (1982) and FAME extracted and purified as described previously (Tocher and Harvie 1988). FAME were separated and quantified by gas-liquid chromatography (Fisons GC8600, Fisons Ltd., Crawley, U.K.) using a 30 m × 0.32 mm capillary column (CP wax 52CB; Chrompak Ltd., London, U.K). Hydrogen was used as carrier gas and temperature programming was from 50 °C to 180 °C at 40 °C/min and then to 225 °C at 2 °C/min. Individual methyl esters were identified by comparison to known standards and by reference to published data (Ackman 1980).

Preparation of isolated hepatocytes, caecal enterocytes and gill cells

Fish were killed by a blow to the head and livers, intestinal tract and gills dissected immediately. The gall bladder was removed carefully from the liver and the main blood vessels trimmed. The liver was perfused via the hepatic vein with solution A (calcium and magnesium-free Hanks balanced salt solution (HBSS) +10 mM HEPES +1 mM EDTA) to clear blood from the tissue. The pyloric caeca were cleaned of adhering adipose tissue before being dissected from the intestinal tract and lumenal contents rinsed away with solution A. The gills were rinsed in solution A and blotted on tissue to remove excess blood and medium. Essentially the same method based on collagenase digestion and sieving has been utilized successfully to isolate gill cell, hepatocyte- and enterocyte-enriched preparations (Tocher et al. 2000, 2002, 2003). Briefly, the tissues were chopped finely with scissors and about 0.5 g of chopped tissue was incubated with 20 ml of solution A containing 0.1% (w/v) collagenase at 20 °C for 45 min. The digested tissues were filtered through 100 μm nylon gauze and the cells collected by centrifugation at 300× g for 2 min. The cell pellets were washed with 20 ml of solution A containing 1% w/v fatty acid-tree bovine serum albumin (FAF-BSA) and re-centrifuged. The washing was repeated with a further 20 ml of solution A without FAF-BSA. Hepatocytes and enterocytes were resuspended in 10 ml of Medium 199 containing 10 mM HEPES and 2 mM glutamine, whereas gill cells were resuspended in 1 ml of HBSS containing 2 mM $CaCl_2$. One hundred μl of cell suspensions were mixed with 400 μl of Trypan Blue, cells counted and their viability assessed using a haemocytometer. The viability at isolation was > 97% and > 94%, for hepatocytes and enterocytes respectively, and in both cell types only decreased by between 5 and 10% over the period of the incubation. One hundred μl of cell suspensions were retained for protein determination according to the method of Lowry et al. (1951) after incubation with 0.4 ml of 0.25% (w/v) SDS/1M NaOH for 45 min at 60 °C.

Table 1. Dietary formulations and proximate compositions

Component	LFO	LVO	HFO	HVO	LFO	LVO	HFO	HVO	LFO	LVO	HFO	HVO
Pellet size (mm)	2				3				6			
LT Fishmeal	50.0		66.0		65.0		50.0		35.2		43.5	
Wheat	15.0		13.0		14.0		12.0		22.0		13.5	
Soybean meal	20.0		0.0		10.0		11.5		12.0		6.5	
Other plant products[1]	5.5		0.0		4.8		2.5		11.8		7.2	
Premixes etc.[2]	2.0		2.0		1.0		1.0		1.0		1.0	
Fish oil[3]	7.5	–	19.0	–	10.0	–	25.4	–	14.6	–	29.2	–
Linseed oil	–	3.8	–	9.5	–	5.0	–	12.7	–	7.3	–	14.6
Rapeseed oil	–	3.8	–	9.5	–	5.0	–	12.7	–	7.3	–	14.6
Protein (%)	53.7	52.0	50.8	51.5	51.5	51.4	48.4	47.9	38.0	37.2	39.7	37.6
Fat (%)	14.1	13.6	26.3	24.4	16.2	17.9	28.9	28.8	16.9	16.9	36.9	36.5
Moisture (%)	4.4	5.7	3.9	4.8	8.0	7.7	3.2	3.1	7.9	8.7	1.8	5.8

[1]Rapeseed meal, wheat flour, wheat and corn glutens.
[2]Vitamin and mineral pre-mixes, Finnstim, pigments according to feed company specifications.
[3]Capelin or herring oils.

Incubation of hepatocyte and enterocyte preparations with [1-^{14}C]18:3n-3

Six ml of each hepatocyte or enterocyte suspension were dispensed into a 25 cm^2 tissue culture flask. Hepatocytes and enterocytes were incubated with 0.3 μCi ($\sim$ 1 μM) [1-^{14}C] 18:3n-3, added as a complex with FAF-BSA in HBSS (albumin:fatty acid ratio of 7-8:1) prepared as described previously (Ghioni et al. 1997). After addition of isotope, the flasks were incubated at 20 °C for 2 h. After incubation, the cell layer was dislodged by gentle rocking and the cell suspension transferred to glass conical test tubes and 1 ml of each suspension withdrawn into a 2 ml microcentrifuge tube for β-oxidation assay as described below. The cell suspensions remaining in the glass conical centrifuge tubes were used for the desaturation/elongation assay as described below.

Assay of hepatocyte and enterocyte fatty acyl desaturation/elongation activities

The cell suspensions were centrifuged at 500× g for 2 min, the supernatant discarded and the hepatocyte or enterocyte 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) 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 isohexane 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, U.K.). Results were corrected for counting efficiency and quenching of ^{14}C under exactly these conditions.

Assay of hepatocyte and enterocyte fatty acyl oxidation activities

Measurement of fatty acid oxidation by estimation of acid-soluble radioactivity after incubating intact cells with labelled fatty acids has been described previously (Frøyland et al. 1996, 2000; Madsen et al. 1998; Torstensen et al. 2000; Tocher et al. 2002). Briefly, after incubation with [1-^{14}C]18:3n-3, 1 ml of hepatocyte or enterocyte suspension was homogenized with a hand-held tissue disrupter (Ultra-Turrax T8/S8N-5G probe, IKA-Werke GmbH & Co., Slaufen, Germany) and centrifuged at 10000× g for 10 min. Five hundred μl of the supernatant was taken into a clean 2 ml microcentrifuge tube and 100 μl of ice-cold 6% FAF-BSA

solution in water was added. After mixing thoroughly, the protein was precipitated by the addition of 1.0 ml of ice-cold 4 M perchloric acid ($HClO_4$), and the tubes centrifuged at 10000× g for 10 min. Five hundred μl of the supernatant was transferred to a scintillation vial, 4 ml of scintillant added and radioactivity in the acid-soluble fraction determined as described above for desaturation/elongation assay.

Assay of gill cell prostaglanding F production

Gill cell suspensions were incubated for 10 min at 20 °C in a shaking water bath before the addition of A23187 to a final concentration of 10 μM. Incubation was continued for 15 min after which the cells were removed by centrifugation (12000× g, 2 min) and the supernatant transferred to tubes containing 150 μl ethanol and 50 μl 2 M formic acid. The supernatants were centrifuged (12000× g, 2 min) to remove any precipitate before being extracted using octadecyl silica (C18) 'Sep-Pak' minicolumns (Millipore (UK), Watford) as described in detail by Bell et al. (1994). The final extract was dissolved in 0.1 ml methanol and stored at −20 °C prior to analysis by enzyme immunoassay. Samples were dried under nitrogen and redissolved in immunoassay buffer. Measurement of total PGF was performed using an enzyme immunoassay kit for $PGF_{2\alpha}$ according to the manufacturer's protocols (SPI-Bio, Massy, France).

Materials

[1-^{14}C]18:3n-3 (50–55 mCi/mmol) was obtained from NEN (DuPont (U.K.) Ltd., Stevenage, U.K.). HBSS, Medium 199, HEPES buffer, glutamine, collagenase (type IV), FAF-BSA, BHT, A23187, silver nitrate and perchloric acid were obtained from Sigma Chemical Co. (Poole, U.K.). Thin-layer chromatography (TLC) plates, 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

All the data are presented as means ± SD (n = 3 or 4). The effects of dietary treatment on HUFA synthesis and β-oxidation in hepatocytes and enterocytes, and liver and pyloric caecal fatty acid compositions at the end of the 2 year trial were determined by one-way analysis of variance (ANOVA) followed, where appropriate, by Tukey's comparison test. The effects of dietary oil type (FO v. VO) and oil level (H v. L) on liver and gill fatty acid compositions and hepatocyte HUFA synthesis and gill cell PG production at selected points in the time course were determined by two-way ANOVA. Percentage data and data which were identified as non-homogeneous (Bartlett's test) were subjected to arcsine transformation before analysis. Differences were regarded as significant when $P < 0.05$ (Zar 1984).

Results

Dietary fatty acid compositions

The diets containing VO were characterized by having increased proportions of 18:1n-9, 18:2n-6 and 18:3n-3 and reduced proportions of saturated fatty acids, long chain monoenes (20:1 and 22:1) and n-3 HUFA (20:5n-3 and 22:6n-3) compared to diets containing FO (Table 2). These differences were slightly more prominent in the diets with higher oil contents due to the reduction in the relative contribution of fatty acids from the non-oil components of the diet, the fish and plant meals. The diets aimed to reflect current feeding practices and so the precise level of long-chain monoenes and n-3HUFA in the diets containing FO varied depending on which fish oil (herring or capelin) was utilized by the individual feed company at the time of diet production.

Effects of diet on fatty acid compositions of liver and pyloric caeca

The differences in the fatty acid compositions of the diets were reflected in the fatty acid compositions of the tissues of the fish at the end of the two year dietary trial. Thus, the fatty acid compositions of total polar lipids of livers from salmon fed the LVO and HVO diets were characterized by increased proportions of 18:1n-9, 18:2n-6 and 18:3n-3 and reduced proportions of 16:0 and total saturated fatty acids, long chain monoenes (20:1 and 22:1), 20:4n-6, 20:5n-3 and 22:6n-3 compared to fish fed diets containing FO (Table 3). Again, the differences tended to be greatest between fish fed the high oil contents, HFO and HVO. Increased percentages of elongated and $\Delta 6$ desaturated products of 18:2n-6 and 18:3n-3, that is 20:2n-6 and 20:3n-6, and 18:4n-3, 20:3n-3 and 20:4n-3, respectively, were observed in fish fed the VO diets (Table 3). Essentially the same pattern was observed

Table 2. Fatty acid compositions (percentage of weight) of diets used in freshwater (FW) and seawater (M6, 6 mm pellet and M9, 9 mm pellet)

	LFO			HFO			LVO			HVO		
	FW	M6	M9	FW	M6	M9	FW	M6	M9	FW	M6	M9
14:0	5.1	4.8	5.7	5.6	5.6	6.3	3.0	0.9	1.4	1.5	0.6	0.7
16:0	14.4	16.6	12.2	14.7	16.9	11.9	11.0	8.0	8.1	8.0	7.0	6.6
18:0	2.0	2.9	1.3	2.3	2.6	1.1	2.2	3.1	2.5	2.4	2.9	3.0
Total saturates[1]	22.6	25.7	20.1	23.6	26.5	20.2	17.0	12.7	13.1	12.7	11.3	11.0
16:1n-7[2]	4.3	5.5	7.6	4.9	6.3	8.5	2.2	1.1	1.7	1.2	0.7	0.8
18:1n-9	15.2	16.2	11.2	14.0	15.0	11.1	24.2	28.5	30.4	30.5	32.6	33.6
18:1n-7	2.5	2.1	2.9	2.5	2.4	3.2	2.9	1.8	2.2	1.8	2.1	1.9
20:1n-9[3]	8.9	4.1	16.6	9.8	5.8	18.4	5.4	0.9	3.9	3.1	1.2	2.0
22:1n-11[4]	12.6	5.9	14.6	13.5	8.0	15.8	7.3	0.3	3.9	3.7	0.7	1.8
24:1	1.2	0.9	0.9	0.9	1.1	0.8	0.8	0.2	0.4	0.4	0.3	0.3
Total monoenes	44.6	34.8	53.8	45.6	38.7	57.7	42.8	32.8	42.4	40.8	37.6	40.3
18:2n-6	5.4	10.4	6.2	3.2	2.9	3.1	11.3	22.9	17.8	13.4	19.1	17.0
20:4n-6	0.4	0.5	0.3	0.5	0.6	0.3	0.1	0.2	0.1	0.1	0.1	0.1
Total n-6PUFA[5]	7.0	11.7	7.2	5.0	4.3	4.0	12.0	23.2	18.1	14.0	19.3	17.1
18:3n-3	2.6	1.8	1.0	1.4	1.7	0.8	16.7	24.1	18.2	26.3	27.5	26.9
18:4n-3	2.7	2.3	2.8	3.2	2.7	3.0	1.3	0.4	0.5	0.7	0.2	0.4
20:4n-3	0.8	0.6	0.4	0.9	0.6	0.4	0.3	0.1	0.1	0.1	0.1	0.1
20:5n-3	6.2	8.6	6.7	6.7	9.6	6.5	3.0	2.1	3.0	1.6	1.4	1.6
22:5n-3	1.0	1.0	0.5	0.9	1.0	0.5	0.3	0.2	0.2	0.1	0.2	0.1
22:6n-3	11.3	11.9	6.0	11.3	13.1	5.4	6.0	3.5	3.7	3.3	2.3	2.1
Total n-3PUFA[6]	24.6	26.2	17.5	24.5	28.8	16.6	27.6	30.5	25.8	32.2	31.6	31.2
Total PUFA[7]	32.4	39.5	26.1	30.4	34.8	22.1	40.0	54.5	44.5	46.4	51.2	48.7
(n-3)/(n-6)	3.5	2.2	2.4	4.9	6.8	4.1	2.3	1.3	1.4	2.3	1.6	1.8

Results are means of two determinations. [1], totals include 15:0, 17:0, 20:0 and 22:0, present at up to 0.5%; [2]also contains n-9 isomer; [3]also contains n-11 and n-7 isomers; [4]also contains n-9 and n-7 isomers; [5]totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6 present at up to 0.5%; [6]totals include 20:3n-3 present at up to 0.2%; [7]totals include C_{16}PUFA present at up to 1.5% in FO diets and up to 0.4% in VO diets; HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil; PUFA, polyunsaturated fatty acids.

in the fatty acid composition of the pyloric caeca although the differences tended to he more pronounced both between FO and VO diets and also between low and high oil contents (Table 4).

Effects of diet on HUFA synthesis and fatty acid oxidation in isolated hepatocytes and caecal enterocytes at the end of trial

HUFA synthesis, as measured by the conversion of [1-^{14}C]18:3n-3 to desaturated and elongated products, was greater in both isolated hepatocytes and caecal enterocytes in fish fed VO (Figure 1A). Rates of conversion were generally higher in hepatocytes compared to enterocytes, except for fish fed diet HFO, which showed the lowest HUFA synthesis activity in hepatocytes, whereas the lowest activity in enterocytes was obtained with fish fed the LFO diet (Figure 1A). Oxidation of [1-^{14}C]18:3n-3, as measured by the recovery of acid-soluble radioactivity, was 3–4-fold higher than conversion by the desaturation/elongation pathway in both hepatocytes and enterocytes but, in contrast to that pathway, fatty acid oxidation was not affected by diet in either cell type (Figure 1B).

Time course of hepatocyte HUFA synthesis and liver polar lipid fatty acid compositions

In general, the activity of the fatty acid desaturation/elongation pathway tended to decrease throughout the two year cycle, with the activities when first determined in parr being 2–3 fold higher than the activities measured at the end of the trial (Figure 2). However, this pattern was interrupted around seawater

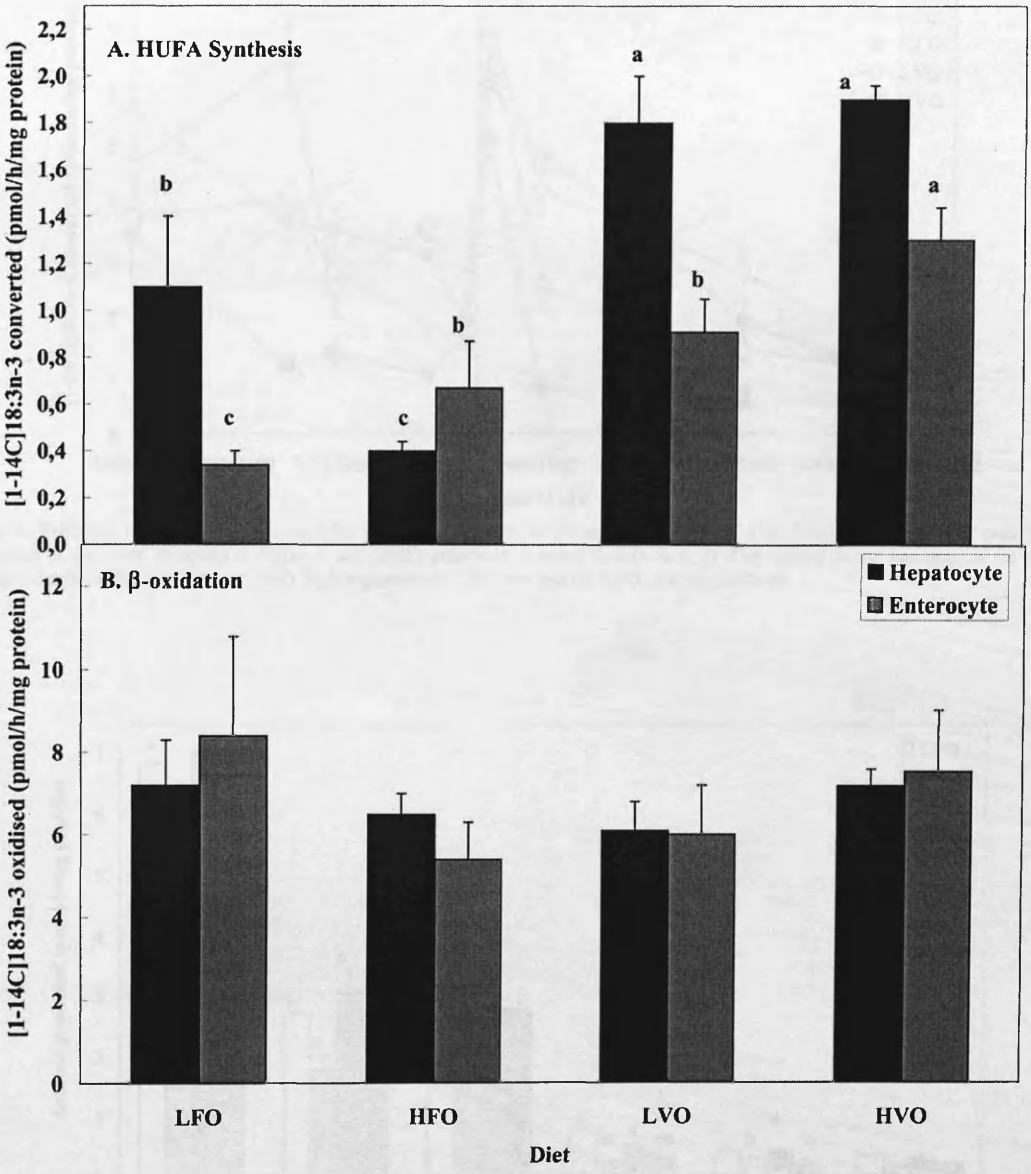

Figure 1. Metabolism of [1-^{14}C] 18:3n-3 in isolated hepatocytes and caecal enterocytes at the end of the dietary trial. HUFA synthesis (A) was determined by measuring total fatty acid desaturation/elongation activity and represents the rate of conversion (pmol.h^{-1}.mg protein^{-1}) of [1-^{14}C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3). Fatty acid β-oxidation activity (B) was determined by the recovery (pmol.h^{-1}.mg protein^{-1}) of radioactivity from [1-^{14}C]18:3n-3 as acid soluble products. All results are means ± S.D. (n = 3). Columns for a specific activity and tissue with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$). HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil.

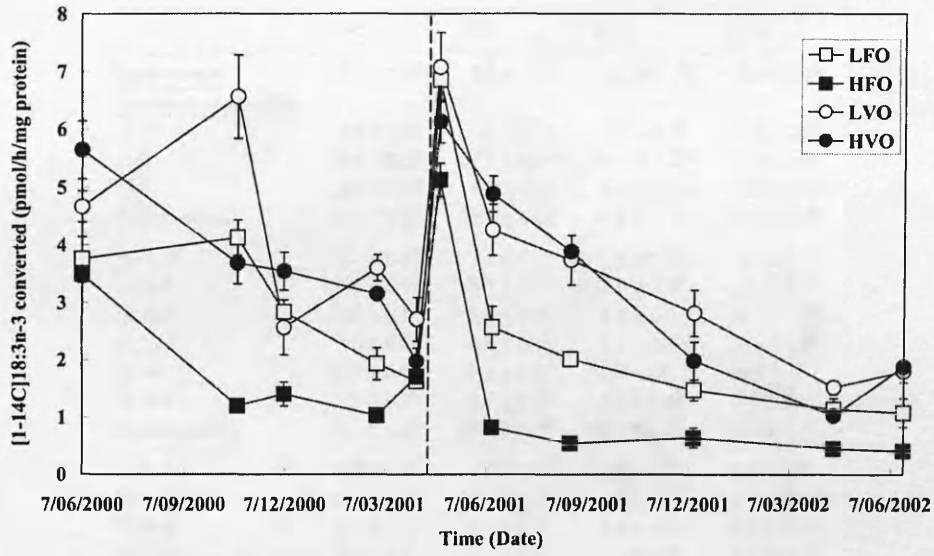

Figure 2. Evolution of HUFA synthetic capacity in isolated hepatocytes throughout the dietary trial. Total HUFA synthetic capacity was determined as described in legend to Figure 1. and results presented as means ± S.D. (n = 3). The vertical dotted line denotes the point of seawater transfer. HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil.

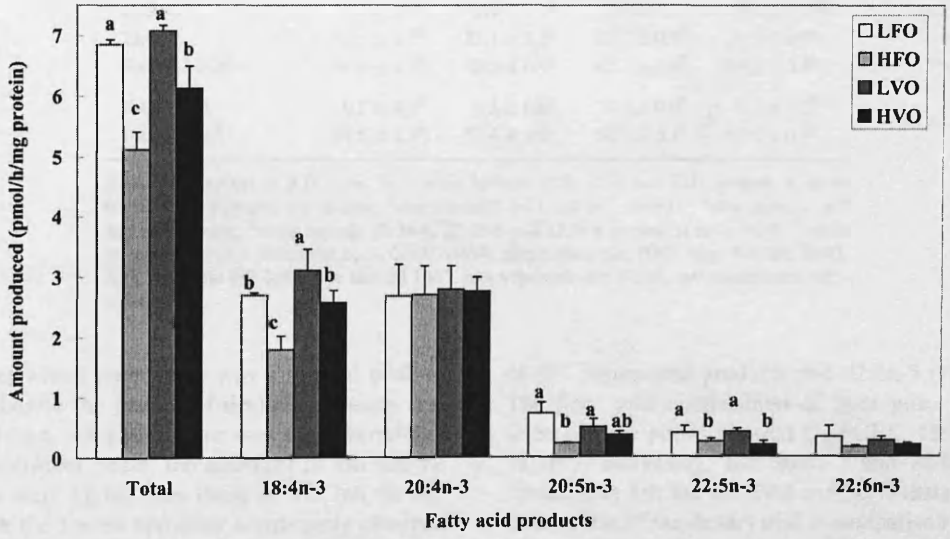

Figure 3. Individual fatty acid products of the desaturation and elongation of [1-^{14}C]18:3n-3 in hepatocytes immediately after seawater transfer (peak activity, 25 April 2001). Results are means ± S.D. (n = 3) and represent the rate production (pmol.h^{-1}.mg protein^{-1}) of individual fatty acids as determined by the recovery of radioactivity in each fatty acid fraction. Columns within a specific fatty acid with different superscript letters are significantly different as determined by one-way ANOVA followed by the Tukey post test ($P < 0.05$). HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil.

Table 3. Total lipid content (percentage of wet weight) and fatty acid composition (percentage of weight) of total polar lipid of liver at the end of the dietary trial (final time point)

	LFO	HFO	LVO	HVO
Lipid content	3.0 ± 0.6^{c}	3.8 ± 0.6^{bc}	4.7 ± 0.7^{ab}	5.2 ± 0.8^{a}
Fatty acid composition				
14:0	1.6 ± 0.1^{b}	2.1 ± 0.2^{a}	0.6 ± 0.0^{c}	0.4 ± 0.0^{c}
16:0	18.8 ± 0.5^{a}	17.7 ± 0.8^{a}	13.8 ± 1.2^{b}	$12.6 \pm 0.7b$
18:0	5.0 ± 0.2^{b}	4.7 ± 0.2^{c}	5.9 ± 0.2^{a}	5.6 ± 0.4^{ab}
Total saturated[1]	26.0 ± 0.6^{a}	25.2 ± 0.8^{a}	20.8 ± 1.3^{b}	19.1 ± 0.8^{b}
16:1n-7[2]	1.9 ± 0.1^{b}	2.5 ± 0.2^{a}	0.9 ± 0.0^{c}	0.7 ± 0.0^{c}
18:1n-9	9.6 ± 0.2^{b}	8.7 ± 0.6^{b}	17.8 ± 1.5^{a}	18.1 ± 0.7^{a}
18:1n-7	2.0 ± 0.1^{b}	2.4 ± 0.1^{a}	1.4 ± 0.0^{c}	1.4 ± 0.1^{c}
20:1n-9[3]	3.5 ± 0.7^{a}	4.6 ± 0.5^{a}	1.2 ± 0.2^{b}	1.1 ± 0.2^{b}
22:1n-11[4]	0.6 ± 0.1^{b}	0.9 ± 0.1^{a}	0.3 ± 0^{c}	$v0.3 \pm 0^{c}$
24:1n-9	1.5 ± 0.1^{a}	1.4 ± 0.1^{a}	1.1 ± 0.1^{b}	1.1 ± 0.1^{b}
Total monoenes	19.1 ± 1.2^{b}	20.7 ± 1.2^{ab}	22.7 ± 1.7^{a}	22.7 ± 1.3^{a}
18:2n-6	2.9 ± 0.2^{c}	1.4 ± 0.1^{d}	8.8 ± 0.3^{b}	9.6 ± 0.3^{a}
20:2n-6	0.5 ± 0.1^{b}	0.3 ± 0.0^{b}	1.2 ± 0.1^{a}	$1.1 \pm 0.2^{\pm a}$
20:3n-6	0.4 ± 0.1^{b}	0.2 ± 0.0^{b}	1.0 ± 0.1^{a}	0.9 ± 0.1^{a}
20:4n-6	2.5 ± 0.1^{a}	2.5 ± 0.2^{a}	1.7 ± 0.2^{b}	1.2 ± 0.1^{c}
Total n-6 PUFA[5]	6.8 ± 0.2^{b}	4.8 ± 0.2^{c}	13.2 ± 0.3^{a}	13.1 ± 0.3^{a}
18:3n-3	0.4 ± 0.1^{c}	0.4 ± 0.0^{c}	6.4 ± 0.4^{b}	11.1 ± 0.9^{a}
18:4n-3	0.2 ± 0.0^{b}	0.3 ± 0.0^{b}	0.7 ± 0.2^{a}	0.9 ± 0.2^{a}
20:3n-3	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}	0.8 ± 0.1^{b}	1.2 ± 0.1^{a}
20:4n-3	0.8 ± 0.0^{c}	1.1 ± 0.1^{bc}	1.2 ± 0.2^{b}	1.8 ± 0.1^{a}
20:5n-3	9.4 ± 0.9^{ab}	10.6 ± 0.7^{a}	8.0 ± 0.2^{b}	8.7 ± 0.8^{b}
22:5n-3	3.3 ± 0.3^{a}	3.6 ± 0.1^{a}	2.5 ± 0.1^{b}	2.1 ± 0.1^{b}
22:6n-3	32.8 ± 1.7^{a}	32.1 ± 1.3^{a}	23.1 ± 0.9^{b}	18.5 ± 0.6^{c}
Total n-3 PUFA	46.9 ± 1.1^{a}	48.2 ± 0.9^{a}	42.7 ± 0.8^{b}	44.2 ± 0.6^{b}
Total DMA	0.3 ± 0.1^{a}	0.3 ± 0.0^{a}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}
Total PUFA[6]	54.5 ± 1.2^{bc}	53.8 ± 1.0^{c}	56.3 ± 0.5^{b}	57.5 ± 0.9^{a}

Results are means ± S.D. (n= 3).[1], totals include 15:0, 17:0 and 20:0, present at up to 0.3%; [2]also contains n-9 isomer; [3]also contains n-11 and n-7 isomers; [4]also contains n-9 and n-7 isomers; [5]totals include 18:3n-6, 22:4n-6 and 22:5n-6 present at up to 0.3%; [6]totals include C_{16}PUFA present at up to 0.6%; DMA, dimethylacetals; HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil LVO, low vegetable oil; PUFA, polyunsaturated fatty acids.

transfer at which point there was a marked peak of activity, before the pattern of declining activity was re-established. Although there was some variability in the freshwater phase, the activities in fish fed the VO diets were higher than those in fish fed the FO diets with the lowest activities consistently obtained in fish fed the HFO diet (Figure 2). HUFA synthesis was significantly affected by oil type and also often by oil level as determined by two-way ANOVA (Table 5). Irrespective of diet and sampling point, the main products of the pathway were the Δ6 desaturated products 18:4n-3 and 20:4n-3, with smaller amounts of Δ5 desaturated products and 22:6n-3 (Figure 3). The fatty acid composition of liver polar lipids at selected time points showed C_{18}PUFA, 18:2n-6 and 18:3n-3, increasing, and 20:5n-3 and 22:6n-3, decreasing, in fish fed the LVO and HVO diets over the time-course of the dietary trial in comparison with fish fed the FO diets (Figure 4). As with hepatocyte HUFA synthesis, both oil type and oil level significantly affected fatty acid composition of liver throughout the dietary trial (Table 5).

Table 4. Total lipid content (percentage of wet weight) and fatty acid composition (percentage of weight) of total polar lipid of pyloric caeca at the end of the dietary trial (final time point)

	LFO	HFO	LVO	HVO
Lipid content	2.9 ± 0.6^{b}	5.2 ± 1.5^{a}	2.5 ± 0.4^{b}	3.9 ± 0.8^{ab}
Fatty acid composition				
14:0	2.7 ± 0.2^{b}	5.5 ± 0.3^{a}	1.6 ± 0.1^{c}	1.0 ± 0.1^{d}
16:0	19.0 ± 0.6^{a}	18.1 ± 0.9^{a}	14.5 ± 1.1^{b}	9.8 ± 0.7^{c}
18:0	5.6 ± 0.2^{ab}	4.2 ± 0.4^{c}	6.2 ± 0.7^{a}	4.5 ± 0.2^{bc}
Total saturated[1]	27.6 ± 0.8^{c}	28.4 ± 1.2^{a}	22.6 ± 1.8^{b}	15.5 ± 1.0^{c}
16:1n-7[2]	2.6 ± 0.4^{b}	5.7 ± 0.7^{a}	1.2 ± 0.3^{c}	0.8 ± 0.0^{c}
18:1n-9	9.2 ± 0.3^{c}	11.0 ± 0.7^{c}	19.0 ± 3.1^{b}	31.0 ± 0.5^{a}
18:1n-7	2.6 ± 0.1^{b}	3.0 ± 0.1^{a}	2.1 ± 0.1^{c}	2.1 ± 0.1^{c}
20:1n-9[3]	4.4 ± 0.5^{b}	7.2 ± 0.9^{a}	2.4 ± 0.2^{c}	2.4 ± 0.2^{c}
22:1n-11[4]	3.5 ± 0.6^{b}	7.5 ± 1.4^{a}	1.7 ± 0.1^{c}	1.6 ± 0.1^{c}
24:1n-9	1.0 ± 0.8	1.0 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
Total monoenes	23.3 ± 0.4^{b}	35.5 ± 0.9^{a}	27.0 ± 2.9^{b}	38.5 ± 0.3^{a}
18:2n-6	2.1 ± 0.1^{c}	2.5 ± 0.5^{c}	7.5 ± 1.3^{b}	13.4 ± 0.2^{a}
20:2n-6	0.4 ± 0.0^{b}	0.3 ± 0.0^{b}	0.8 ± 0.0^{a}	0.8 ± 0.0^{a}
20:3n-6	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.4 ± 0.1^{a}	0.5 ± 0.1^{a}
20:4n-6	2.1 ± 0.1^{a}	1.2 ± 0.2^{b}	1.6 ± 0.1^{b}	0.5 ± 0.0^{c}
Total n-6 PUFA[5]	5.4 ± 0.2^{c}	4.7 ± 0.4^{c}	10.6 ± 1.4^{b}	15.5 ± 0.1^{a}
18:3n-3	0.6 ± 0.0^{c}	1.0 ± 0.2^{c}	8.1 ± 1.1^{b}	15.8 ± 0.4^{a}
18:4n-3	0.8 ± 0.1^{bc}	1.8 ± 0.4^{a}	0.5 ± 0.1^{c}	1.3 ± 0.1^{ab}
20:3n-3	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.8 ± 0.0^{a}	1.0 ± 0.1^{a}
20:4n-3	0.7 ± 0.1	1.0 ± 0.3	0.6 ± 0.0	1.0 ± 0.2
20:5n-3	8.8 ± 0.6^{a}	8.2 ± 0.9^{a}	5.9 ± 1.2^{b}	2.2 ± 0.3^{c}
22:5n-3	2.2 ± 0.2^{a}	2.1 ± 0.6^{a}	1.4 ± 0.2^{ab}	0.7 ± 0.1^{b}
22:6n-3	30.1 ± 0.7^{a}	16.5 ± 1.3^{b}	18.9 ± 2.3^{b}	8.4 ± 0.2^{c}
Total n-3 PUFA	43.4 ± 0.7^{a}	30.8 ± 1.1^{bc}	36.3 ± 3.9^{b}	30.4 ± 1.1^{c}
Total DMA	0.4 ± 0.2	0.7 ± 0.1	0.6 ± 0.4	0.2 ± 0.1
Total PUFA	48.7 ± 0.9^{a}	35.5 ± 1.4^{b}	46.9 ± 3.9^{a}	45.9 ± 1.2^{a}

Results are means ± SD (n = 3). [1]totals include 15:0 present at up to 0.5%; [2]also contains n-9 isomer; [3]also contains n-11 and n-7 isomers; [4]also contains n-9 and n-7 isomers; [5]totals include 18:3n-6 and 22:5n-6 present at up to 0.4%; DMA, dimethylacetals; HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil; PUFA, polyunsaturated fatty acid.

Time course of gill prostaglandin F (PGF) production and gill polar lipid fatty acid compositions

In general, there was a trend for the capacity of PGF production by isolated gill cells to be greatest at mid-winter samplings in December, with lower activities recorded at the mid-summer samplings in June (Figure 5). However, the most striking feature overall was the very low levels of PGF production capacity measured around the period of parr-smolt transformation and seawater transfer. The effects of dietary treatment were also less pronounced at the summer samplings and, especially, seawater transfer (Figure5). However, the most prominent dietary effect was that PGF production capacity was generally lower in fish fed the VO diets, at least in the seawater phase, with the lowest level of PG production generally observed in fish fed the HVO diet (Figure 5). Gill PGF production was affected by dietary oil level between seawater transfer and final sampling with levels of PGF lower in fish fed the diets with higher oil content (Figure 5 & Table 5). In contrast, dietary oil level generally did not significantly affect the levels of the PG precursor fatty acids, 20:4n-6 and 20:5n-3 and the 20:5n-3/20:4n-6

Table 5. Significance of effects of dietary oil type and oil content on HUFA synthesis in hepatocytes and PG synthesis in gill cells and fatty acid compositions of liver and gill at specific times during the two year cycle.

	Dietary oil type		Dietary oil level		Interaction	
Fatty acid	F value	significance	F value	significance	F value	significance
LIVER						
6-Dec-00						
C_{18}PUFA	1140.0	<0.0001	92.5	<0.0001	162.5	<0.0001
20:5n-3	1.7	0.2268	1.7	0.2268	6.9	0.0307
22:6n-3	109.9	<0.0001	21.4	0.0017	6.9	0.0306
HUFA synthesis	26.9	0.0008	1.6	0.2472	44.7	0.0002
25-Apr-01						
C_{18}PUFA	608.4	<0.0001	36.7	0.0003	69.2	<0.0001
20:5n-3	27.3	0.0008	20.5	0.0019	12.1	0.0083
22:6n-3	408.8	<0.0001	69.1	<0.0001	11.2	0.0101
HUFA synthesis	73.4	<0.0001	139.7	<0.0001	56.6	<0.0001
11-Dec-01						
C_{18}PUFA	718.8	<0.0001	32.3	0.0005	103.4	<0.0001
20:5n-3	5.6	0.0450	3.4	0.102	1.7	0.2237
22:6n-3	239.5	<0.0001	15.4	0.0044	5.0	0.0560
HUFA synthesis	65.3	<0.0001	25.0	0.001	0.0	1.0000
18-Jun-02						
C_{18}PUFA	2772.0	<0.0001	49.4	0.0001	140.0	<0.0001
20:5n-3	16.5	0.0036	5.5	0.0475	0.4	0.5554
22:6n-3	285.7	<0.0001	14.1	0.0056	8.4	0.0202
HUFA synthesis	138.6	<0.0001	10.1	0.0132	16.3	0.0038
GILL						
6-Dec-00						
Gill 20:4n-6	0.0	0.6938	4.2	0.0755	4.2	0.0755
Gill 20:5n-3	8.1	0.0216	0.1	0.7599	2.5	0.1525
Gill EPA/AA	37.5	0.0003	13.5	0.0063	1.5	0.2555
Gill PGF	25.3	0.0010	4.4	0.0687	70.1	<0.0001
25-Apr-01						
Gill 20:4n-6	0.3	0.6290	0.3	0.6290	0.7	0.4268
Gill 20:5n-3	8.9	0.0175	0.1	0.8099	0.6	0.4769
Gill EPA/AA	0.8	0.3972	7.2	0.0278	0.0	1.0000
Gill PGF	17.3	0.0031	21.7	0.0016	6.0	0.0400
11-Dec-01						
Gill 20:4n-6	138.9	<0.0001	1.7	0.2268	42.9	0.0002
Gill 20:5n-3	6.2	0.0380	0.6	0.4604	1.2	0.3093
Gill EPA/AA	0.1	0.7831	2.0	0.1923	0.1	0.7831
Gill PGF	62.8	<0.0001	47.0	0.0001	0.6	0.4629
18-Jun-02						
Gill 20:4n-6	30.0	0.0006	1.2	0.3052	30.0	0.0006
Gill 20:5n-3	1.9	0.2048	1.2	0.3143	0.6	0.4651
Gill EPA/AA	1.1	0.3216	4.5	0.0675	4.5	0.0675
Gill PGF	5.8	0.0423	0.0	0.9661	3.9	0.0838

Data from Figures 2,4,5 and 6 at specific time points were subjected to two-way ANOVA as described in the Methods section. AA, arachidonic acid; EPA, eicosapentaenoic acid; PGF, prostaglandin F; HUFA, highly unsaturated fatty acids; PUFA, polyunsaturated fatty acids.

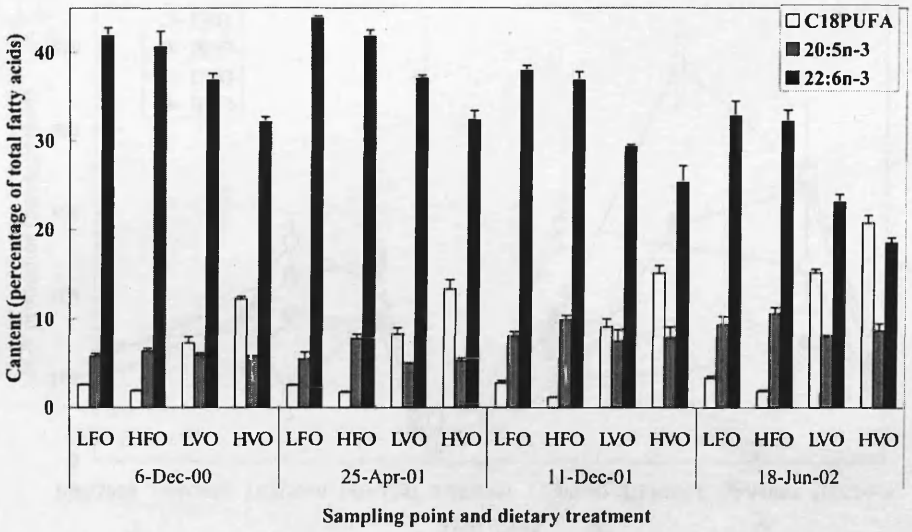

Figure 4. Content of C_{18} PUFA (18:2n-6 and 18:3n-3), and 20:5n-3 and 22:6n-3 in liver polar lipids at selected time points during the dietary trial. Results are presented as percentage of total fatty acids and are means ± S.D. (n = 3). HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil.

ratio at selected time points (winter peaks, seawater transfer and end point) during the trial (Figure 6 & Table 5). The level of gill 20:4n-6 was not significantly affected by oil type at the earlier time points but was significantly lower in fish fed the VO diets in the seawater phase (Figure 6 & Table 5). The level of gill 20:5n-3 increased in all the dietary treatments during the trial, although levels were generally significantly higher in fish fed the diets containing FO (Figure 6 & Table 5). Gill 20:5n-3/20:4n-6 ratio was generally not significantly affected by dietary treatment from seawater transfer onwards (Table 5).

Discussion

Several studies have shown that both dietary oil type and oil content can have significant effects on fatty acid metabolism in Atlantic salmon (Bell et al. 1997; Tocher et al. 2000, 2002, 2003; Torstensen et al. 2000; Rosenlund et al. 2001; Jobling et al. 2002a,b,c). Major consequences of these effects can be altered lipid content and fatty acid composition of the flesh, both of which can compromise the nutritional quality of the fish as food for the human consumer (Sheehan et al. 1996; Johansen and Jobling 1998; Bell et al. 2001a, 2002; Torstensen et al. 2000; Rosenlund et al. 2001). The present study aimed to investigate the effects of dietary oil content and composition on key pathways of fatty acid biochemistry throughout a two year growth cycle. Two important pathways of lipid and fatty acid homeostasis, whose relative activities contribute to final tissue fatty acid compositions, are HUFA synthesis through desaturation and elongation, and fatty acid oxidation through the β-oxidation pathway. It has been reported that a major fate of dietary 18:3n-3 in salmonids was oxidation for energy (Bell et al. 2001b). Thus, 18:3n-3 not only serves as a substrate for the synthesis of the important long-chain n-3 HUFA, but also as an energy source. Therefore, we investigated the effects of the diets on the balance between these two pathways, using [1-^{14}C] 18:3n-3 as fatty acid substrate, in two important organs, liver (hepatocytes) as the major lipid metabolising organ, and intestine (enterocytes), as it is the first organ to encounter dietary fatty acids and has been shown to be a site of significant HUFA synthesis (Bell et al. 2003b). Irrespective of diet, the primary fate of [1-^{14}C]18:3n-3 was β-oxidation rather than HUFA synthesis in both hepatocytes and enterocytes. Dietary oil type had no significant effect on β-oxidation in hepatocytes or enterocytes, as there was no increased oxidation of 18:3n-3 in cells from fish fed VO compared to fish fed FO. Perhaps surprisingly though, dietary oil level also had no significant effect on β-oxidation activity when measured using [1-^{14}C]18:3n-3 as substrate in either hepatocytes or enterocytes. Thus, the effect of the dietary treatments was that the balance between the

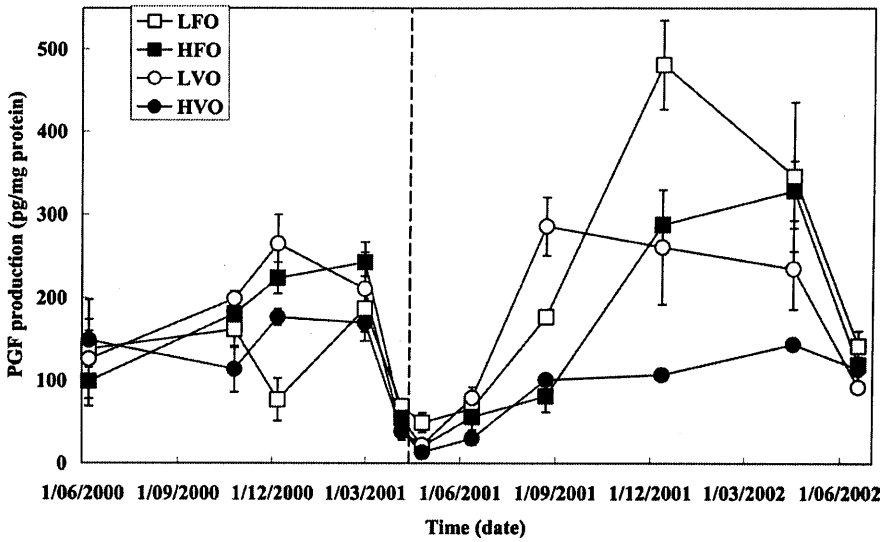

Figure 5. Evolution of prostaglandin F (PGF) synthesis capacity of isolated gill cells throughout the dietary trial. PGF production (pg/mg protein) was determined after stimulation of gill cells with the calcium ionophore, A23187. Results presented as means ± S.D. (n = 3). The vertical dotted line denotes the point of seawater transfer. HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil.

pathways competing for dietary 18:3n-3 was moved in the favour of HUFA synthesis in both liver and intestine in fish fed VO. However, as has been observed previously, the shift in the balance towards HUFA synthesis is not sufficient to maintain n-3HUFA levels or prevent the deposition of excess dietary C_{18} PUFA in the tissues (Bell et al. 2001a, 2002, 2003a: Tocher et al. 2002, 2003).

In the present study, HUFA synthesis in hepatocytes showed a generally decreasing trend over the two year time-course of the trial, interrupted with a peak in activity around seawater transfer, and that the activity was greater throughout in fish fed diets containing VO. The peak in activity had been observed in previous trials, although it's precise timing in relation to seawater transfer can vary (Bell et al. 1997; Tocher et al. 2000). The timing of the peak in the present trial, virtually at transfer, was also observed in the first of our earlier trials (Bell et al. 1997) and supports our hypothesis that the peak in activity coincides with the completion of the parr-smolt transformation and that variation in its temporal relationship to seawater transfer is because transfer is an artificially imposed time point (Tocher et al. 2000). In the earlier parr-smolt trials, hepatic HUFA synthesis declined during the seawater phase, but this was not unexpected in those trials as all fish were shifted to diets containing FO after seawater transfer. In the present trial, the VO diets continued to be fed throughout the seawater phase, but hepatocyte HUFA synthesis activity still declined, albeit the activity was always higher in fish fed the VO diets than in fish fed the diets containing FO. Therefore, once in seawater and as the fish ages the HUFA synthetic capacity appears to decline. In addition, activities were relatively high in the fish when first sampled (~ 3 months post-hatch) and much higher than the activities in 2 year-old fish at the end of the trial. This general decline in HUFA synthesis capacity in hepatocytes with age of the fish was not apparent in the earlier trials which only looked at fish during a window around 20 weeks before and after seawater transfer (Bell et al. 1997; Tocher et al. 2000). Decreasing fatty acid desaturation capacity with age and development has been noted previously in mammals. Specifically, the activity of hepatic Δ6 desaturase has been reported to decrease with aging in rats (Hrelia et al. 1989; Bourre and Piciotti 1992).

Another possible consequence of feeding vegetable oils is disturbance of eicosanoid metabolism. Vegetable oils contain no 20:4n-6, the predominant eicosanoid precursor fatty acid, whereas fish oils usually contain around 1–1.5% of this fatty acid (Ackman 1980; Padley et al. 1994). Therefore, this potential 20:4n-6 deficiency in VO diets must be met by conversion of 18:2n-6 to 20:4n-6. Previously, we had shown that PGF production by gill cells in response

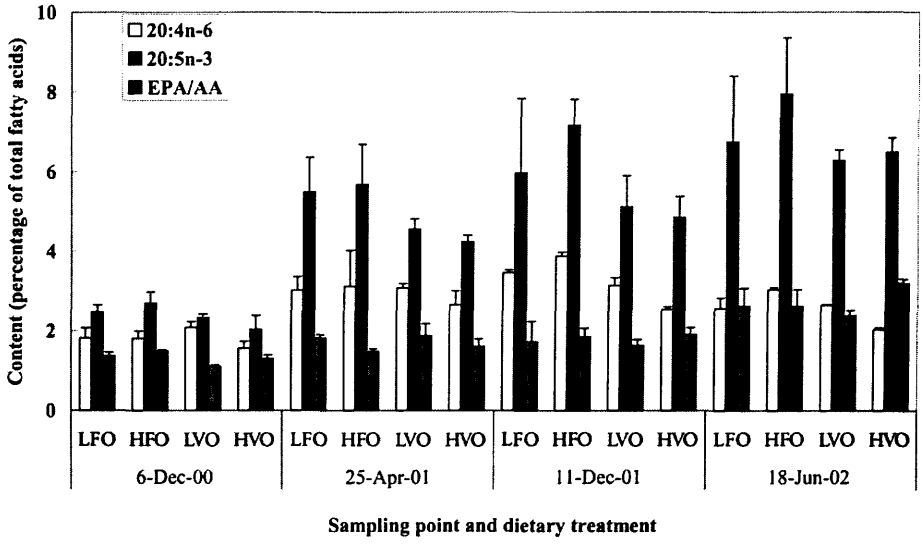

Figure 6. Content of 20:4n-6, 20:5n-3 and the 20:5n-3/20:4n-6 ratio in gill polar lipids at selected time points during the dietary trial. Results are presented as percentage of total fatty acids and are means ± S.D. (n = 3). HFO, high fish oil; HVO, high vegetable oil; LFO, low fish oil; LVO, low vegetable oil.

to calcium ionophore A23187 was low around the period of seawater transfer with peaks of activity before and after transfer (Bell et al. 1997). This pattern was explained in terms of it being a relatively acute response to changes in the physiology of the fish, and the gills in particular. That is, the peak in activity prior to seawater challenge being due to physiological changes in the gills as an integral part of the preadaptive parr-smolt transformation, with the increased PG production after transfer being a response to increased salinity once the fish were in seawater (Bell et al. 1997; Tocher et al. 2000). However, the present trial, over a much longer time course, has shown that the pattern may in fact be seasonal, in that the peaks of PGF production capacity appear to be around midwinter, with low PGF production around midsummer and, especially, seawater transfer. Immune responses of ectothermic animals such as salmon are known to vary seasonally, but generally parameters tend to be suppressed in winter and highest in summer (Slater and Schreck 1998). The very low capacity for PGF production around seawater transfer may have consequences for fish health, suggesting that the fish could be more susceptible to infection or disease at that time. This is known to be a period of increased stress in salmon with increases in cortisol, glucose and IGF-1 recorded around this time, all of which could also contribute to an immuno-suppressive effect (Weyts et al. 1999). Other studies have shown changes in serum proteins, IgM levels and leucocyte populations, that may affect cellular and humoral immunity, take place around parr-smolt transformation in salmon (Maule et al. 1989; Melingen and Wergeland 2000; Melingen et al. 2002). In addition, some diseases, such as infectious pancreatic necrosis virus (IPNV) (Bowden et al. 2002), tend to strike in the period immediately after seawater transfer, whereas fish still in freshwater or successfully transferred are highly resistant, consistent with fish being more susceptible to infection immediately post-seawater transfer. However, the interactions between endocrine, immune and physiological changes at smoltification are complex (Weyts et al. 1999).

In the present study, the dual effects of dietary oil content and composition on the capacity of gill cells to produce PG gave variable results, but a generally consistent feature was that PGF production in response to A23187 was lowest in fish fed the HVO diet. Previously, $PGF_{2\alpha}$ and $PGF_{3\alpha}$ production were both reduced in fish fed FO in comparison to fish fed a 1:1 blend of RO and LO at dietary oil levels that were closer to the low lipid diets used in the present trial, being 19% and 24% pre- and post-transfer, respectively (Bell et al. 1997). In the earlier trial, the 20:5n-3/20:4n-6 ratio in gill polar lipids was generally higher in fish fed the FO diets, consistent with

the $PGF_{2\alpha}$ data, but not the $PGF_{3\alpha}$ data. $PGF_{2\alpha}$ production exceeded $PGF_{3\alpha}$ production by up to 4-fold (Bell et al. 1997), and so total PGF production, as determined in the present trial, will reflect $PGF_{2\alpha}$ production, but there was no strong relationship between PGF production and 20:5n-3/20:4n-6 ratio, or gill cell 20:4n-6 and 20:5n-3 levels in the present study. Relatively poor correlation between gill polar lipid 20:4n-6 and 20:5n-3 levels and gill cell PGF production was noted in another earlier study (Tocher et al. 2000). This is further supported by the effects of dietary oil content on gill PGF production. During the seawater phase there was a clear relationship between dietary oil content and gill PGF production, with lower production in fish fed the high oil diets compared to fish fed the equivalent oil at lower level. This relationship was very significant at the mid-winter sampling (11 Dec 2001) but dietary oil had no significant effect on gill 20:4n-6 or 20:5n-3 levels or the 20:5n-3/20:4n-6 ratio. The conclusion must be that other factors are also important in determining the level of PG production. Among these could be direct effects of other specific fatty acids, such as 20:3n-6 and 20:4n-3, that can compete for binding to the eicosanoid generating enzymes, or more indirect effects of fatty acids on general membrane composition leading to altered fluidity or membrane microenvironments. In addition, modulation of gene expression via changes in oxidant stress, nuclear receptor activation or covalent modification of specific transcription factors could affect eicosanoid metabolism (Jump et al. 1999).

In summary, the present trial has shown that the combination of feeding high energy (oil) diets, and diets in which vegetable oils replace fish oil, throughout the growth cycle of Atlantic salmon have significant effects on various pathways of fatty acid metabolism. Based on these data and the overall performance of the fish during the trial, we can draw three main conclusions. Firstly, although feeding dietary vegetable oil throughout the production cycle significantly altered fatty acid biochemistry, it did not lead to any metabolic effects that were obviously detrimental to general fish health and physiology. Secondly, the combination of high dietary oil content and 100% replacement of FO with VO oil did not significantly exacerbate the effects of feeding salmon VO. Thirdly, there are temporal and/or seasonal patterns of fatty acid metabolism throughout the salmon growth cycle that, although affected by feeding VO, are not greatly, or apparently adversely, altered. Despite this, the changes in fatty acid metabolism induced by high dietary oil and vegetable oil replacement do contribute to possible detrimental effects for the human consumer, such as altered flesh lipid content and fatty acid compositions, and so this area merits continued investigation to minimize any negative impacts that sustainable, environmentally-friendly and cost-effective aquaculture diets could have in the future.

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